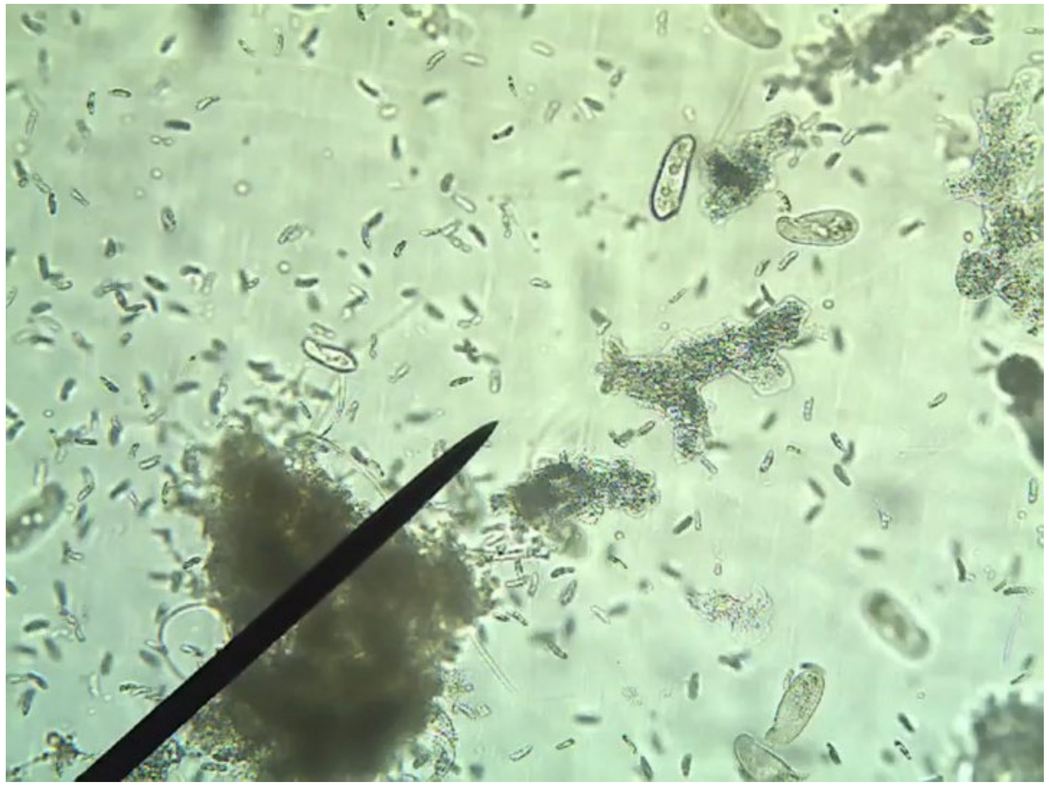
Microscopy of the cell and intracellular structures

Miklós Kellermayer

Light microscopy



Zacharias Jensen
(1580-1638)

Anton van Leewenhoek
(1632-1723)

Robert Hooke
(1635-1703)

Leewenhoek's simple
microscope

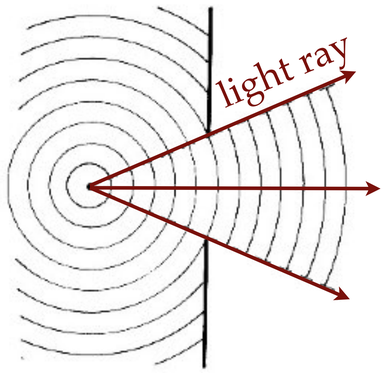
Robert Hooke's
mikroscope

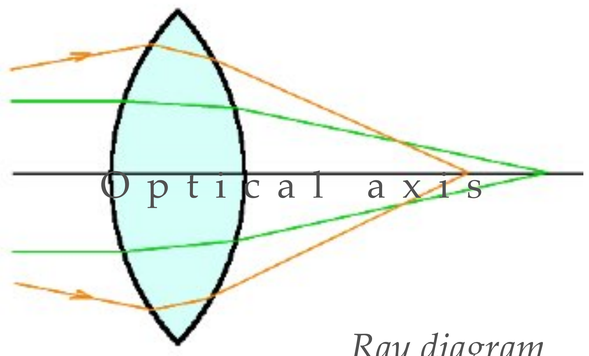
Cells in cork
(Robert Hooke)

Geometric optics

Geometric optics

If light propagates through a slit much larger than its wavelength, then the spreading of the wavefront (phase) may be simplified into a line ("light ray").



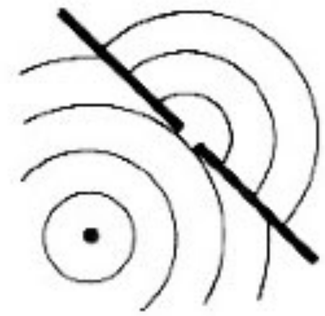


Ray diagram

- Optical (light) ray ("light beam"): abstraction, mathematical line.
- Arrows represent the direction of energy propagation.
- Optical axis: line connecting the midpoint of optical components (e.g., lenses).
- Principle of reversibility: the direction of energy propagation (arrows) may be reversed.

Wave optics

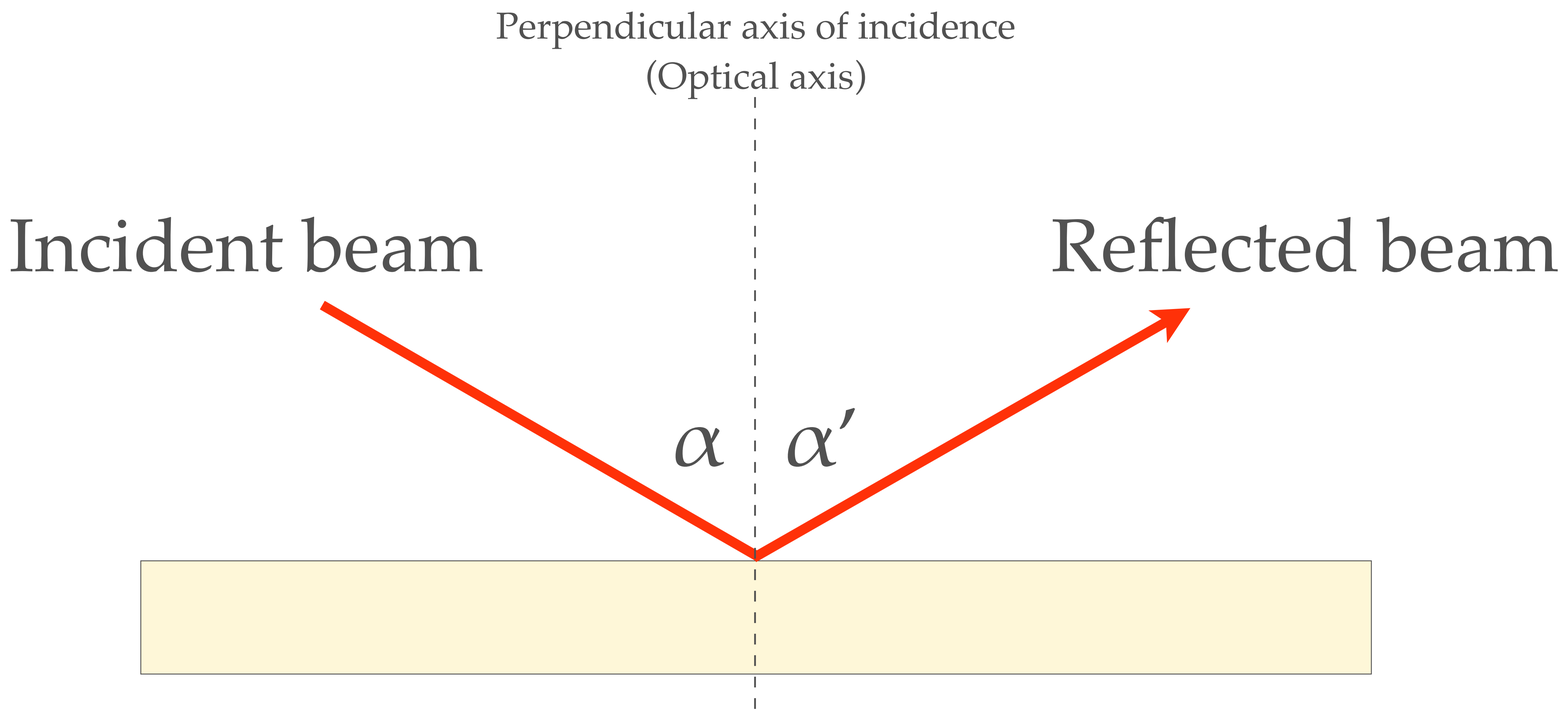
If light propagates through a slit comparable or smaller than its wavelength, then its wave properties must be taken into account.



Speed of propagation of light in ***vacuum***: c=2,99792458 x 10^8 ms^{-1}
In optically denser media the speed of propagation is reduced (c_1).
This may be expressed with the ***absolute refractive index*** (n_1):

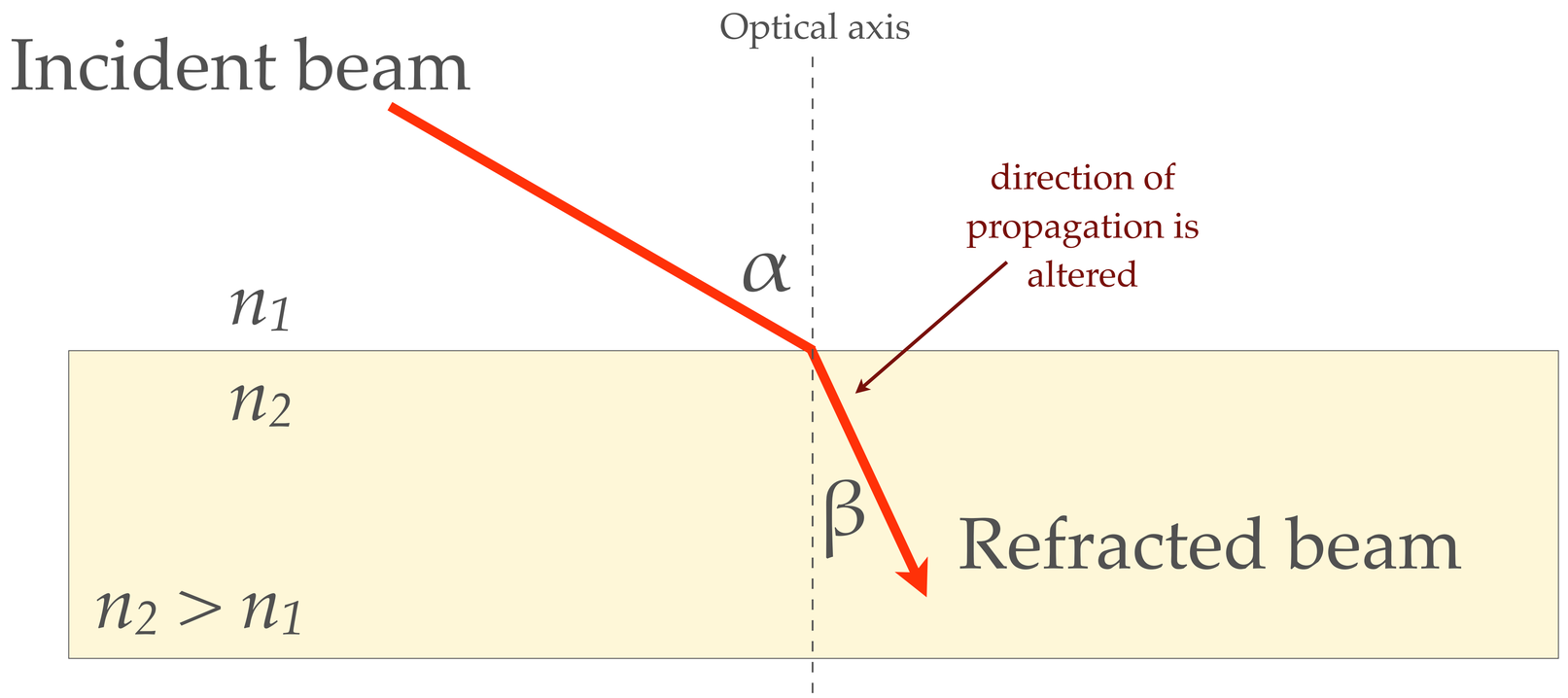
$$n_1 = \frac{c}{c_1}$$

Reflection



- α = angle of incidence; α' = angle of reflection.
- Incident beam, reflected beam and optical axis are in the same plane.
- Incident and reflected angles are identical (α=α‘).

Refraction

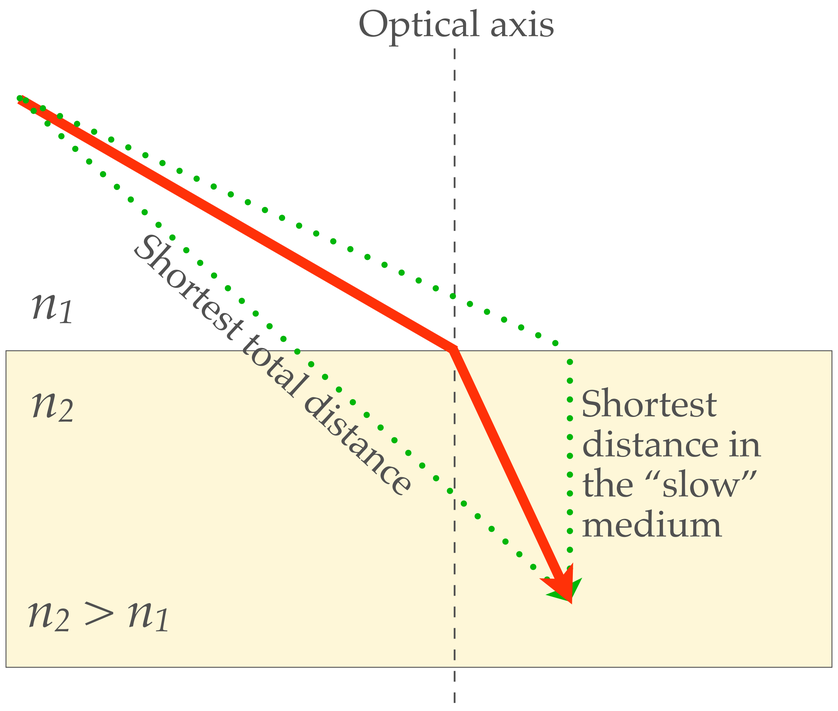


- α = angle of incidence; β = angle of refraction.
- Incident and refracted beams and axis of incidence are in the same plane.
- Snell's law:

$$\frac{\sin\alpha}{\sin\beta} = \frac{c_1}{c_2} = \frac{n_2}{n_1}$$

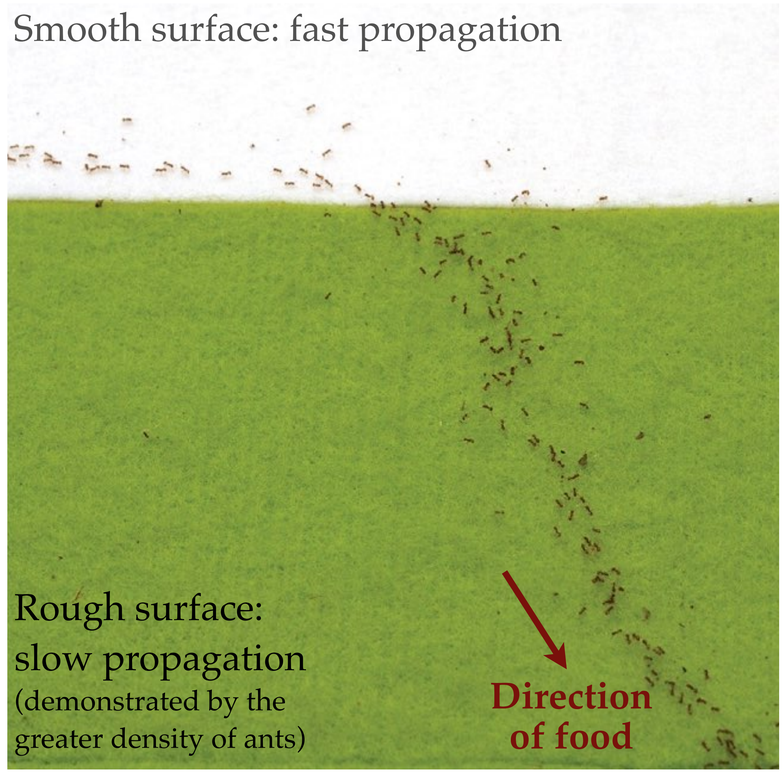
Explanation of refraction:

Fermat's principle of least times



Light "chooses" the path that can be covered in the least time (i.e., fastest).

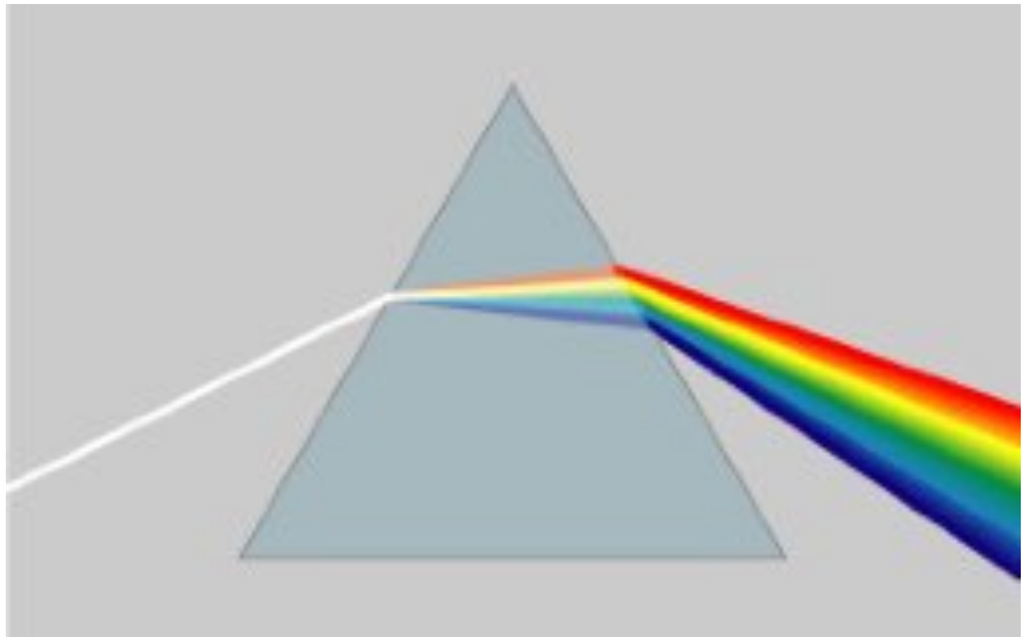
Fermat's principle is at work in other places, too!



Path "selection" by ants (*Wasmannia auropunctata*) at the boundary of media with different "resistances".

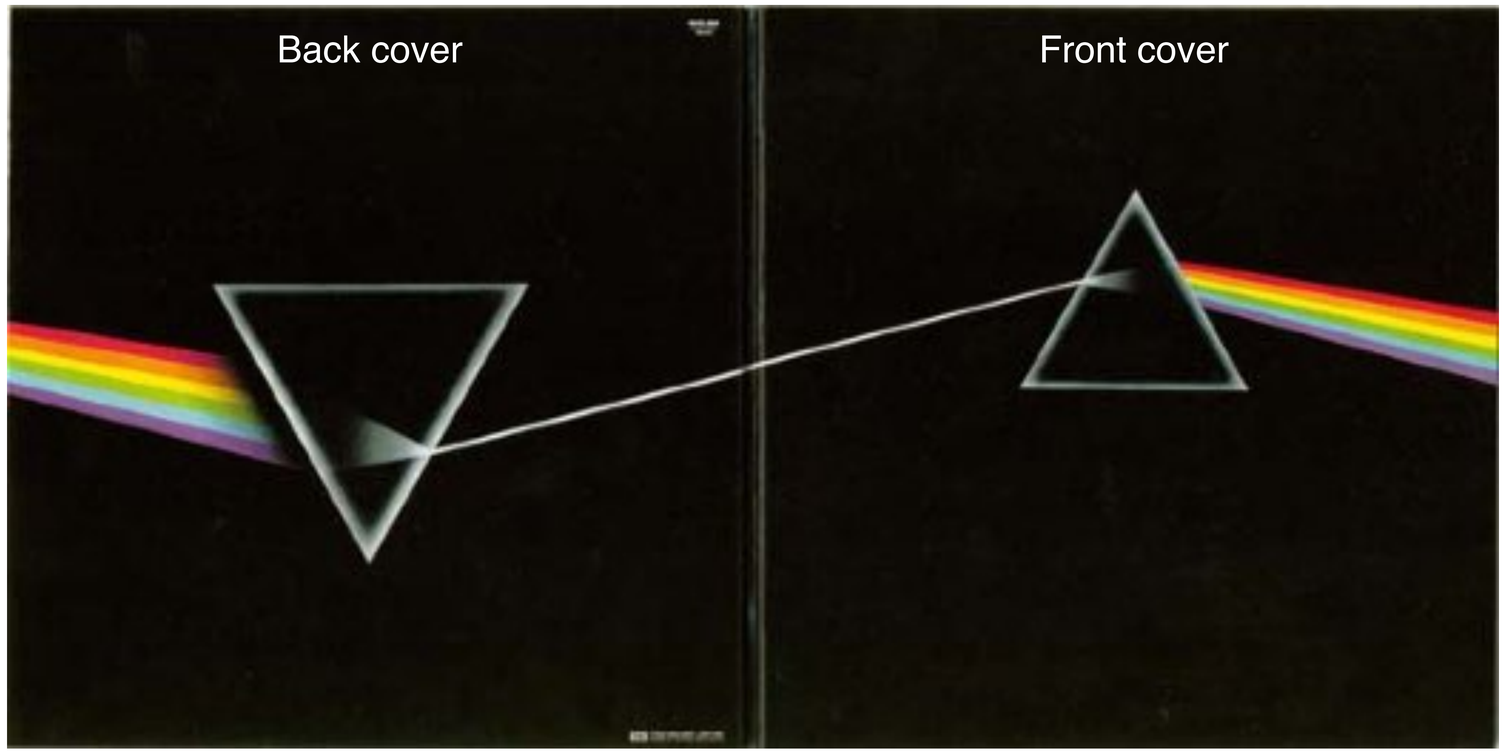
Dispersion

Index of refraction depends on frequency!



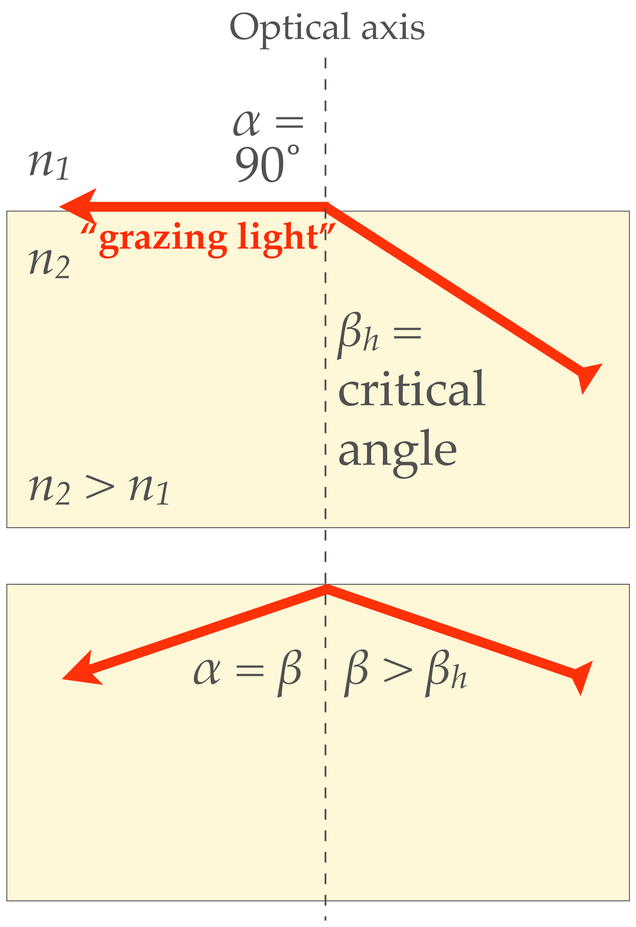
- The greater the frequency of light - the greater the refractive index.
- A prism decomposes white light according to wavelength (physical color).

Dispersion appears in interesting places...

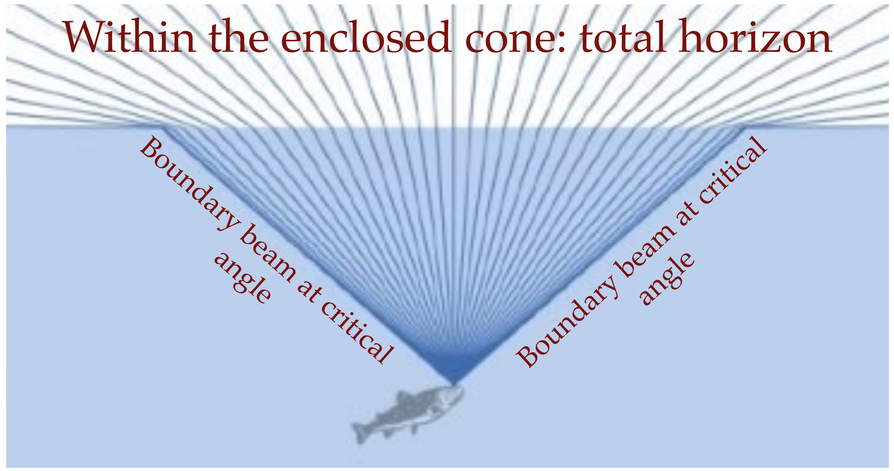


Pink Floyd: The Dark Side of the Moon

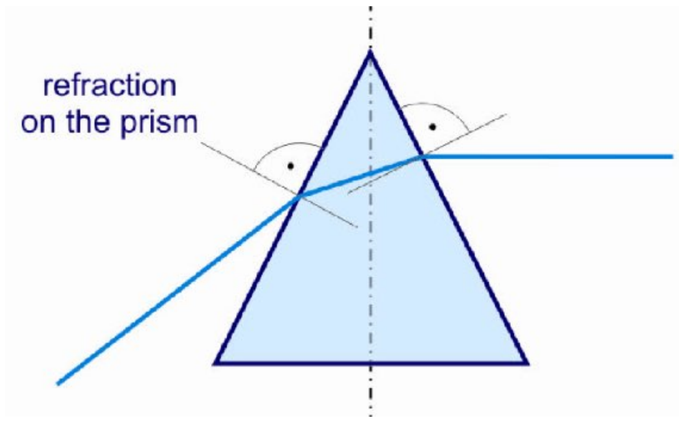
Total internal reflection

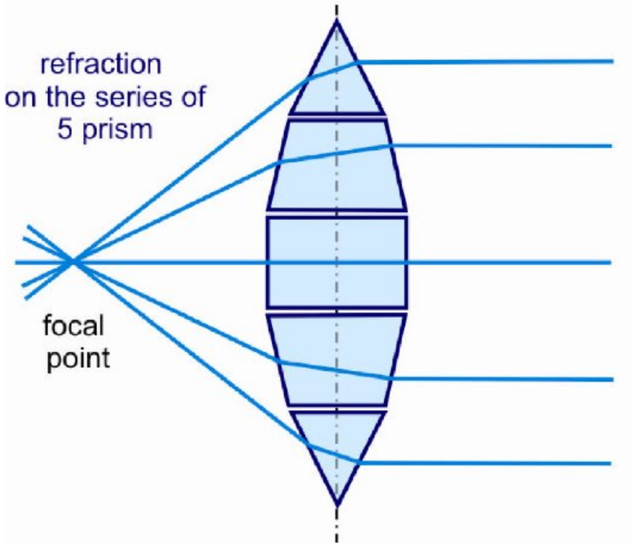


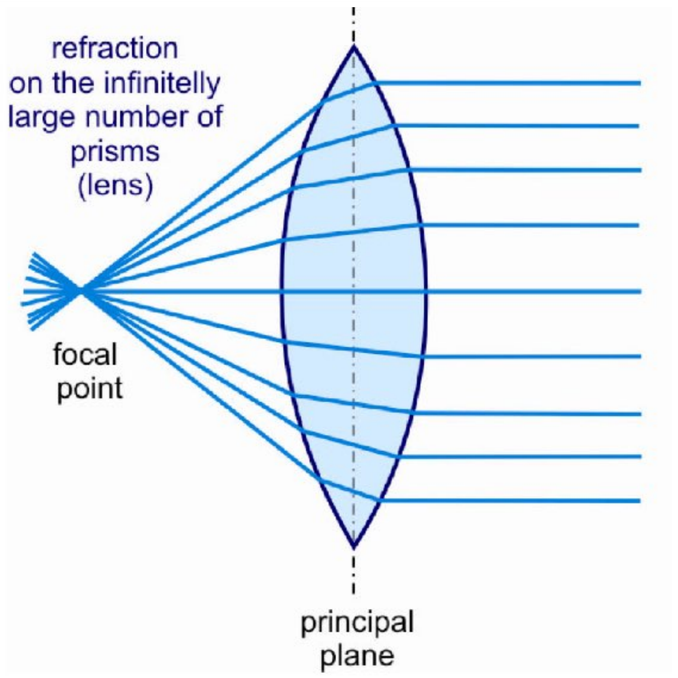
Total reflection within the optical medium of greater refractive index ("total ***internal*** reflection", TIR)



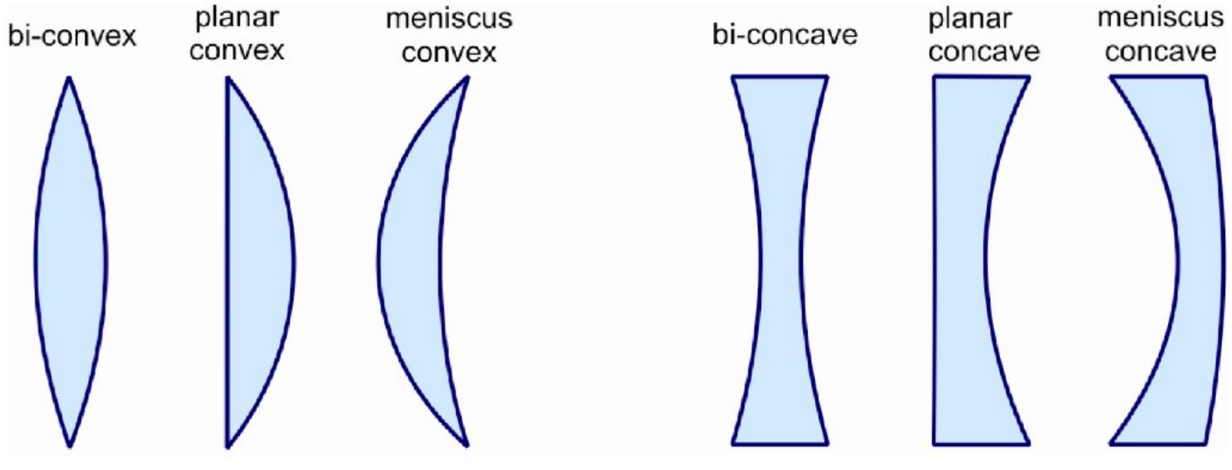
Refraction on curved surface





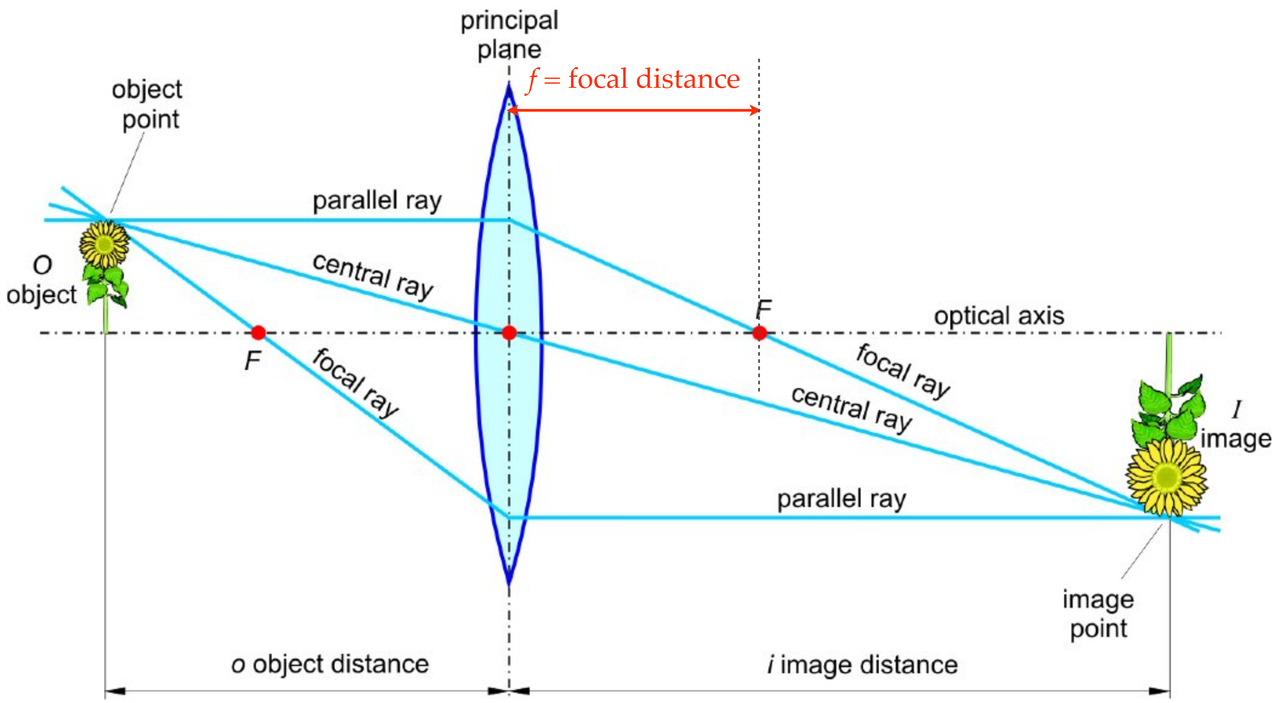


Types of lenses:



Optical imaging

Image formation may be achieved by using a curved refractile surface



- Real image: may be projected onto a surface
- Virtual image: may be mapped by using an accessory lens
- Magnification > 1, if the object is within 2*f* distance

Magnification

$$N = \frac{K}{T} = \frac{k}{t}$$

Lens equation

$$D = \frac{1}{f} = \frac{1}{t} + \frac{1}{k}$$

D=optical power (diopter, m^{-1})

Optical power of refractile surface

$$D = \frac{n - n'}{r}$$

n-n'=difference between the refractive indeces of optical media
r=radius of curvature of refractile surface

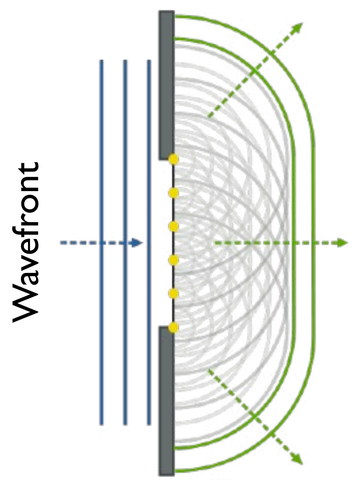
Wave phenomena I.

Diffraction

Huygens-Fresnel principle:
every point of a wavefront is the source of further waves

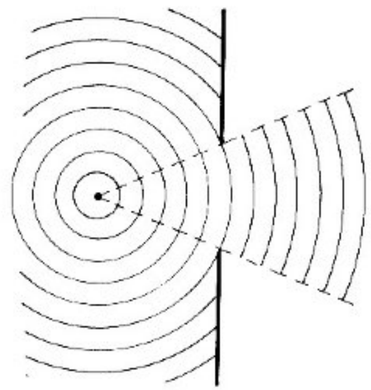
Christiaan **Huygens**
(1629-1695)

Augustin-Jean **Fresnel**
(1788–1827)

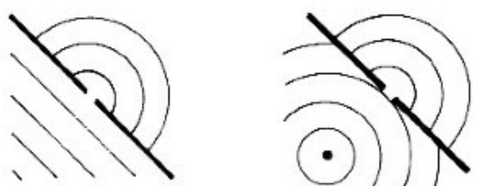


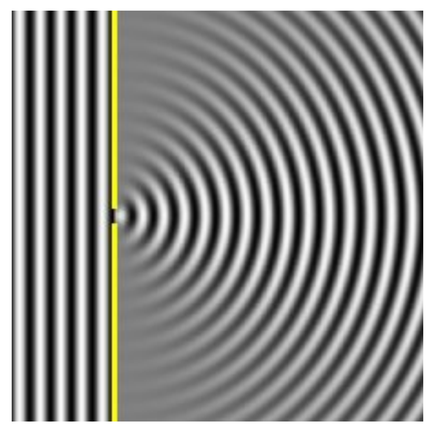
The wave appears in
the "shaded" areas, too.

Slit much greater
than the wavelength
(λ)



Slit much smaller than
wavelength (λ)





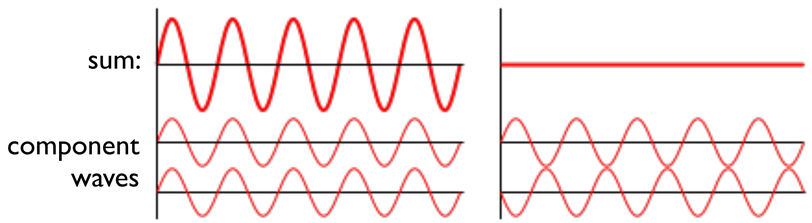
Wave phenomena II.

interference

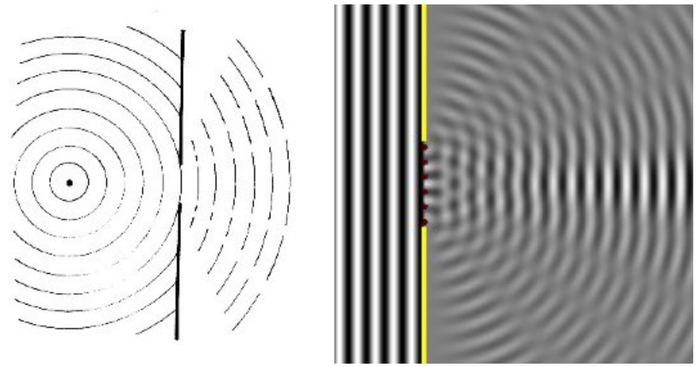
Principle of superposition

Waves in phase (ϕ=0): amplification

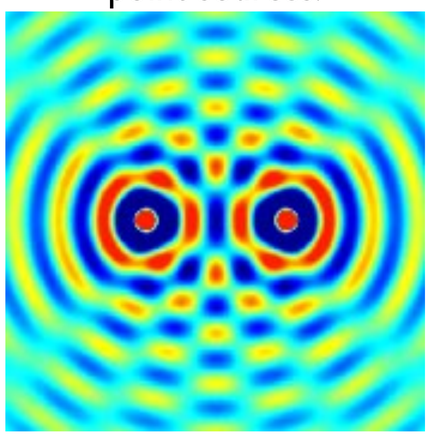
If ϕ=π : cancellation



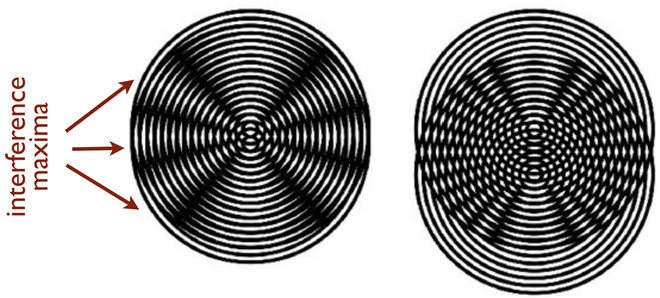
Slit comparable to wavelength
(=pointlike slits separated by distance *d*, where *d*~λ)



Interference of waves emerging from two point sources.



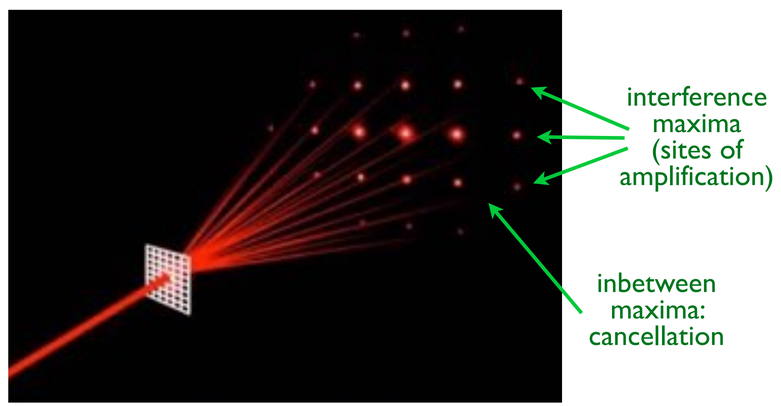
Interference pattern depends on distance (*d*) separating the pointlike slits



small d ~ large distance between maxima

large d ~ small distance between maxima

Diffraction pattern of a 2D optical grating

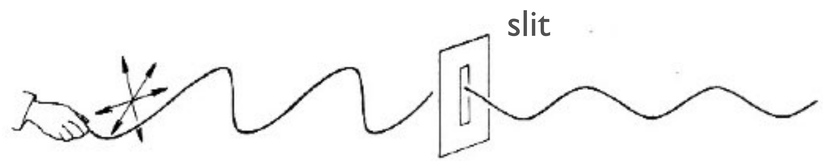


Wave phenomena III.

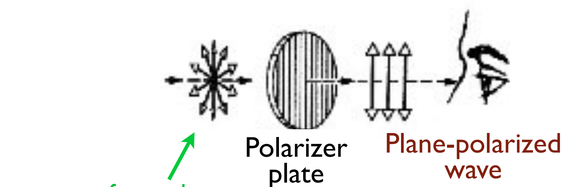
Polarization

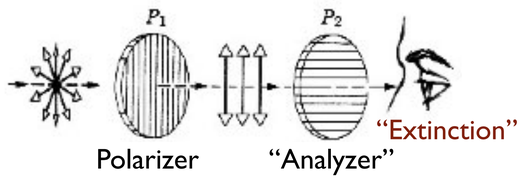
- **Polarization:** oscillation is oriented in some *preferred* direction
- **Birefringence** is related to polarization: anisotropic propagation velocity
- Only *transverse* waves can be polarized.

Polarization of Mechanical waves

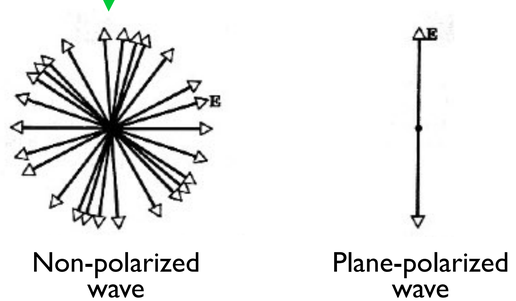


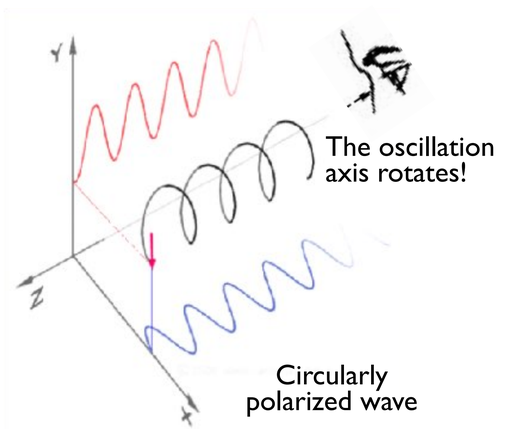
Polarization of Electromagnetic waves



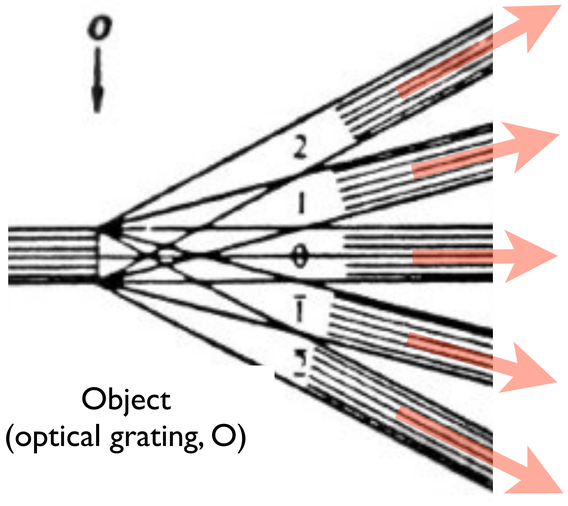


Polarization can be understood by observing the head-on view of the wave:





Resolving power of the light microscope is limited by wave optics

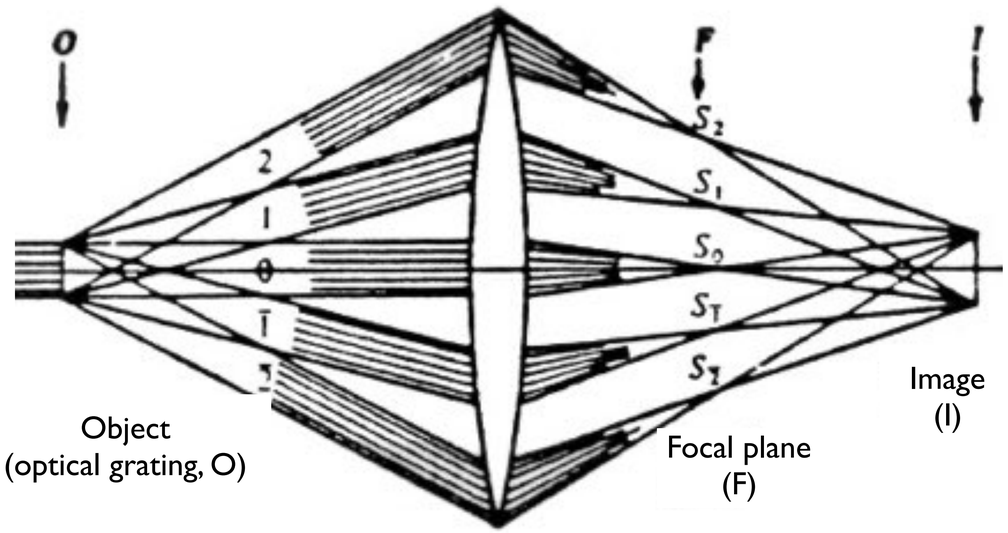


Object
(optical grating, O)

Diffraction maxima: structural information

Principal maximum: light-absorption information
(exponential decay)

Diffraction-limited imaging: its condition is the collection of at least the first diffraction maximum



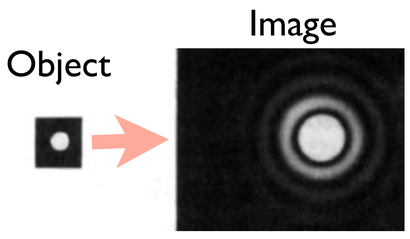
Object
(optical grating, O)

Focal plane
(F)

Image
(I)

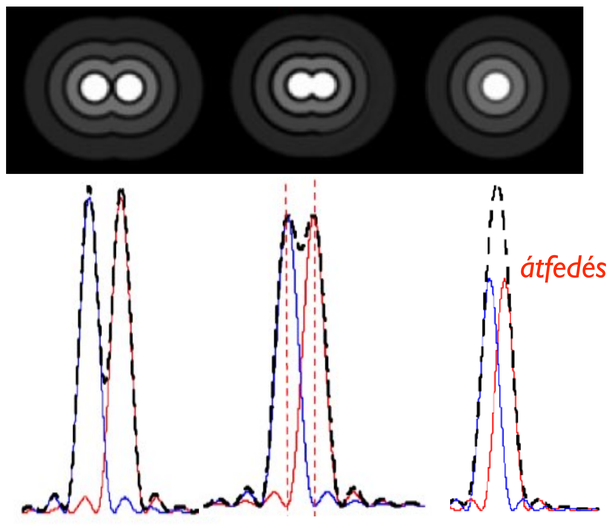
Object

Image



Because of diffraction: the image of a point object is a disk (Airy disk)

Rayleigh criterion



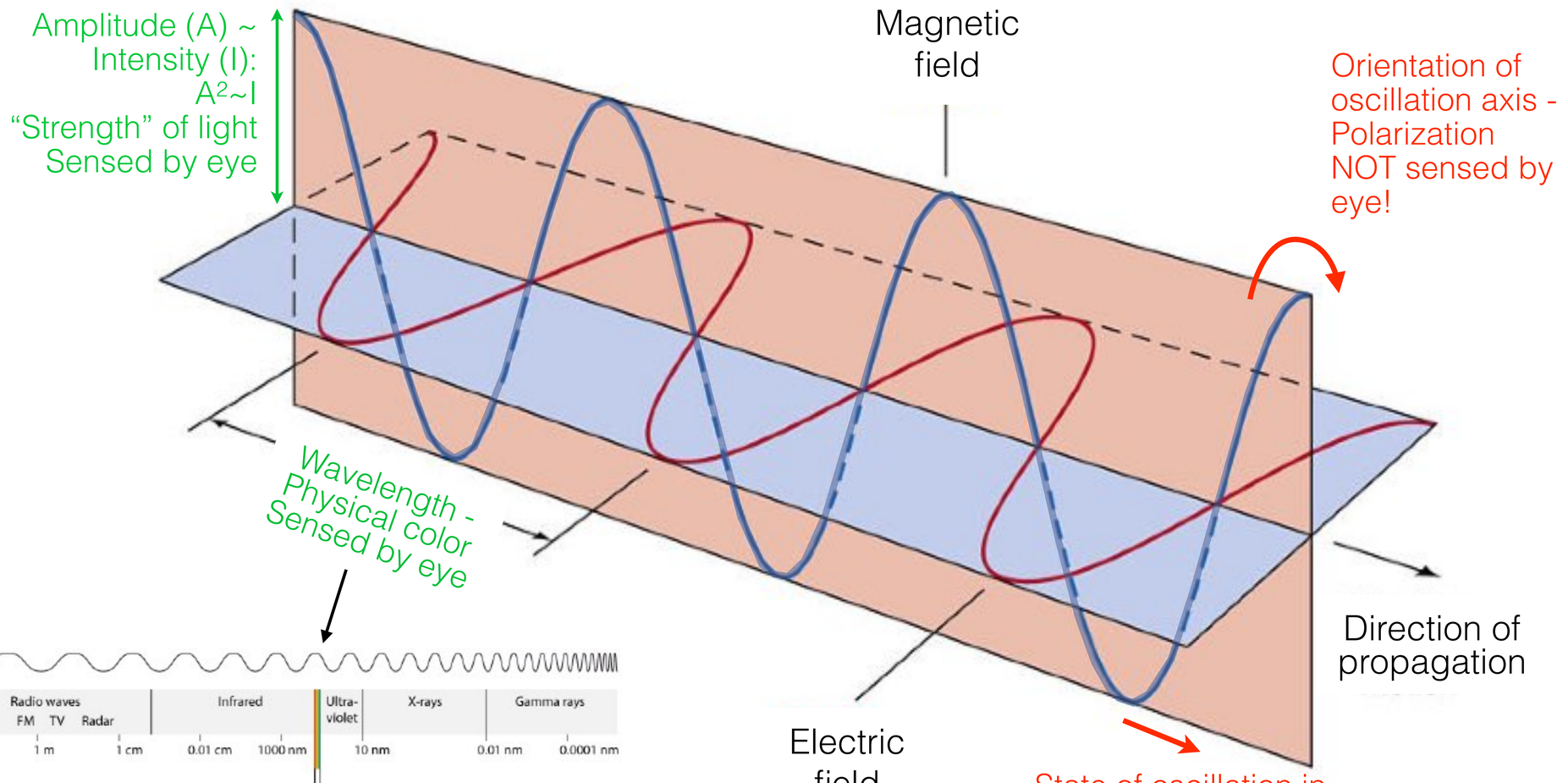
Smallest resolved distance (Abbé-equation):

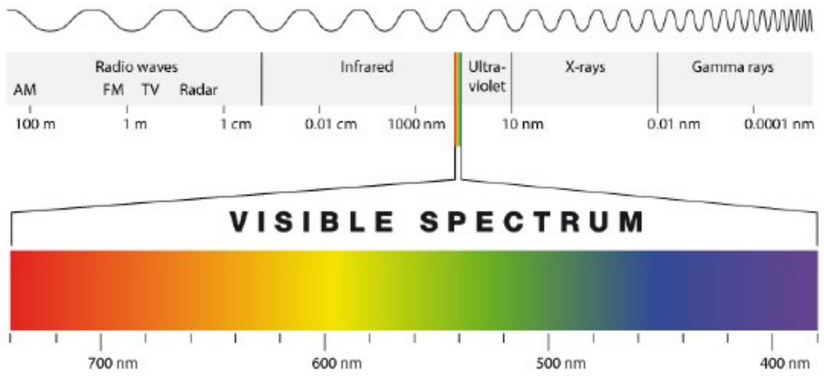
$$d = \frac{0.61\lambda}{n \sin\alpha}$$

λ = wavelength
n = refractive index of immersion medium
α = angle between optical axis and the bounding light ray

Detectable parameters of light

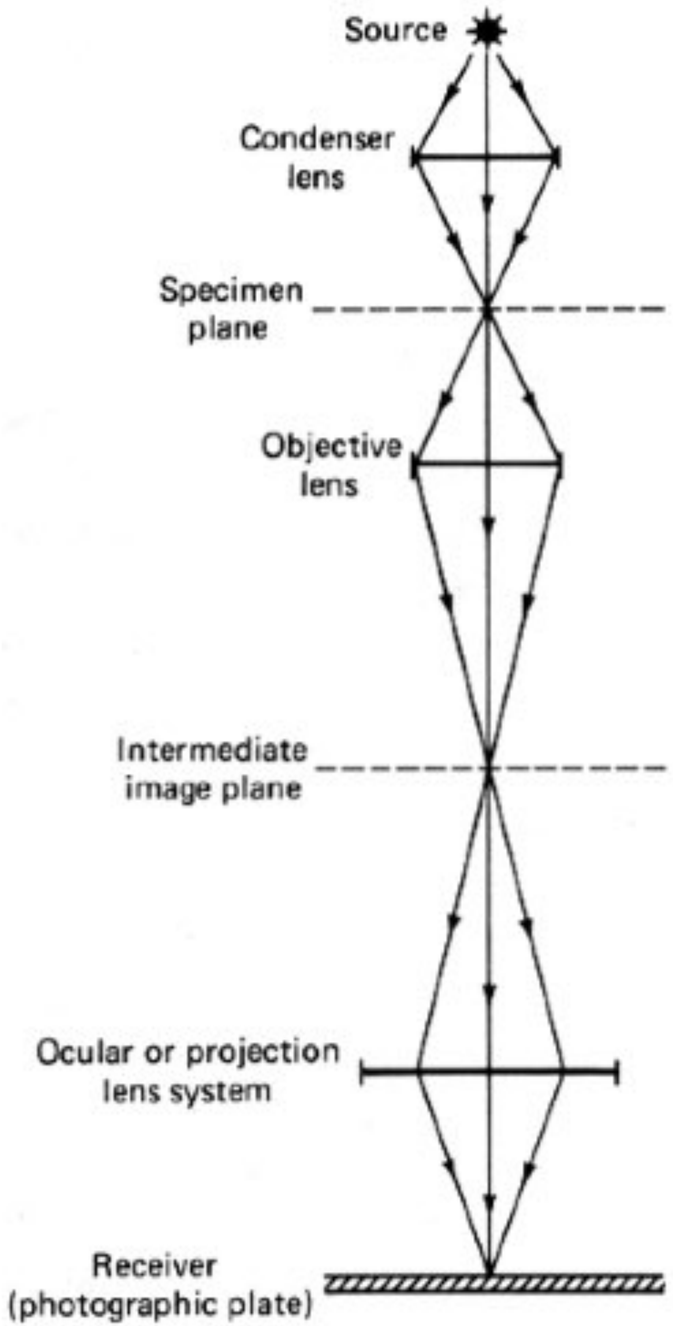
Electromagnetic (transverse) wave





- Eye senses: wavelength and amplitude (~intensity)
- Eye is insensitive to: phase and polarization

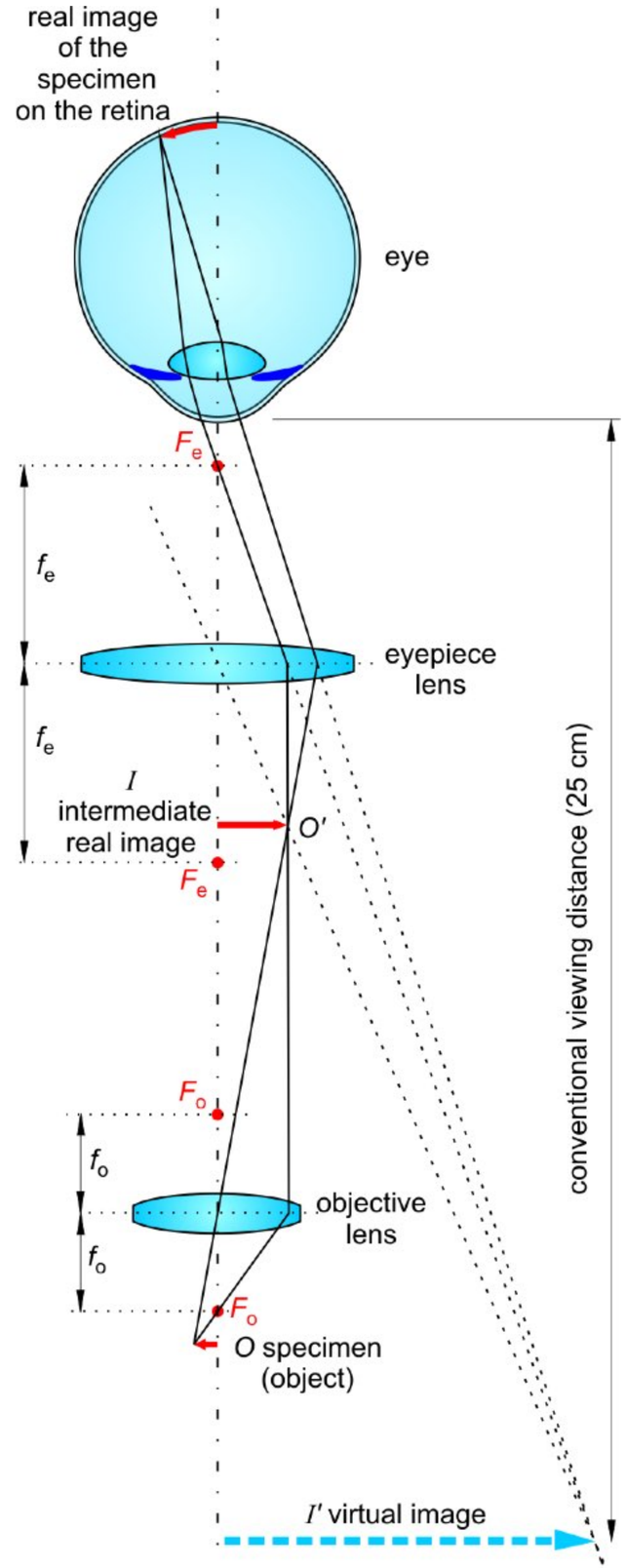
Schematics of the microscope



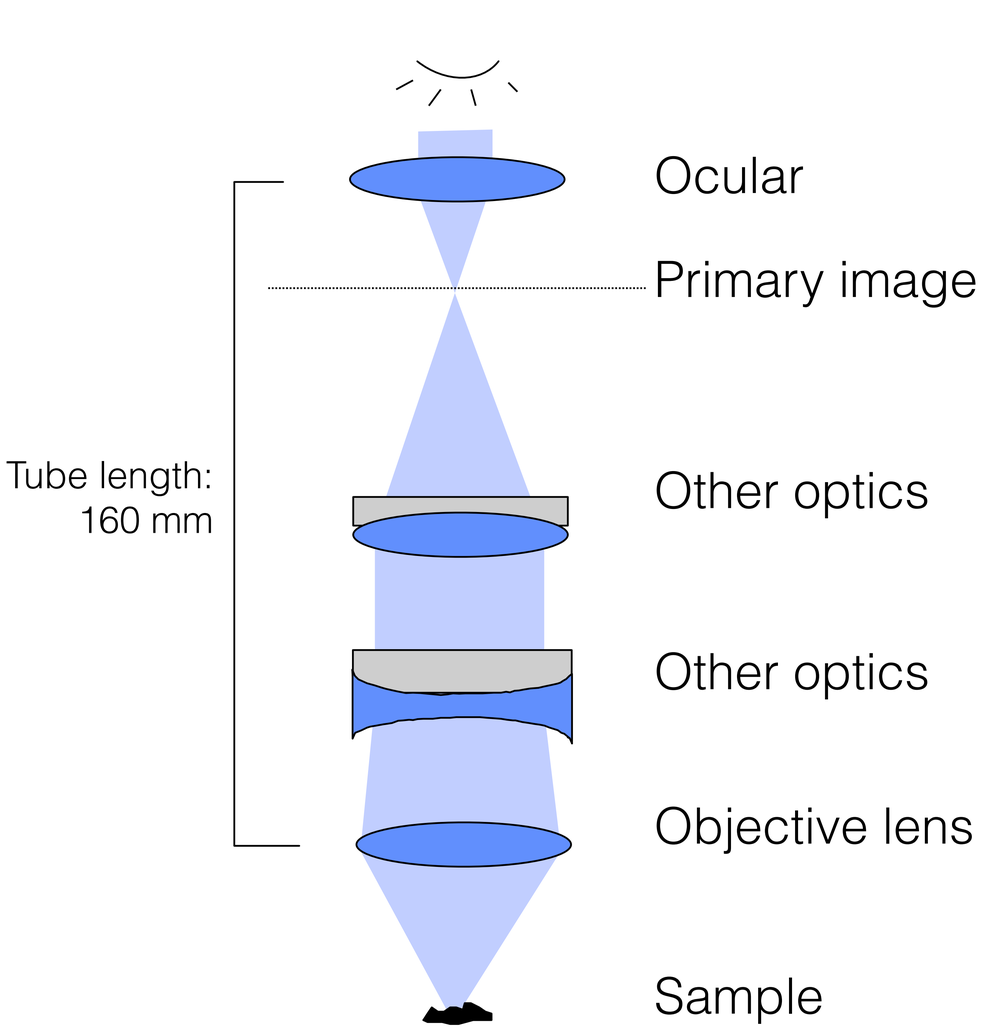
ILLUMINATOR	LIGHT SOURCE COLLECTOR FIELD DIAPHRAGM	
CONDITIONER	CONDENSER IRIS POLARIZER NOMARSKI PRISM FLUORESCENCE EXCITATION FILTER	PHASE ANNULUS DARK-FIELD ANNULUS APERTURE MASK
CONDENSER	Focal length Numerical aperture Aberration Transmission	
SPECIMEN	Slide thickness/immersion Coverslip thickness/immersion Absorb, diffract, fluoresce, retard, etc. Azimuth	
OBJECTIVE LENS	Magnification/focus Numerical aperture Aberration Transmission OTF	
IMAGE FILTER	NOMARSKI PRISM COMPENSATOR ANALYZER FLUORESCENT BARRIER FILTER	OBJECTIVE IRIS PHASE PLATE SSEE FILTER
OCULAR	Magnification Aberration Field size Eye point	
RECEIVER	EYE PHOTOGRAPHIC EMULSION PHOTOMULTIPLIER PHOTODIODE ARRAY VIDEO CAMERA	

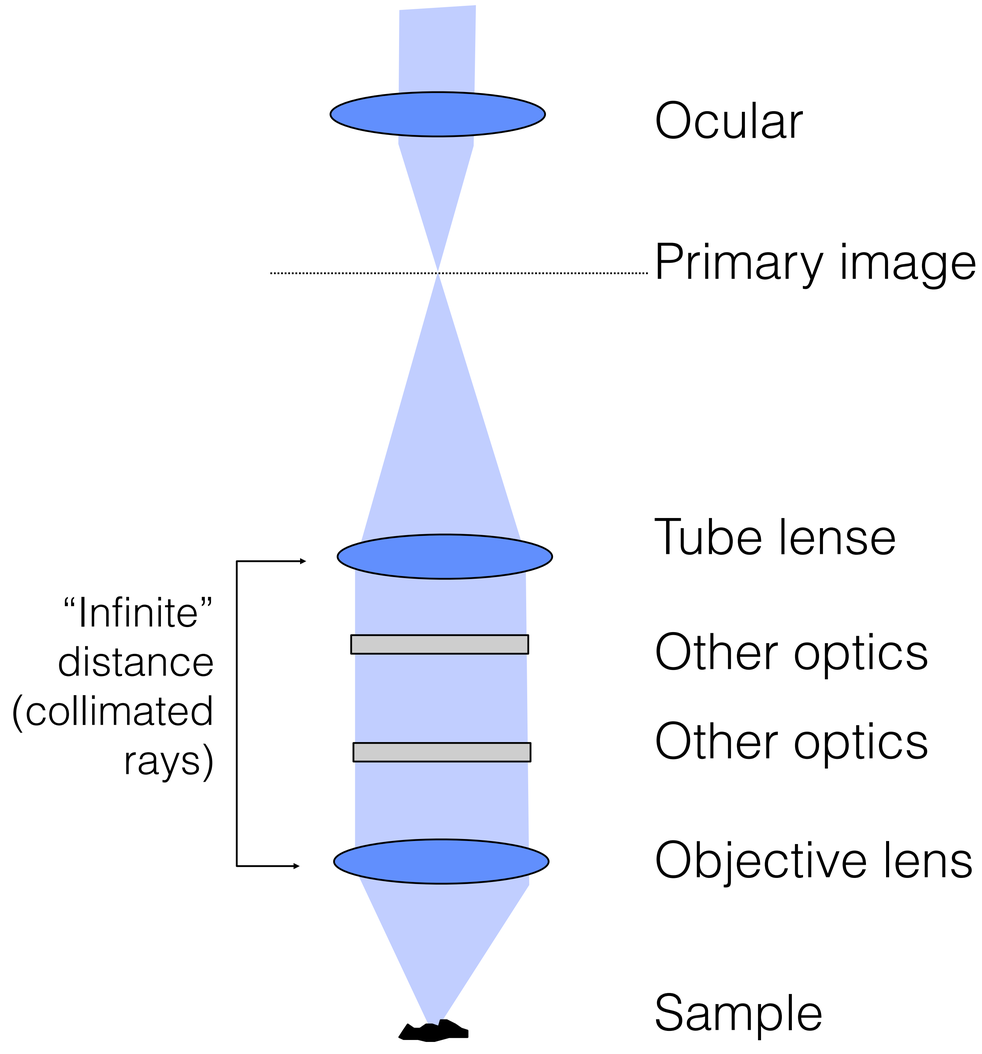
Image formation in the compound microscope

- Magnified, up-side-down, virtual image
- Condition of the formation of projected image: an accessory lens (eye lens) needs to be positioned in the optical path.
- Projection screen: retina

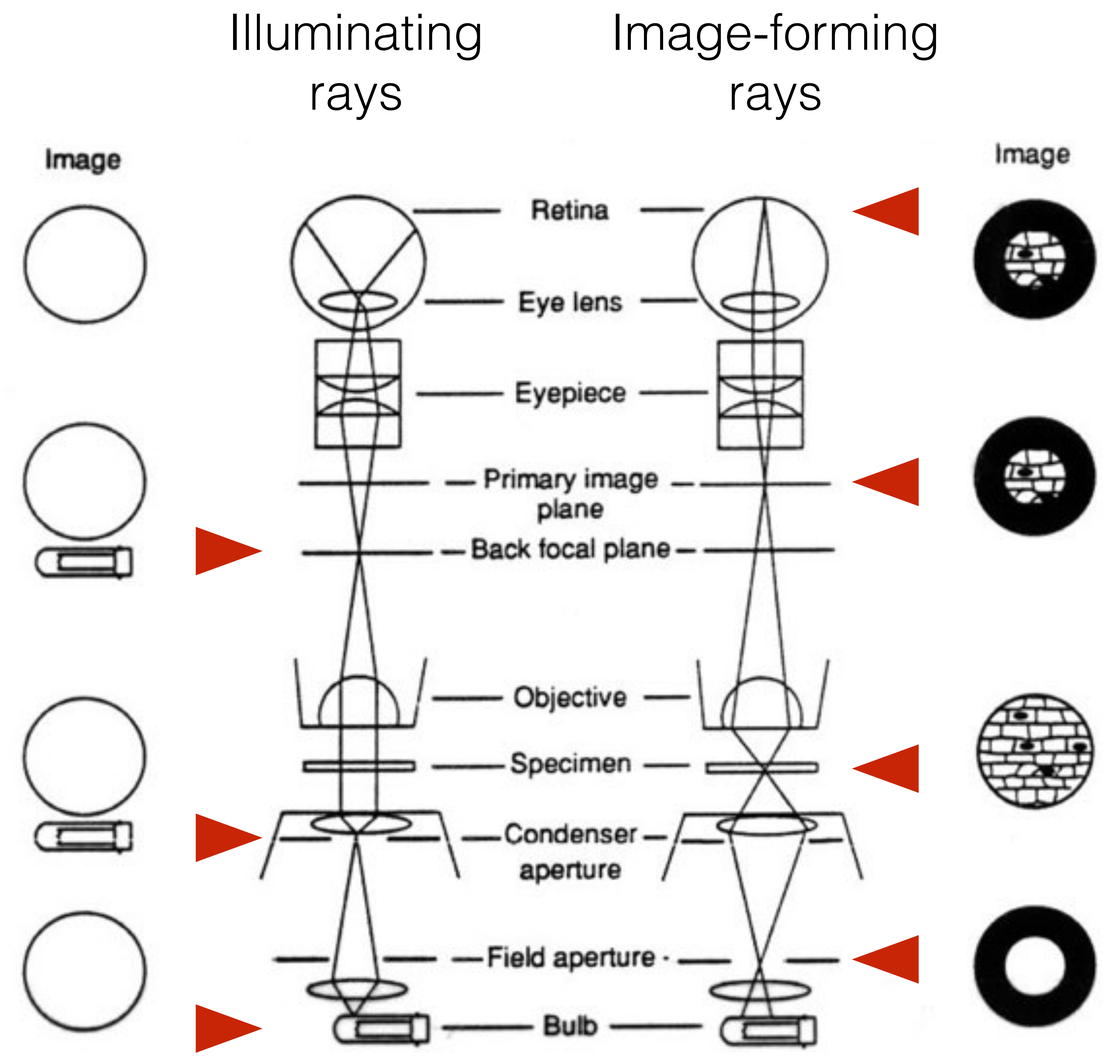


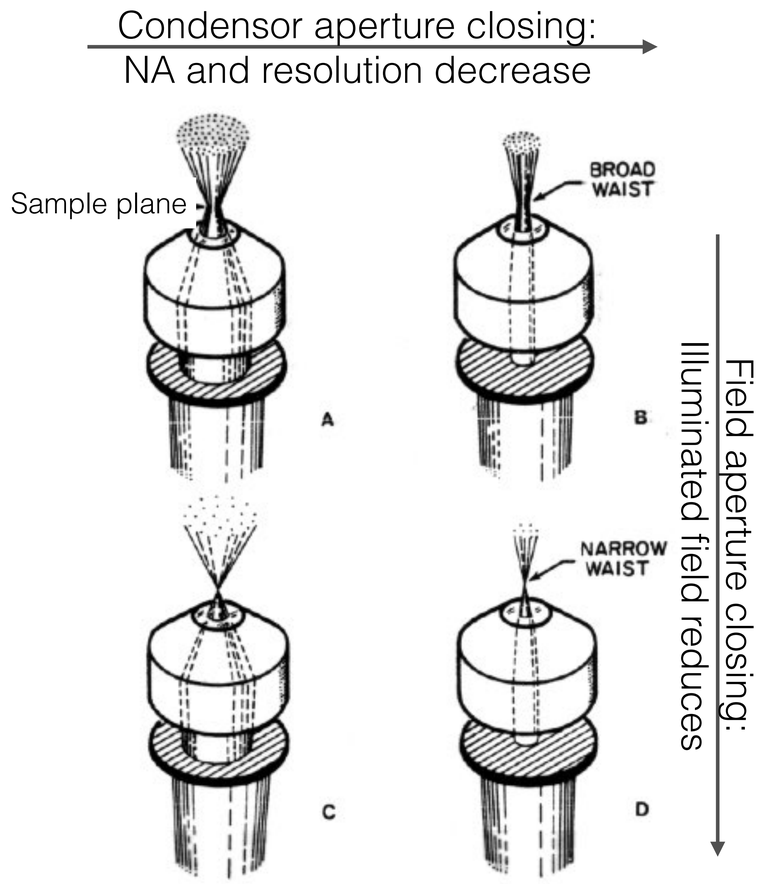
Ordinary vs. infinity-corrected microscope optics





Köhler Illumination



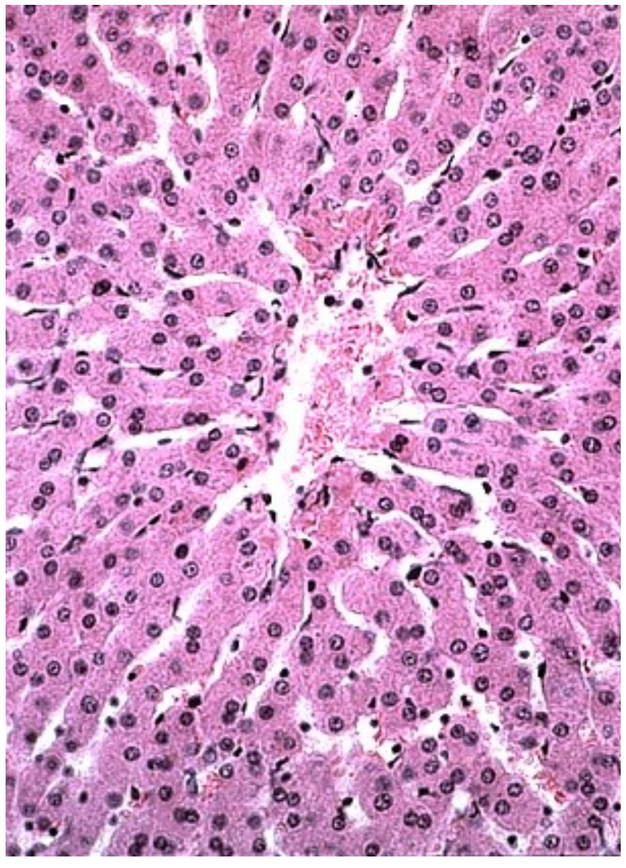


Conjugate planes!

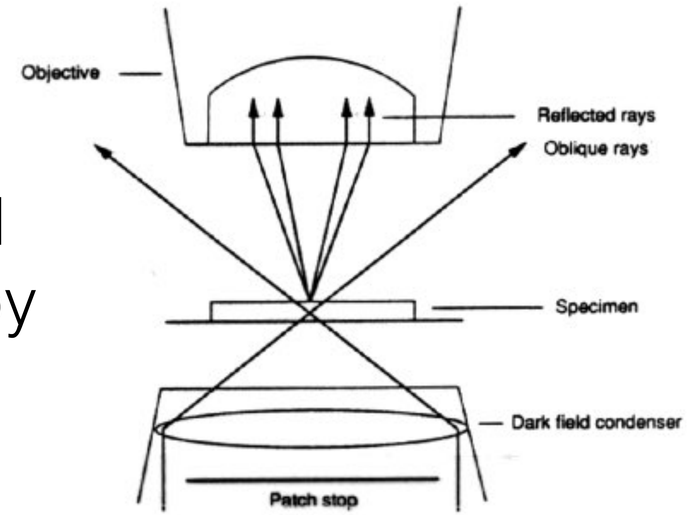
Contrast mechanisms I.

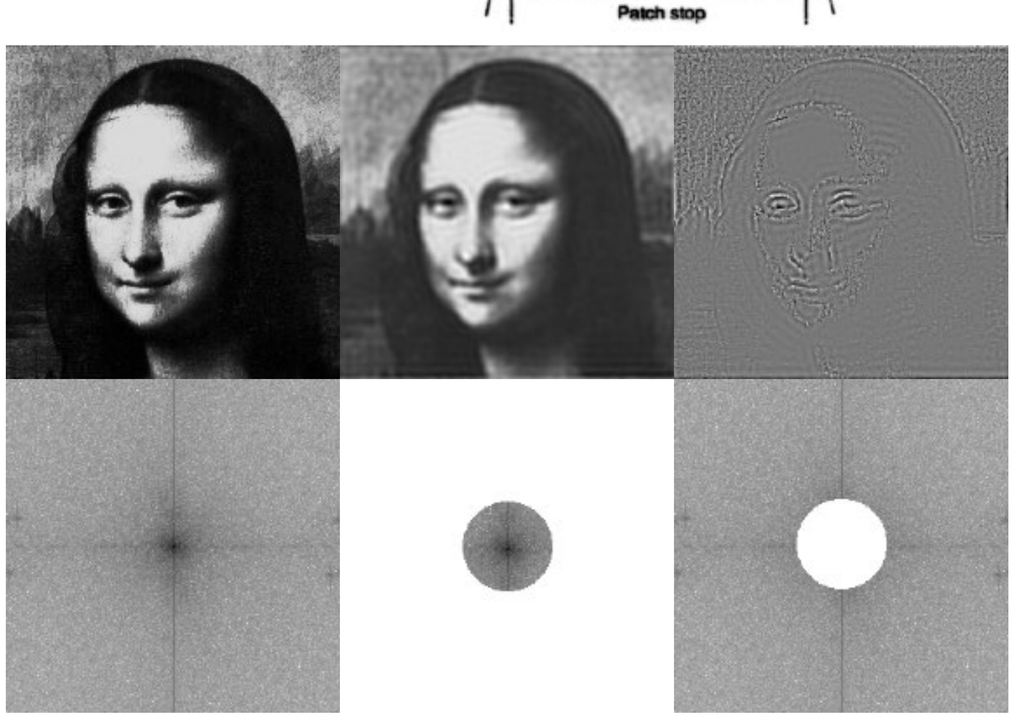
Absorption contrast

Stained sample (e.g., hematoxylin-eosine)



Dark field microscopy



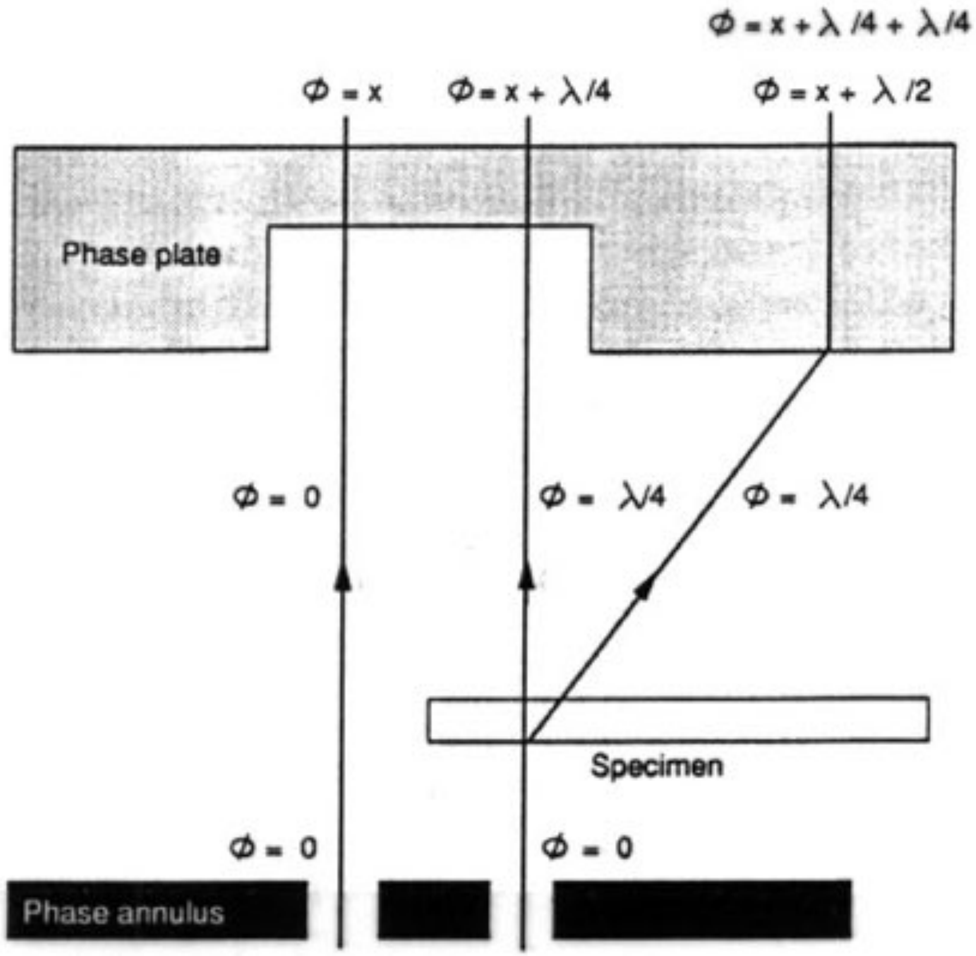


Contrast mechanisms II.

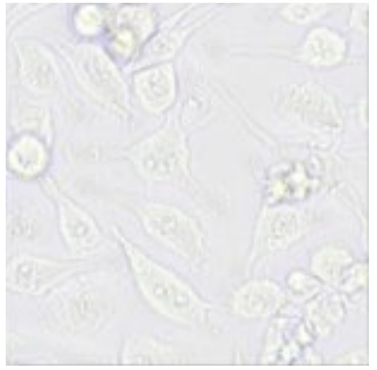
Phase contrast microscopy

Frits Zernike
(1888-1966)
Nobel-prize

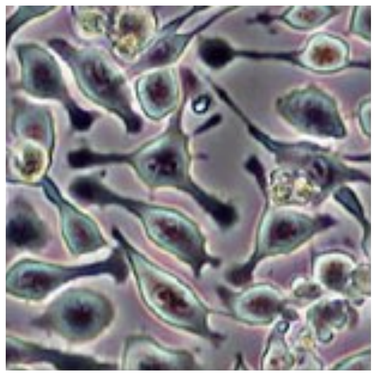
- **Phase**: shows the state of vibration within the entire period (2π).
- Expressed with the **phase angle** (ϕ).
- Phase difference between waves: **phase shift** (retardation or acceleration)



Live (unstained) cells



Bright-field
microscopic image

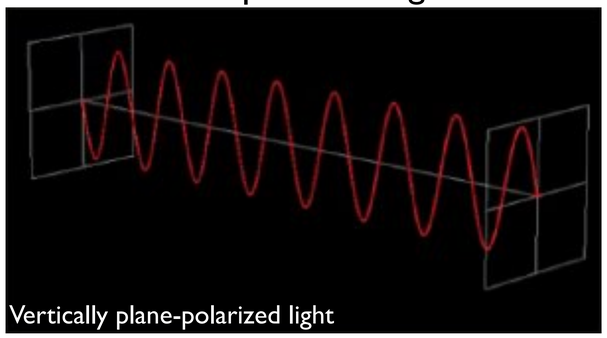


Phase-contast
microscopic image

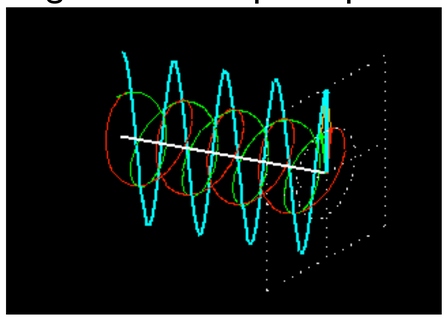
Contrast mechanisms III.

Polarization microscopy

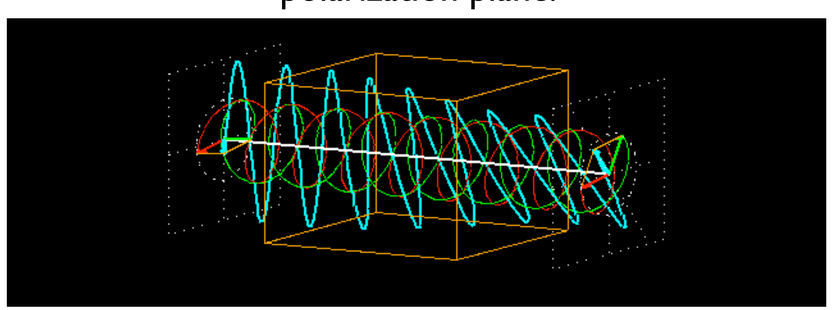
Plane-polarized light

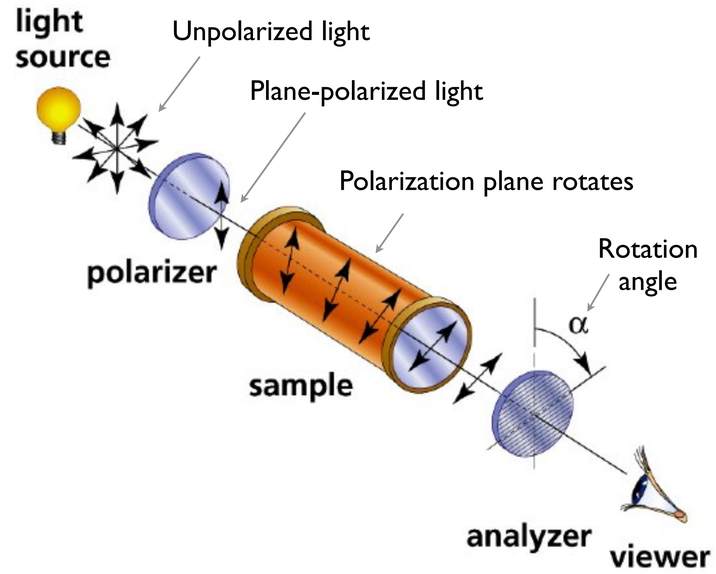


Superposition of right and left circularly polarized light results in plane-polarized light:

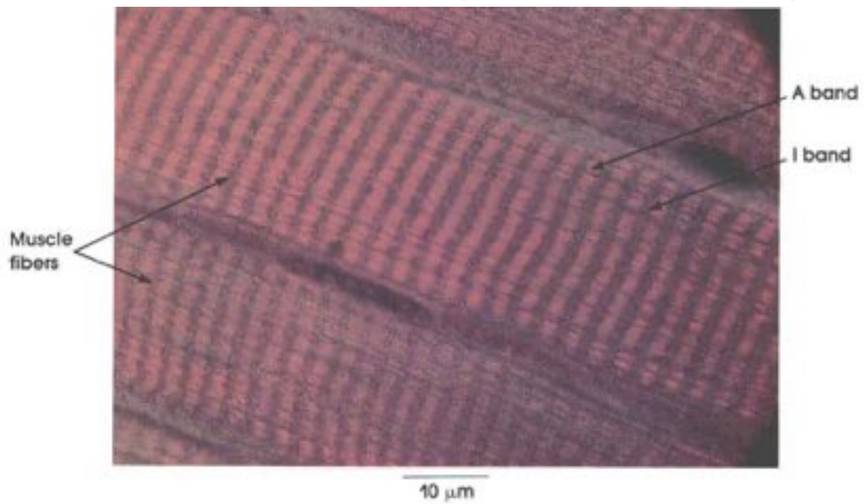


In anisotropic medium the relative phase shift of the different circularly polarized components results in the rotation of the polarization plane:





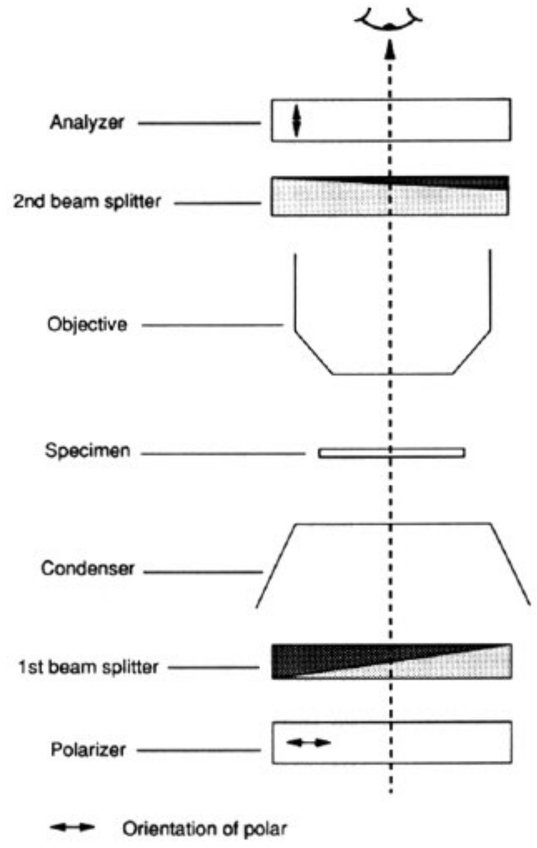
Striated muscle fiber in the polarization microscope

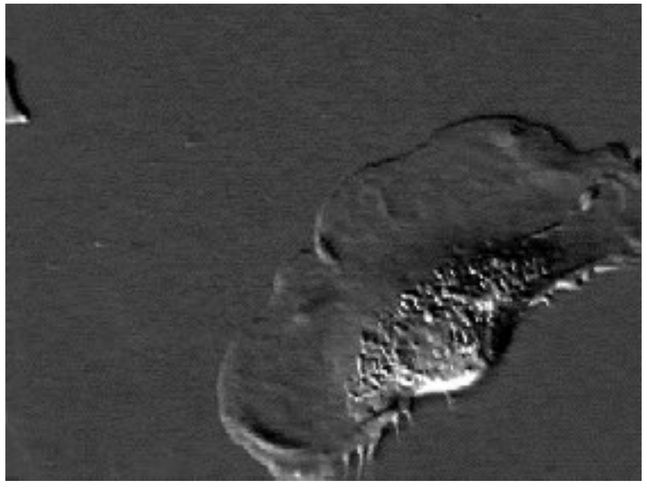


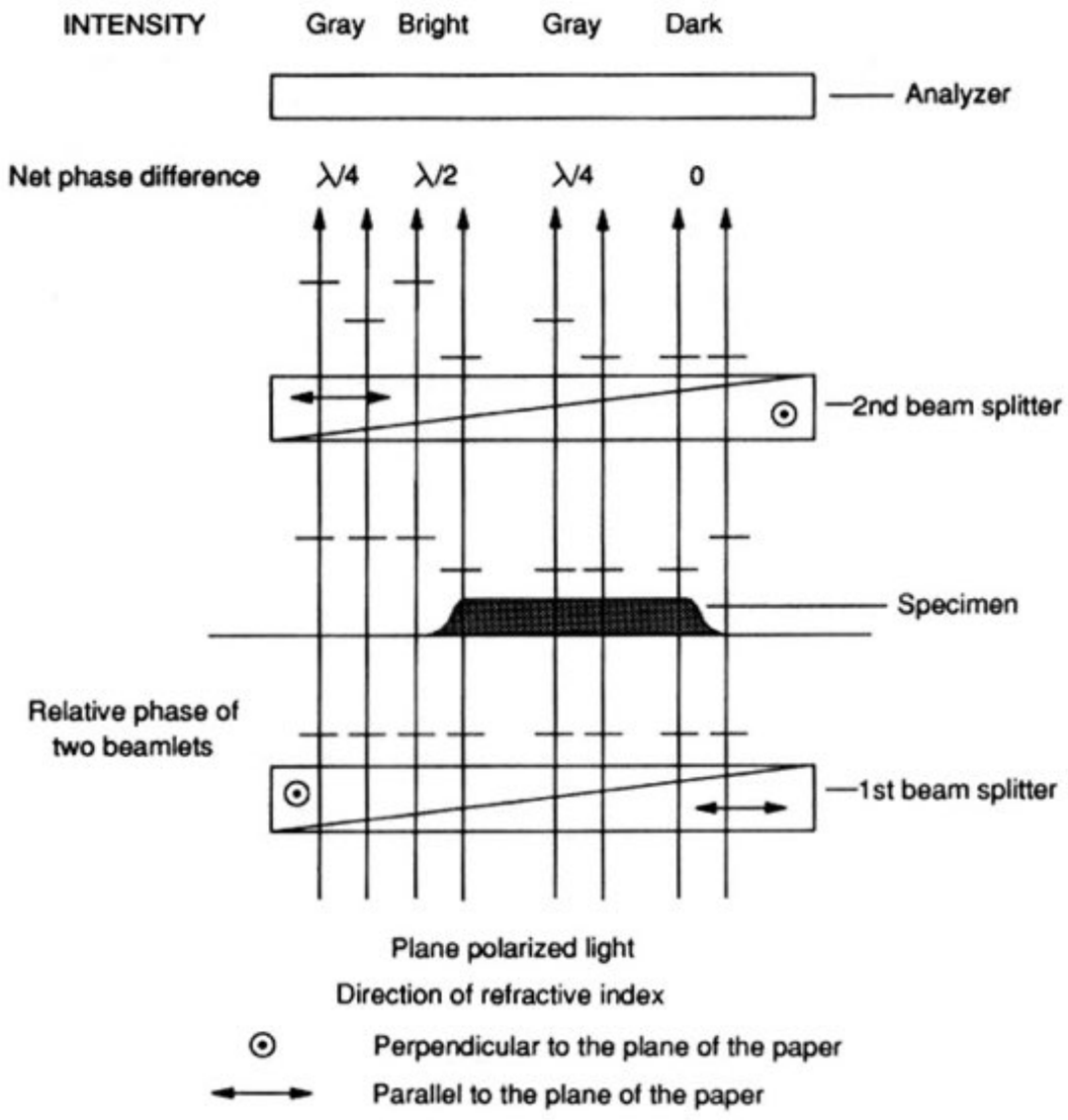
- A-band: anisotropic (birefringent)
- I-band: isotropic

Contrast mechanisms IV.

Differential Interference Contrast (DIC, Nomarski) Microscopy



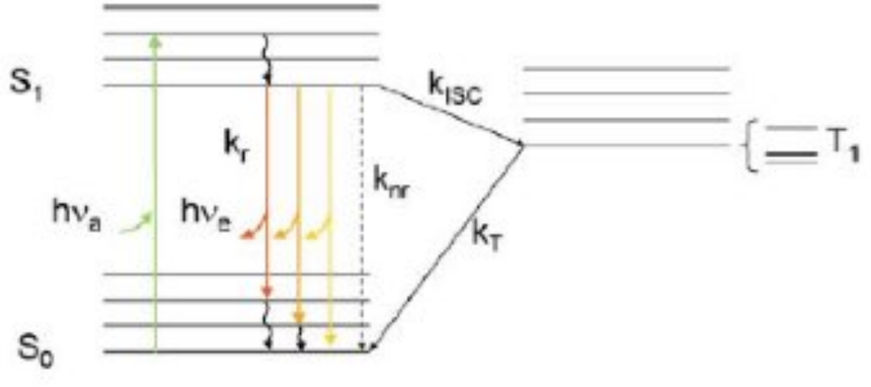




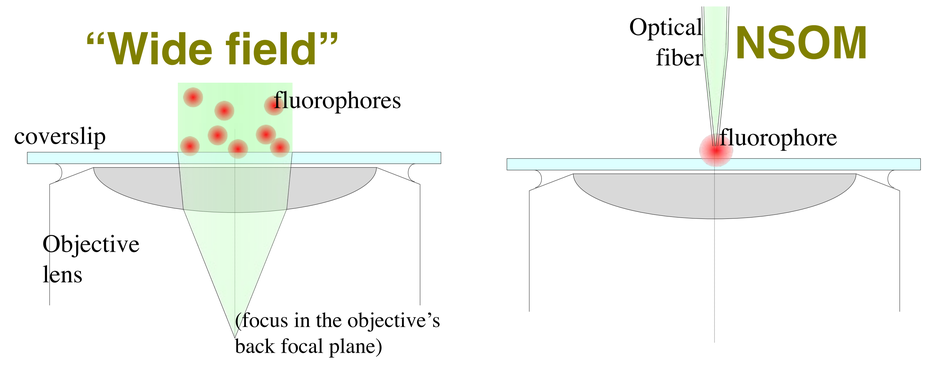
Cotrast mechanisms V.

Fluorescence

Fluorescence transitions

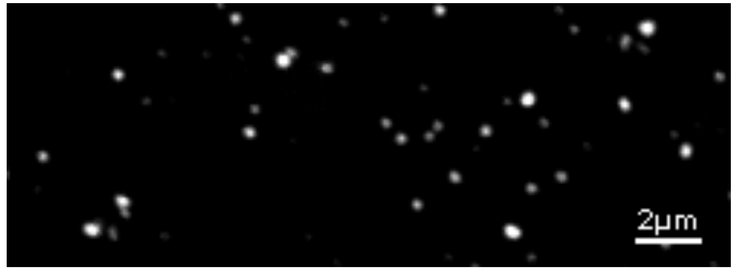


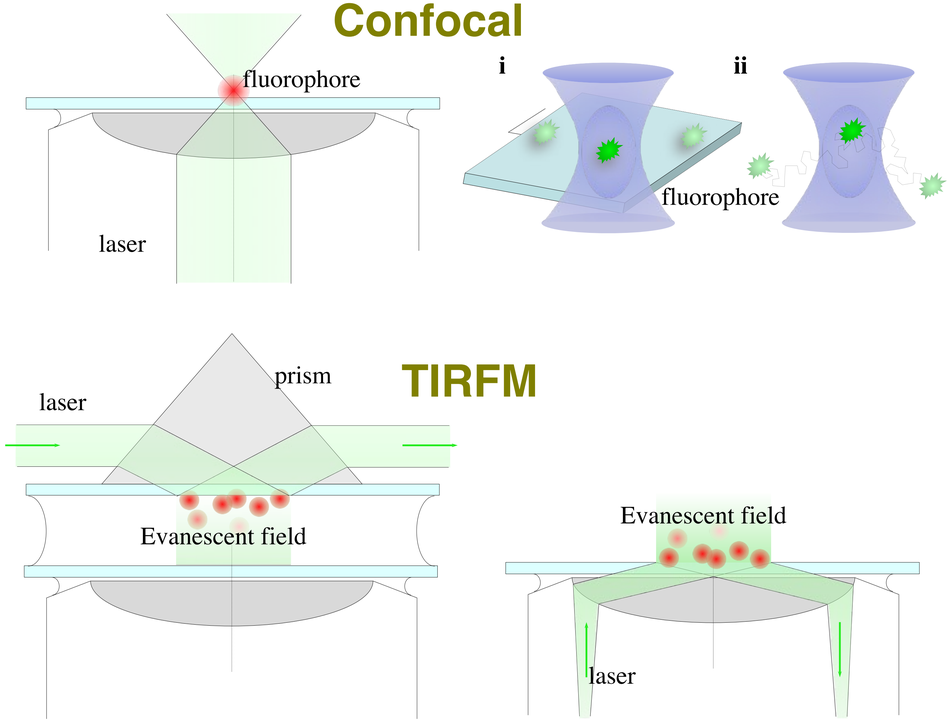
Excitation geometries



Time res.: ns
Spatial res.: diffraction limited (but FRET: nm)

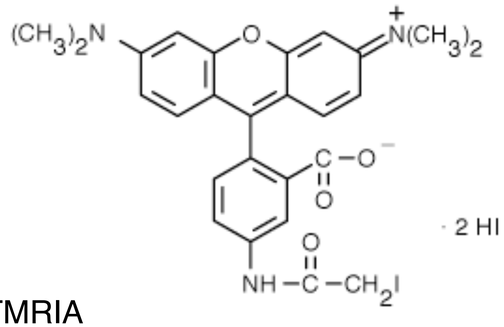
TRITC-labeled titin molecules





Source of fluorescence I.

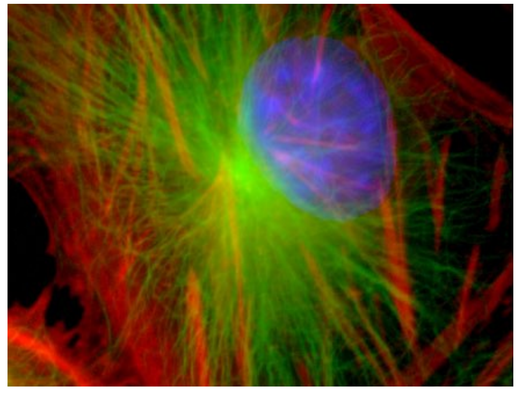
1. Native side-chain labeling



TMRIA

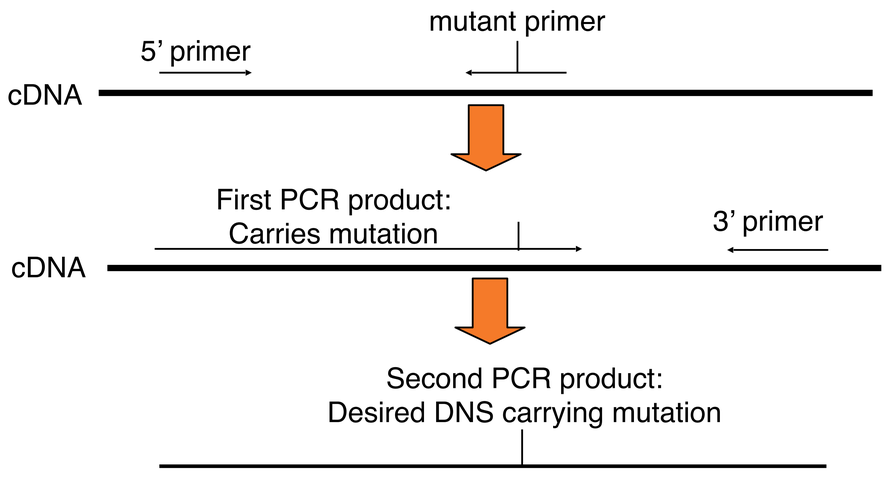
Fluorophore: Dye molecule + Chemical cross/linker

Relative chemical specificity (SH, NH_2)
Relative spatial specificity
Labeling steps: calculation of molar ratio, incubation, removal of unbound dye

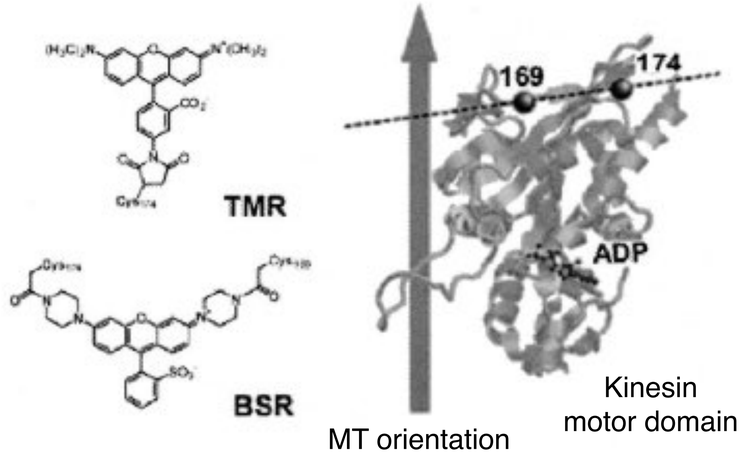


2. Targeted point mutagenesis

Targeted placement of cysteine residues



Special case:
Bifunctional fluorophore



Source of fluorescence II.

3. Conjugation with fluorescent proteins

a. Green Fluorescent Protein, GFP

Size, structure: ~27 kDa, 238 aa, 11-strand ß-barrel

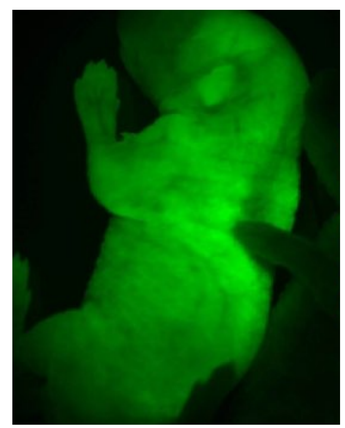
Chromophore: Ser65-Tyr66-Gly67 side chains of central ß-strand

Fluorescence depends on intact 3D structure

Tandem fusion constructs: genes of GFP and protein of interest

Advantages: *in vivo* measruements, spectral variants of GFP mutants.

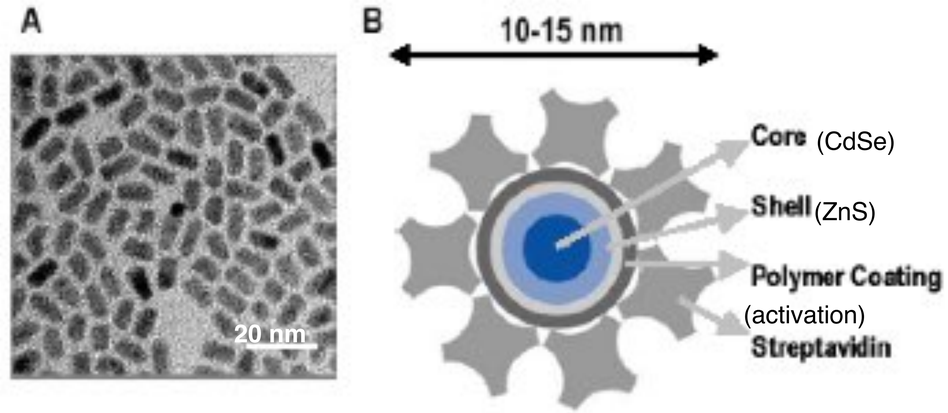
Disadvantages: blinking, terminal labeling, interference with target protein.



b. Photoactivated GFP analogue

c. Kaede: fluorescent protein from coral, shows UV-induced greed-red photoconversion

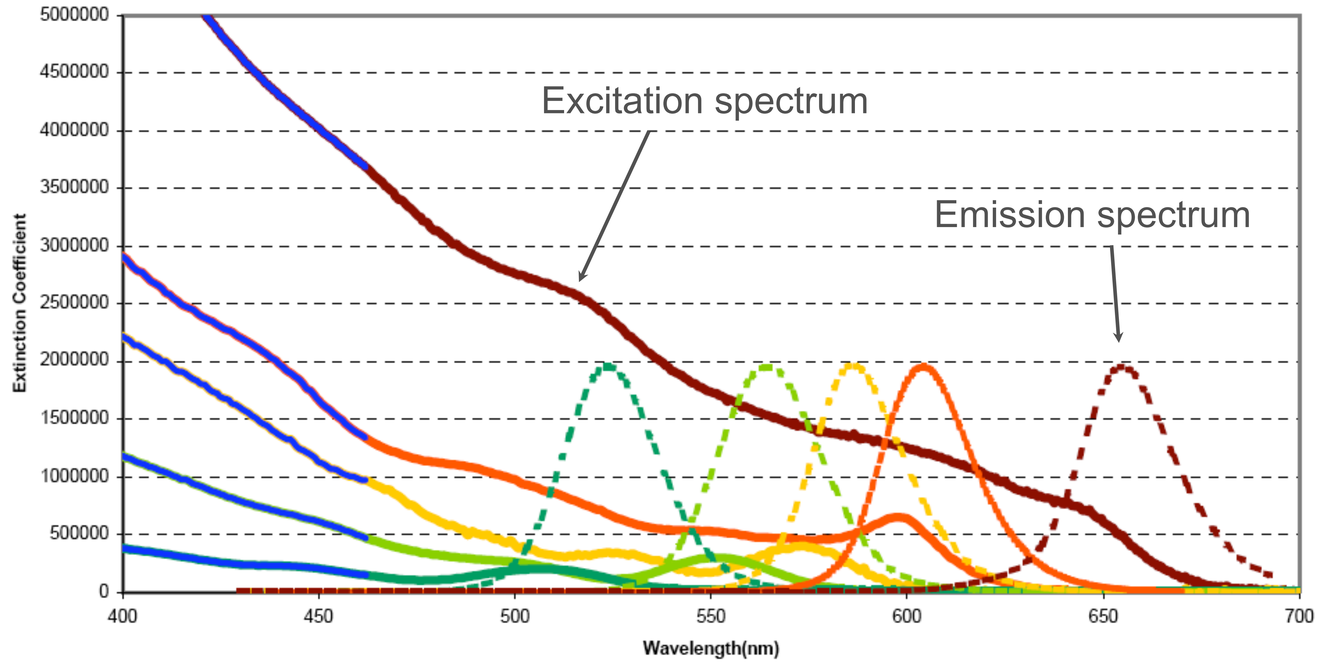
4. Quantum dots



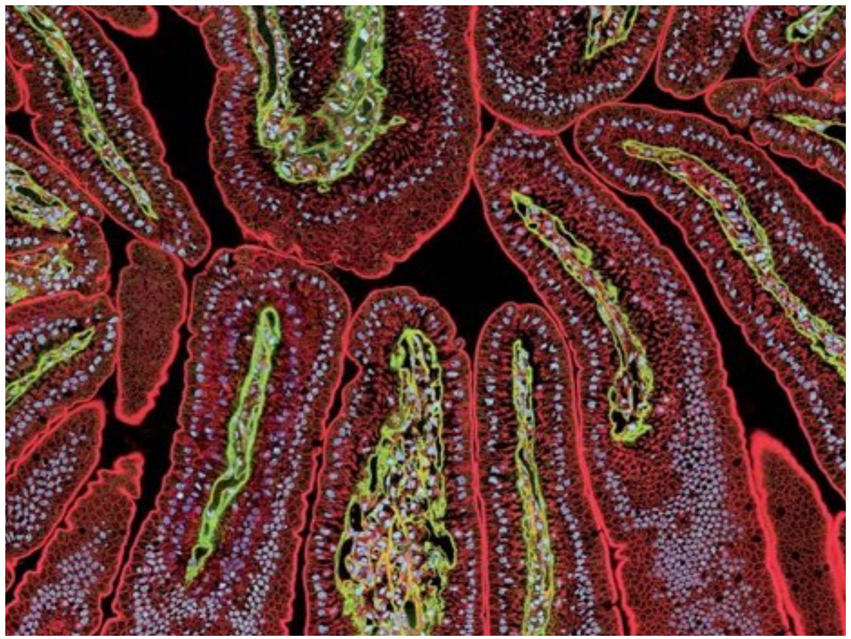
Semiconductor nanocrystals
Emission spectrum depends on particle size

Advantages:
Broad excitation spectrum
Tunable emission spectrum
Highly resistant to photobleaching

Quantum dot labeling



Advantages:
Wide excitation spectrum
Tunable emission spectrum
Resistance to photobleaching

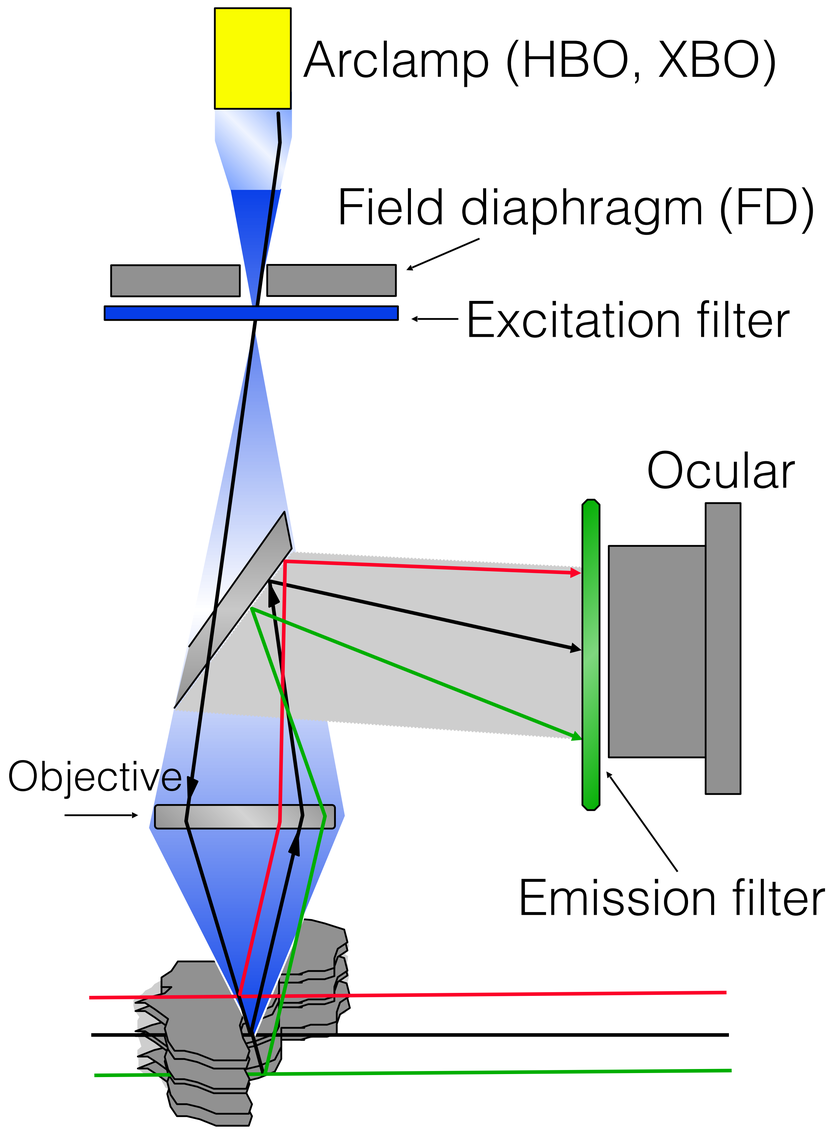


Red: actin
Green: Laminin
Blue: nucleus

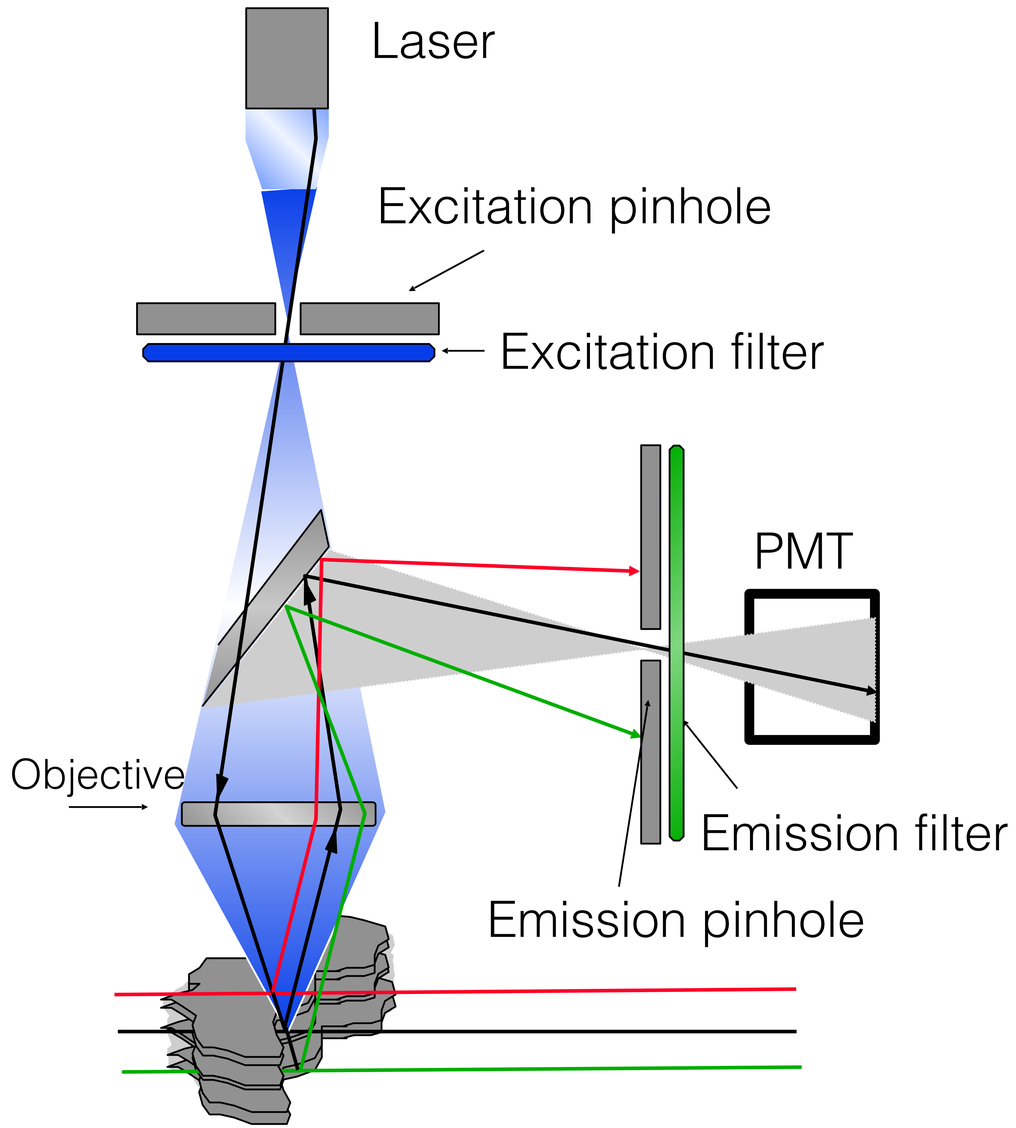
A mouse intestinal section visualized using fluorescent Qdot nanocrystal conjugates. Actin was labeled with a mouse anti-actin monoclonal antibody and visualized using red-fluorescent Qdot 655 goat F(ab')2 anti–mouse IgG. Laminin was labeled with a rabbit anti-laminin polyclonal antibody and visualized using green-fluorescent Qdot 525 goat F(ab')2 anti–rabbit IgG. Nuclei were stained with blue-fluorescent Hoechst 33342

Wide-field vs. confocal fluorescence microscope

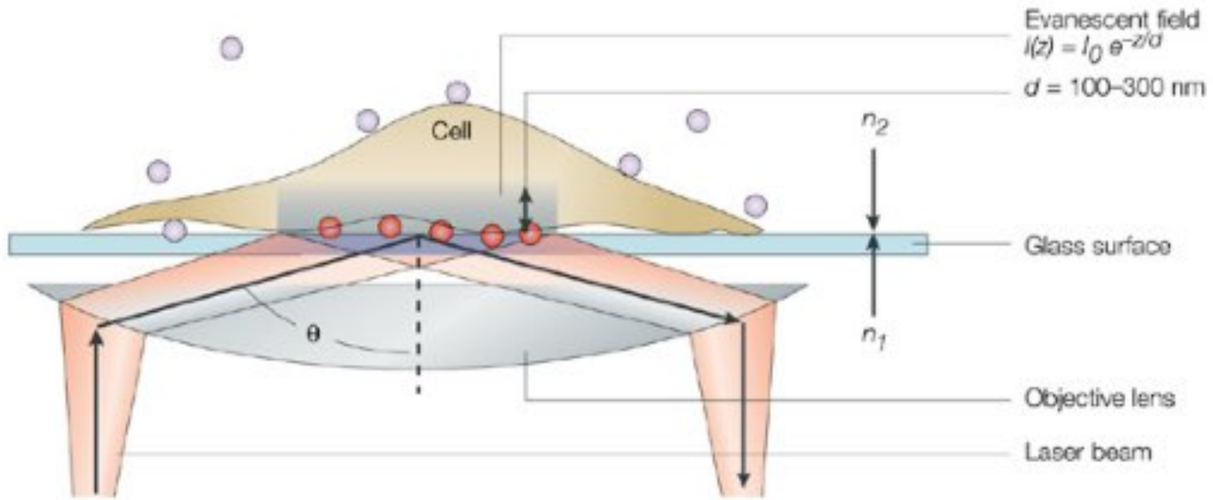
“Wide field”

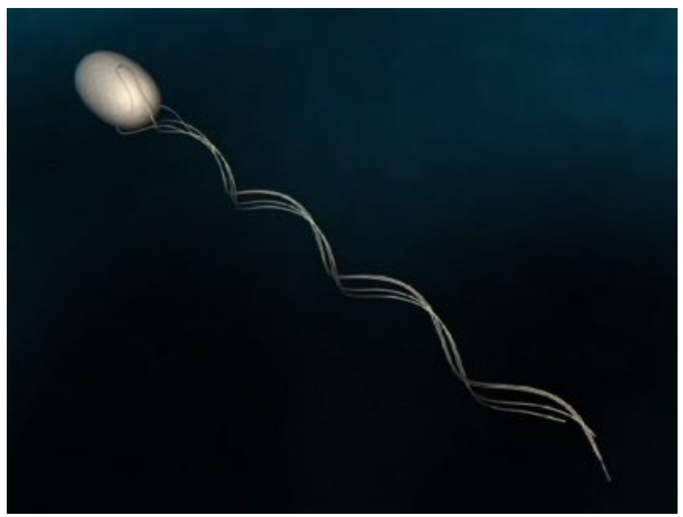


Confocal



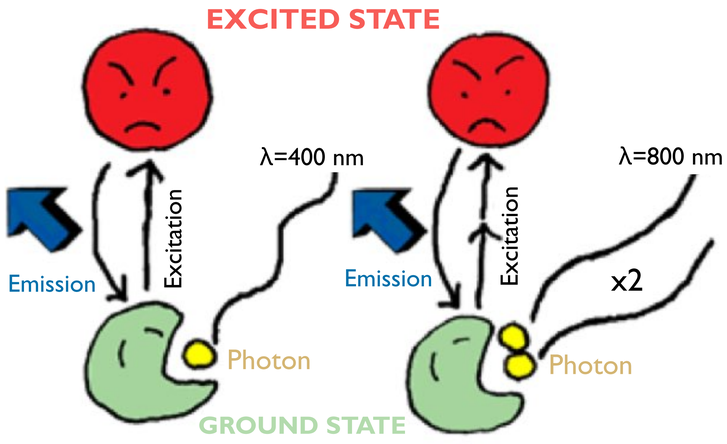
Total Internal Reflection Fluorescence Microscopy (TIRFM)





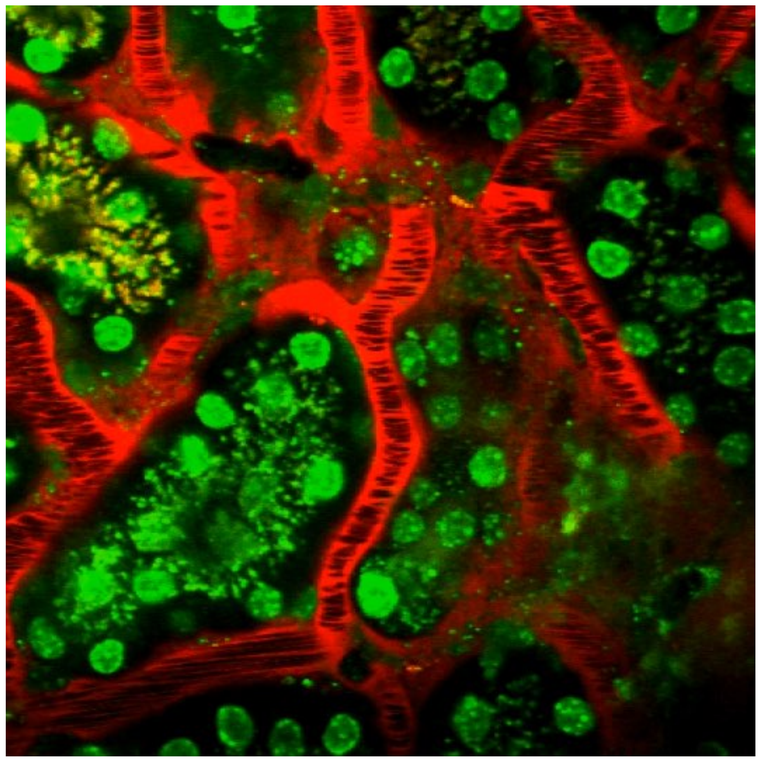
Multiphoton microscopy and its photochemistry application

- Energy of two (or more) photons are added during excitation
- Excitation (hence emission) only in focal point (limited photodamage)
- Excitation with long wavelength (near-IR), short (fs) light pulses
- Large (up to 2 mm) penetration due to long wavelength
- Possibility of launching light-sensitive reactions

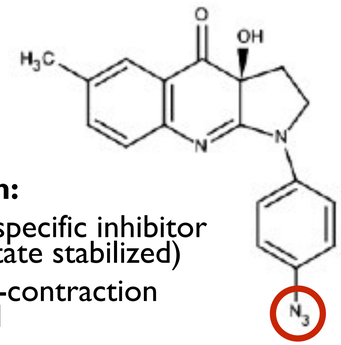


Single-photon fluorescence

Two-photon fluorescence

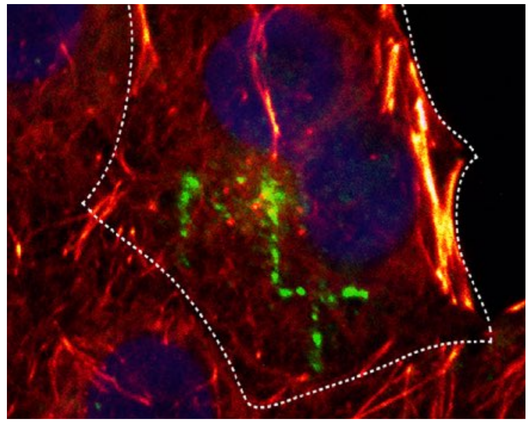


Green: proximal kidney tubules;
Red: albumin (plasma)



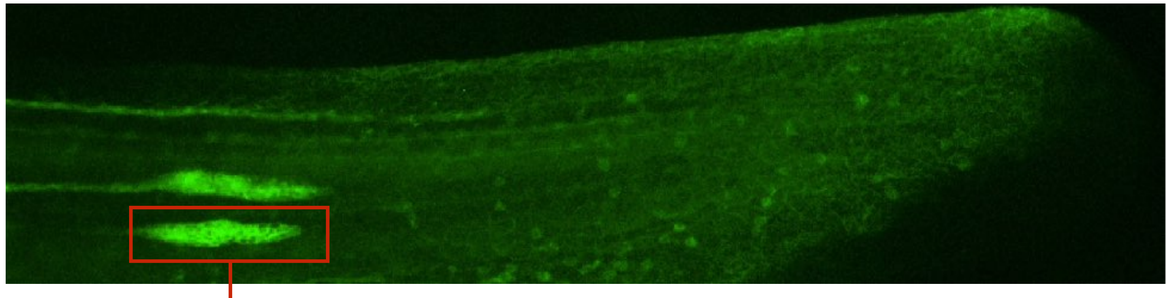
Blebbistatin:

- Myosin II specific inhibitor (ADP-P_i state stabilized)
- Excitation-contraction decoupled



Molecular tattoo: azido-blebbistatin photoactivated with spatial localization (HeLa)

Zebrafish lateral line organ development stops upon 2P exposure



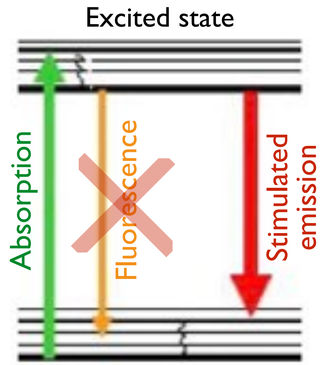
Képiró et al. Chem. Biol. 22, 548, 2015

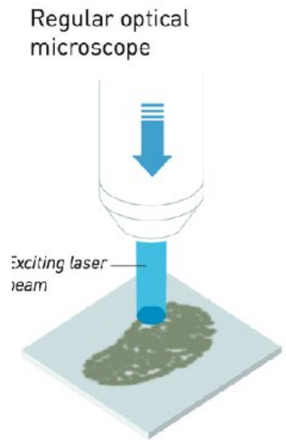
Pointillism (George Seurat, Paul Signac, Vincent van Gogh, Maximilien Luce)

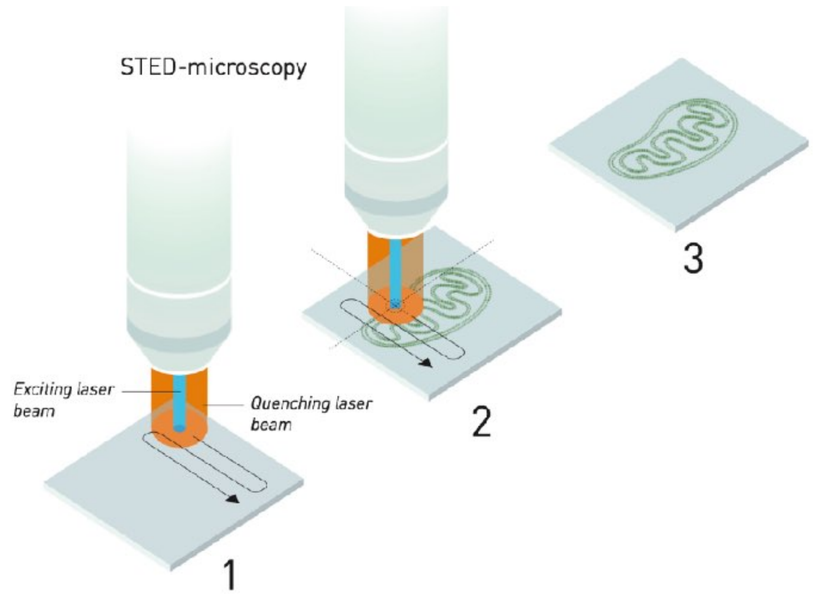
George Seurat: Sunday afternoon on the island of Grand Jatte

STED microscopy
(STimulated Emission Depletion)

Stefan Hell (Nobel-prize 2014)

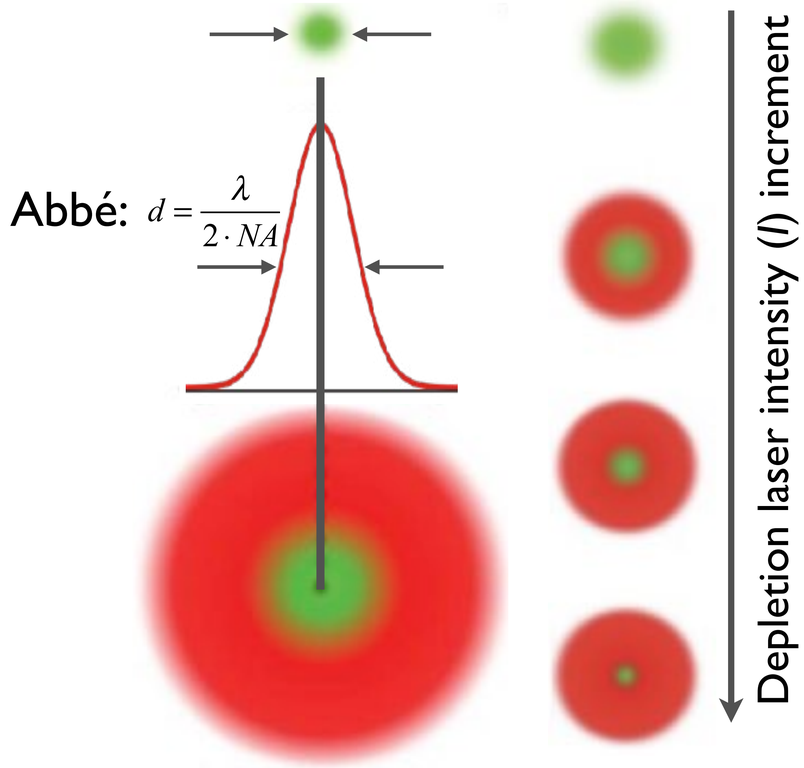


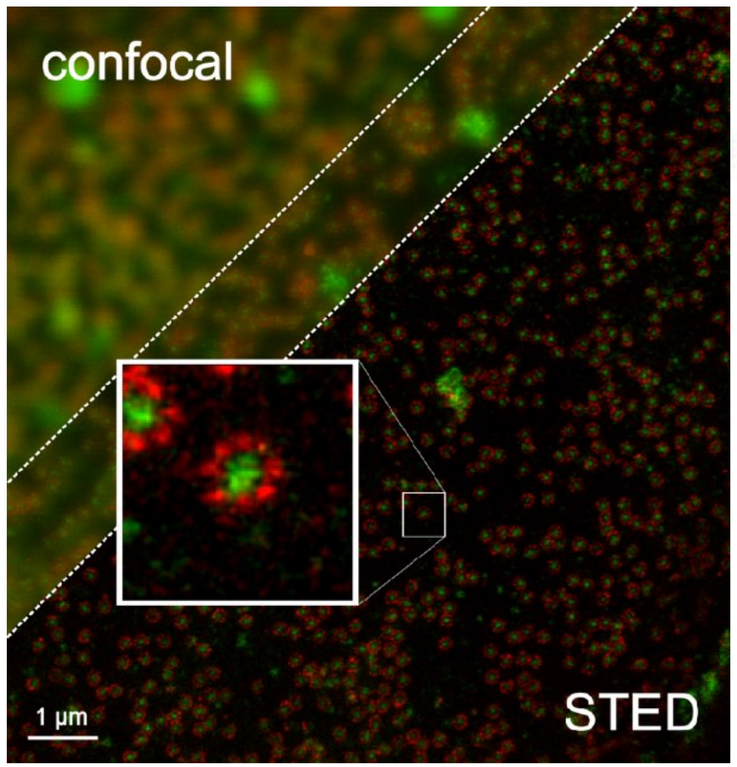


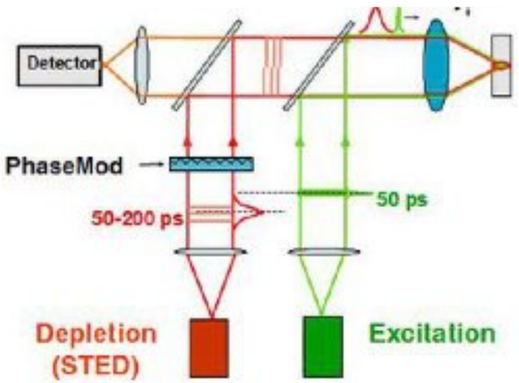


Hell: $d = \frac{\lambda}{2 \cdot NA\sqrt{1 + I/I_S}}$

Abbé: $d = \frac{\lambda}{2 \cdot NA}$

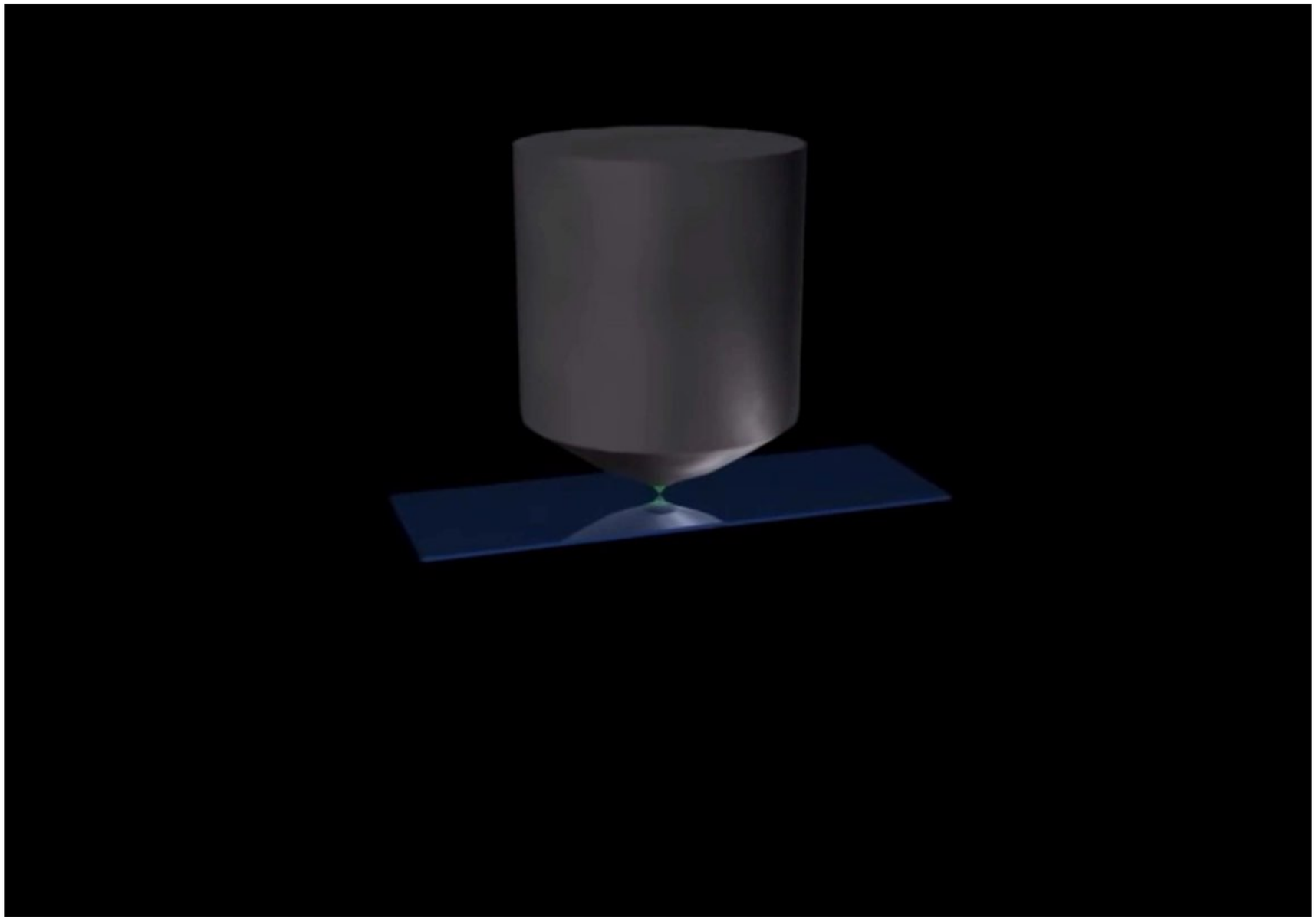






Nuclear pore complexes with STED microscopy

STED microscopy



Jakub Chojnacki

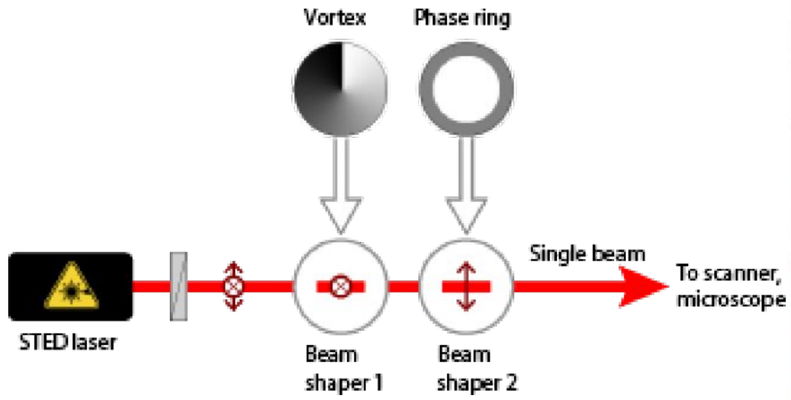
RESCue STED

Problem:
excitation intensity on the fluorophore is enormous (~MW/cm^2)

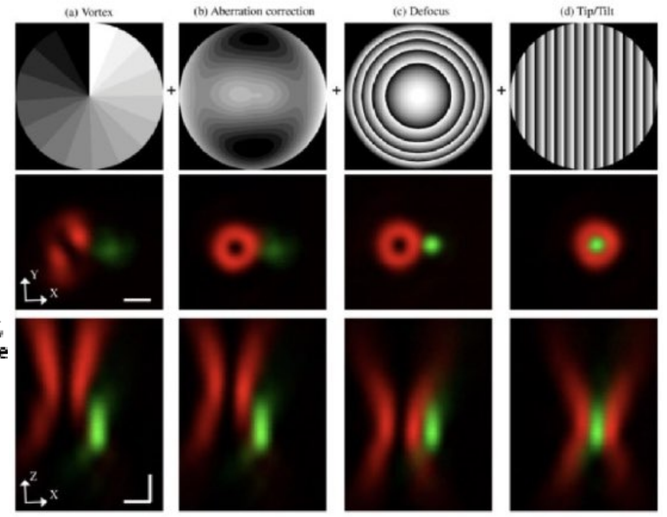
(Abberior Instruments)

3D STED

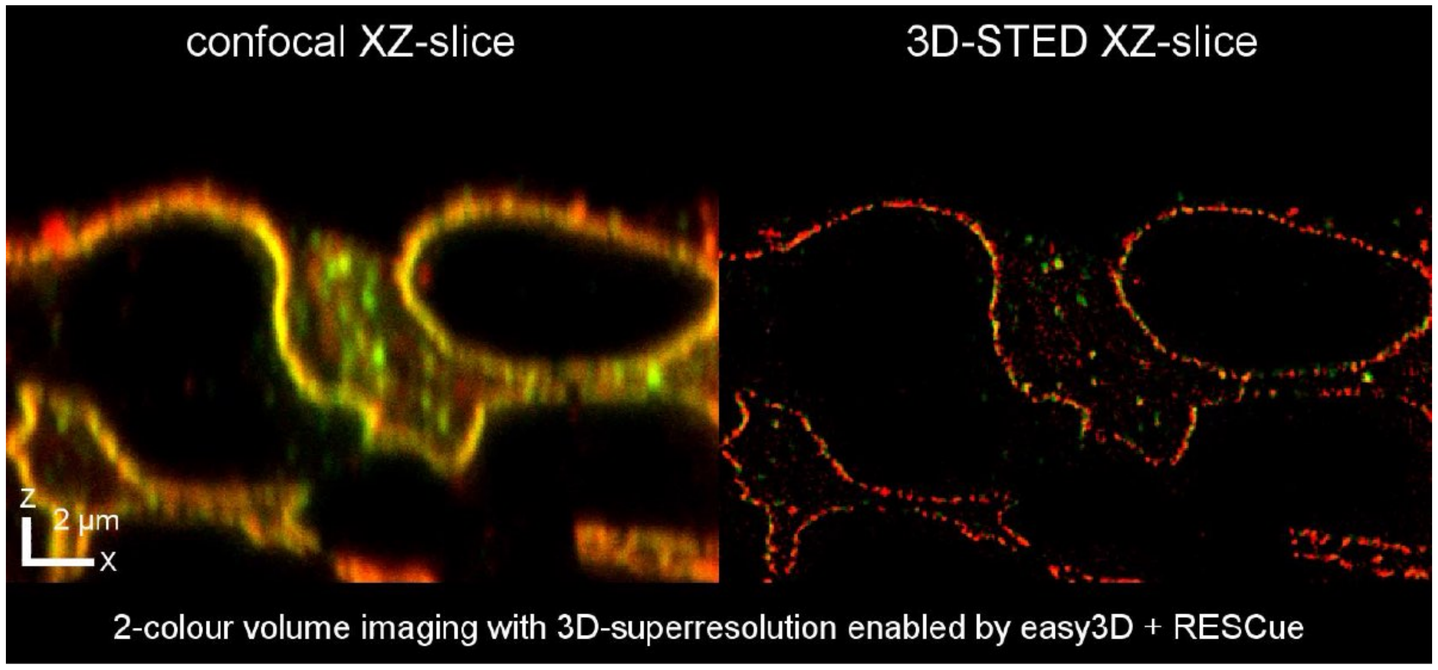
Programmable SLM (Spatial Light Modulator)



“Easy 3D STED”



Aberration correction



Nephrin (red), Podocin (green) (Abberior Instruments)

Optical tweezers: special light microscopic application based on photonic momentum change

Einstein: mass-energy equivalence $E = mc^2$

Planck: law of radiation $E = hf$

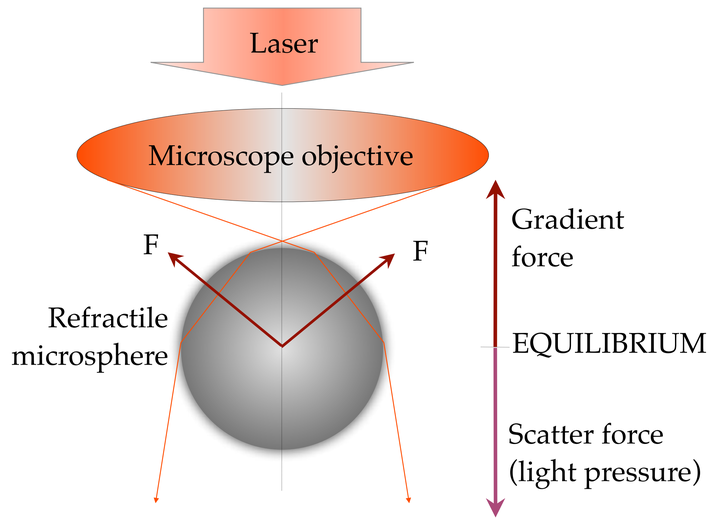
Maxwell: speed of light $c = \lambda f$

Louis-Victor-Pierre-Raymond, 7th duc ***de Broglie*** *(1892-1987)*

$$mc^2 = h \cdot \frac{c}{\lambda}$$

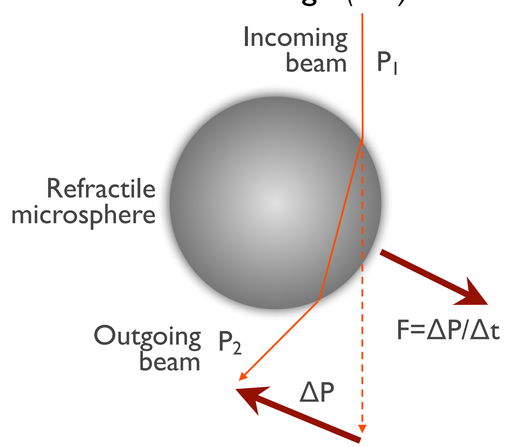
Momentum of the photon: $$P = \frac{h}{\lambda}$$

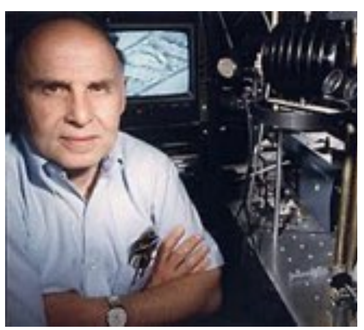
Refractile particles may be ***captured*** *with photonic forces:*



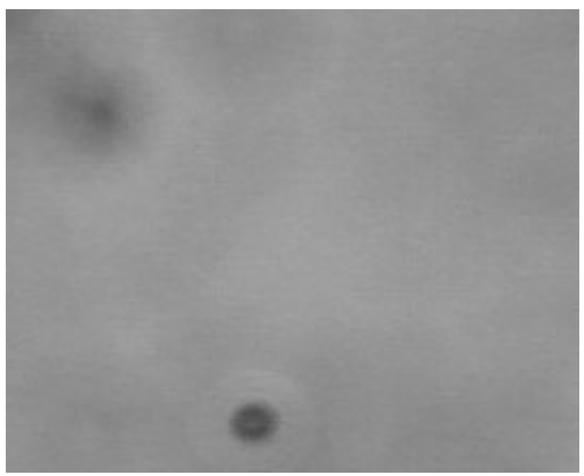
In the ***optical trap*** *a momentum change occurs between the photons and the trapped particle:*

Refraction is accompanied by photonic momentum change (ΔP):





Arthur Ashkin (1970)
Nobel-prize 2018

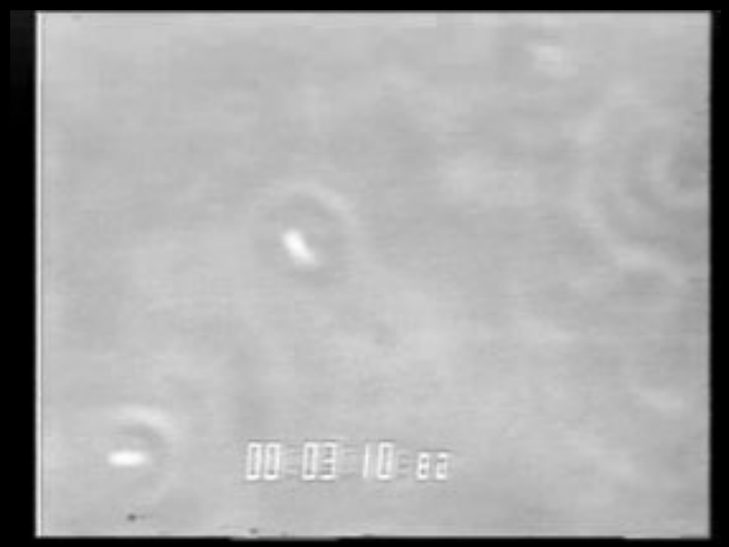


3 μm latex (polystyrene) microspheres in the optical trap

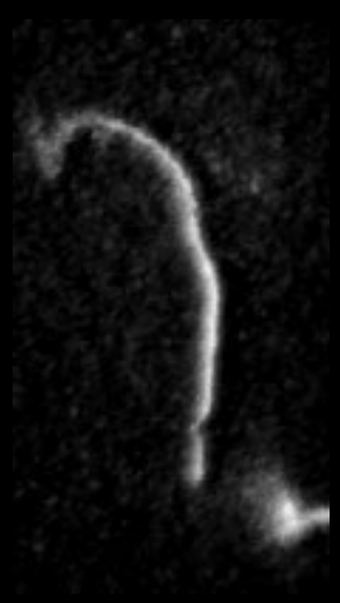
Biomolecule manipulation with light

"Tractor beam", Star Trek

E. coli bacterium

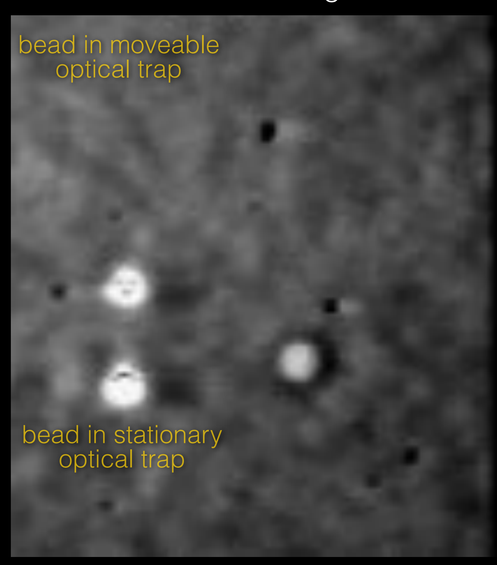


Actin filament

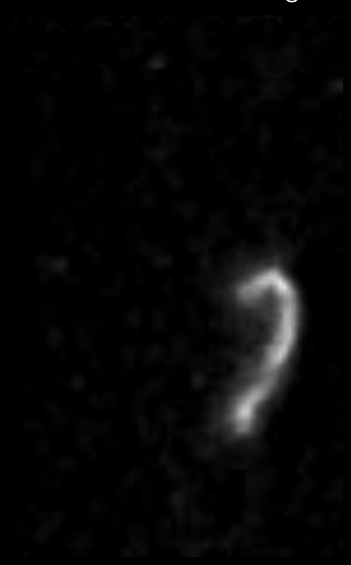


DNA

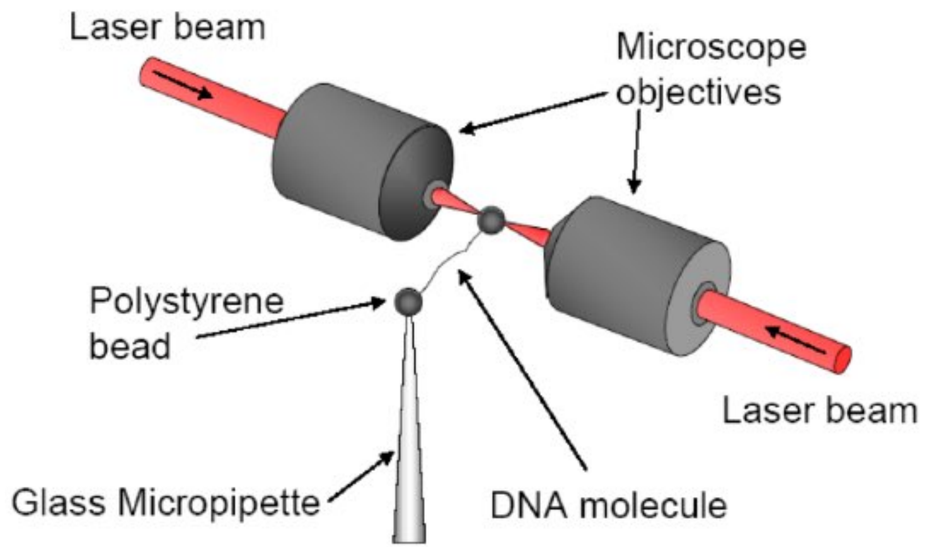
Phase contrast image

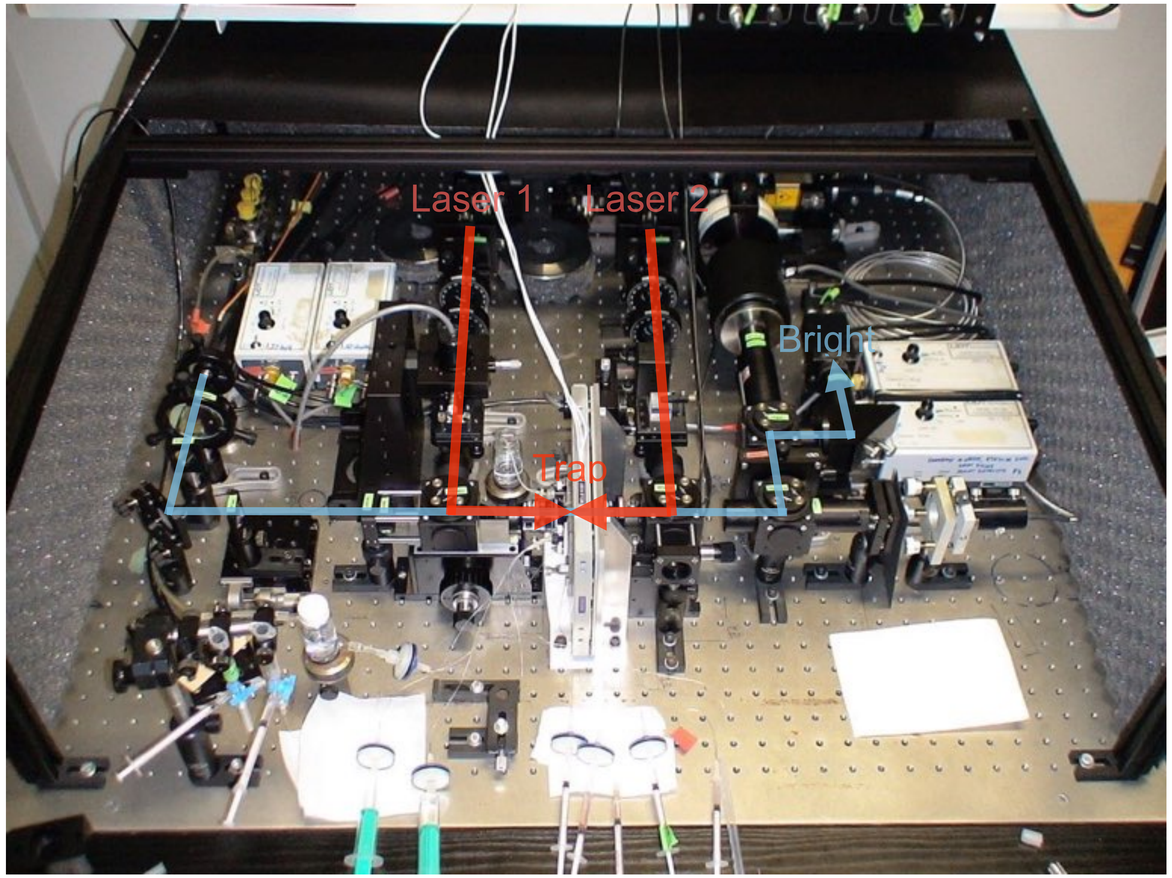


Fluorescence image



Force measurement with optical tweezers

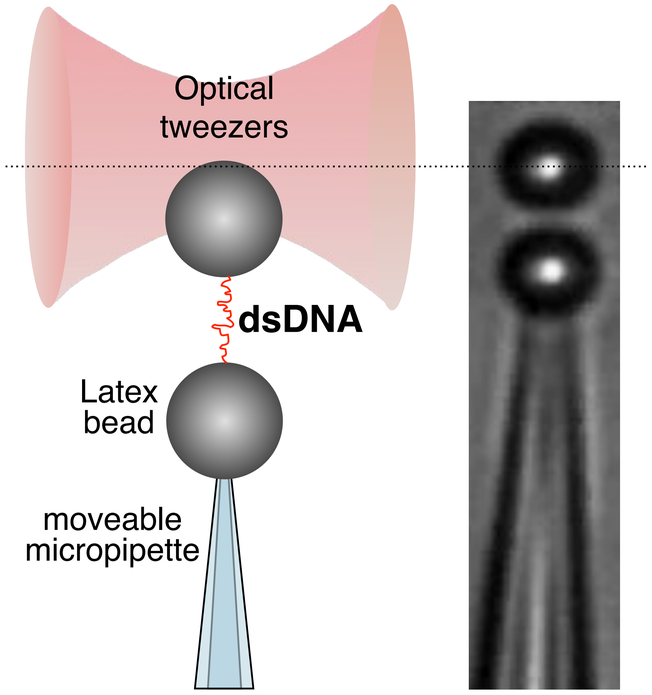




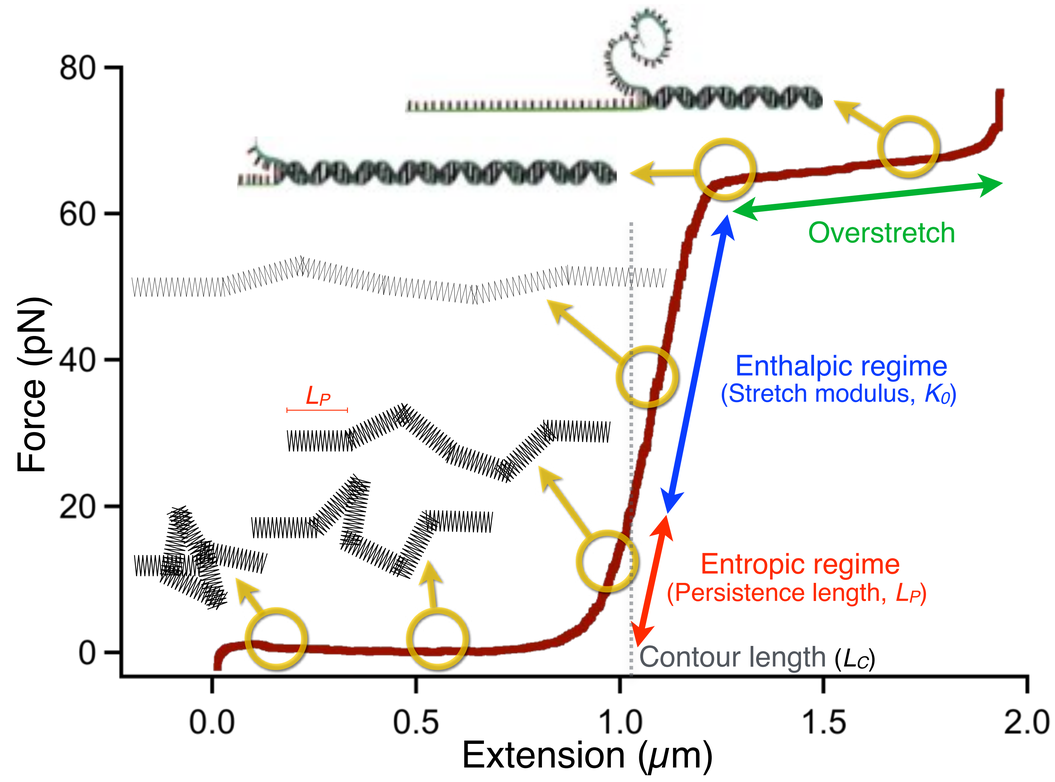
Két lézersugaras optikai csipesz berendezés

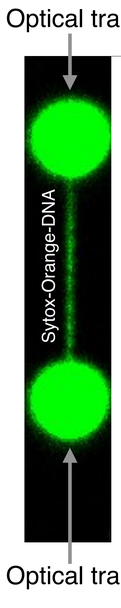
Force calibration
- Direct measurement of photonic momentum change
- Calibration with known force (Stokes force)
- Equipartition theorem

Mechanical extension of dsDNA



dsDNA stretch

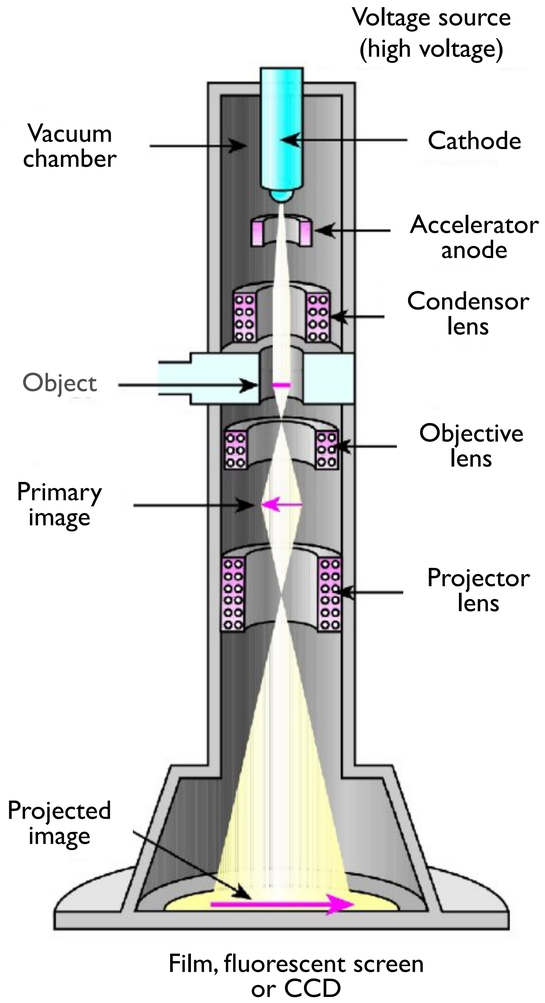




Electron microscopy

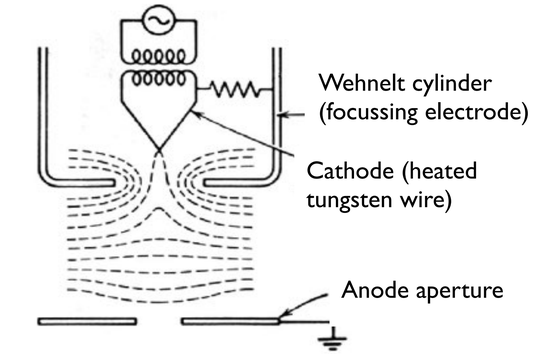
Uses the electron wave as a matter wave

Ernst Ruska (Nobel-prize 1986)

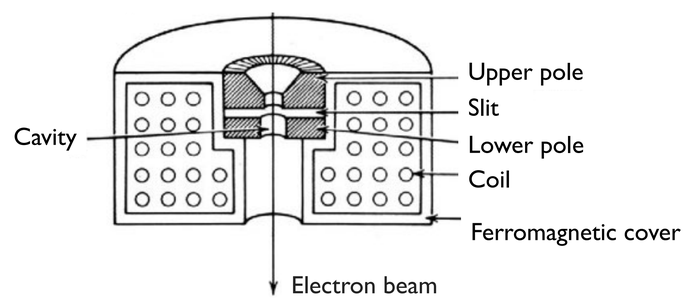


Transmission electron microscope (TEM)

Ray source:
electron gun



Focusing:
diverting the electron
with magnetic lens



$$F = eBV_e \sin\alpha$$

F=force on the electron; e=electron's charge; B=magnetic field; V_e=electron's speed; α=angle between the optical axis and the direction of the magnetic field

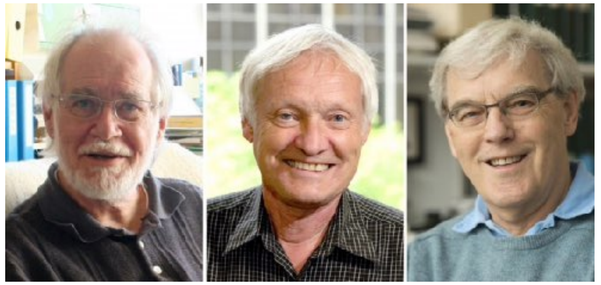
Resolution:

$$d = \frac{\lambda}{\alpha}$$

d=smallest resolved distance
λ="de Broglie" wavelength
α=angle between the optical axis and the direction of the magnetic field

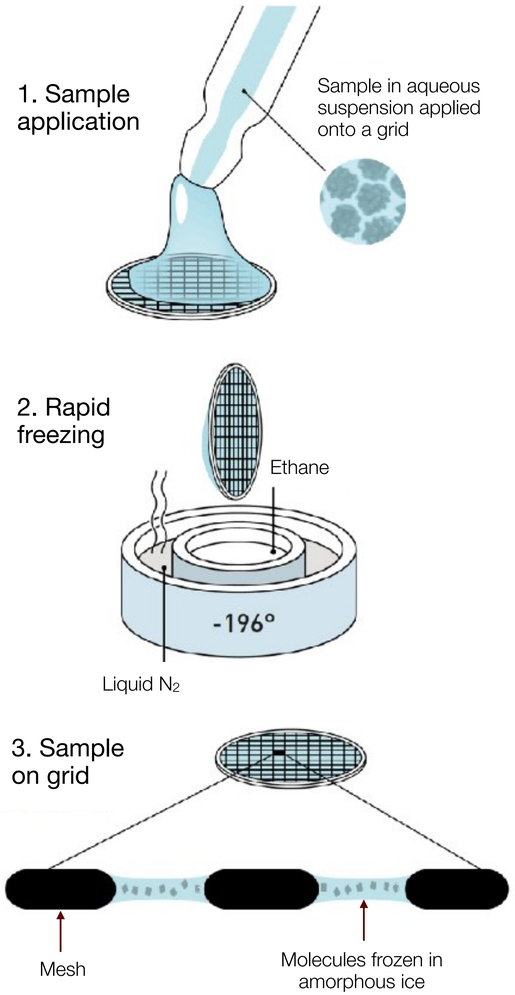
Based on the de Broglie wavelength the theoretical resolution is: d~ 0,005 nm (=5 pm).

Nobel-prize in chemistry 2017: cryoelectron microscopy

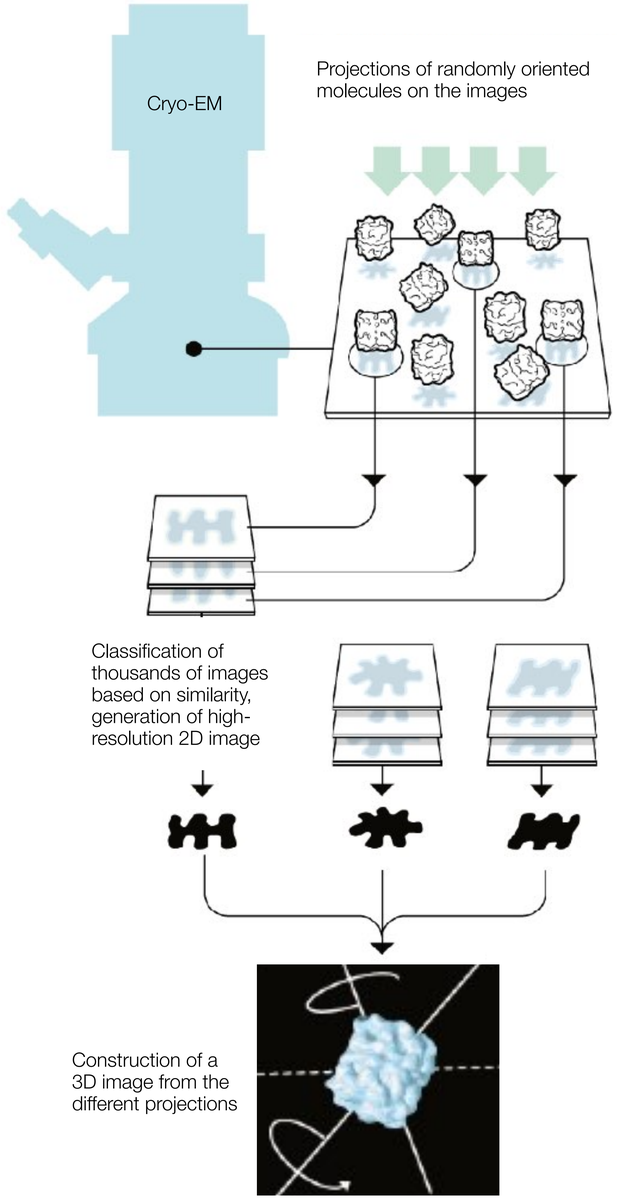


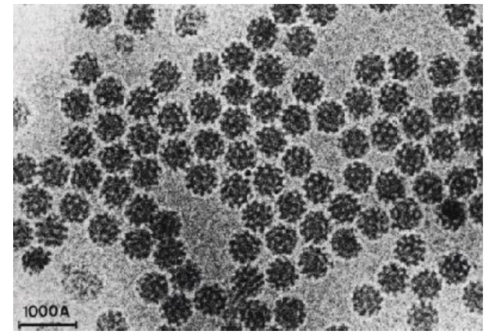
Jacques Dubochet, Joachim Frank, Richard Henderson

Sample preparation

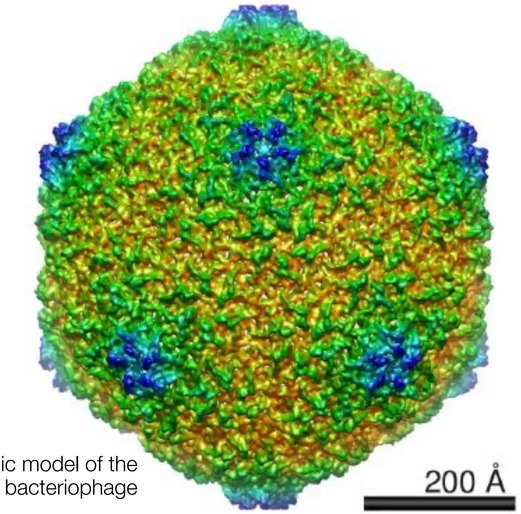


Cryo-EM image acquisition and reconstruction

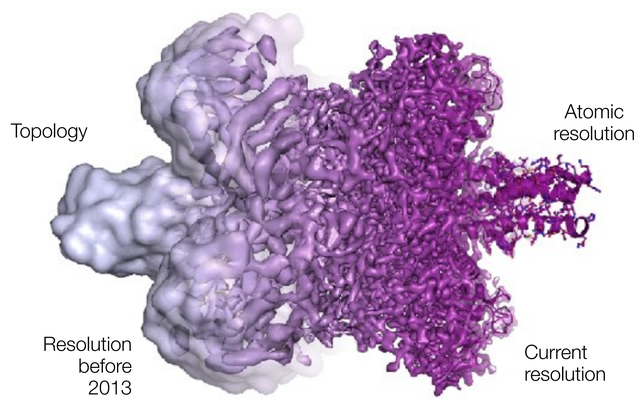




First cryoelectron microscopic image about viruses (Dubochet, 1984)

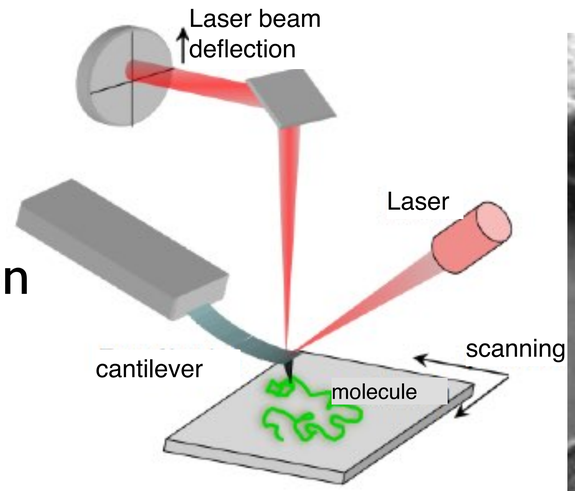


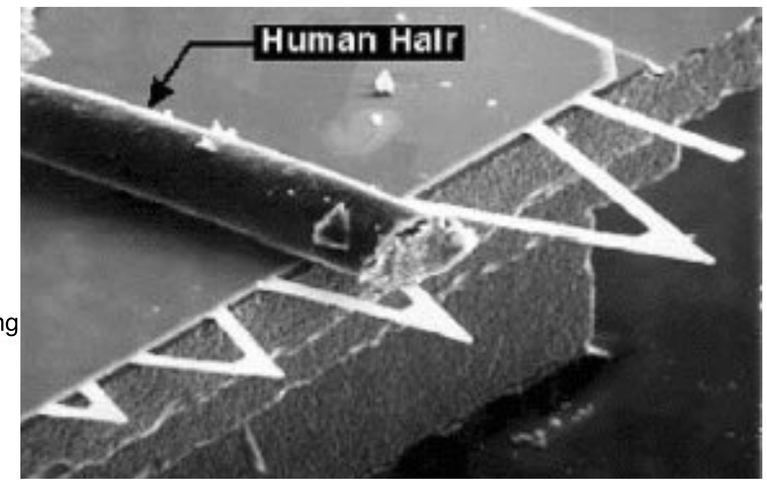
Atomic model of the T7 bacteriophage

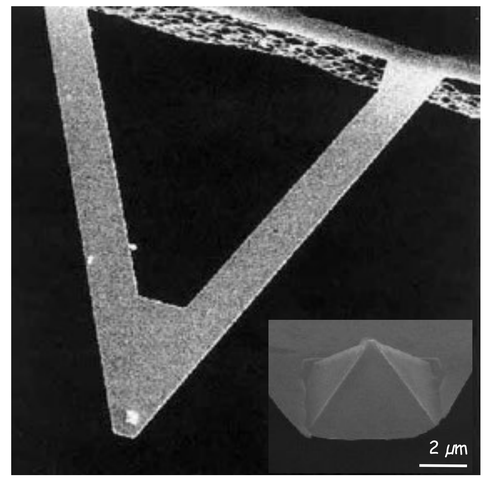


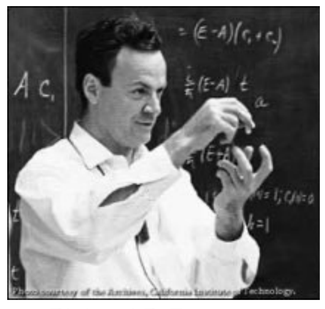
Scanning Probe Microscopies (SPM) I.
Atomic Force Microscope (AFM)

AFM operation



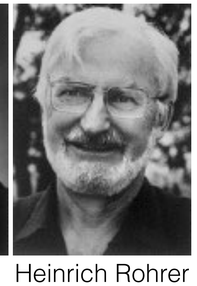




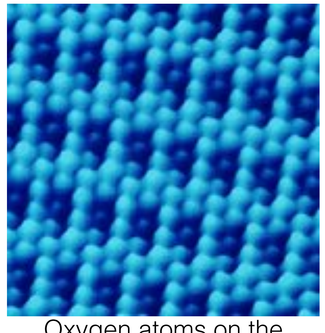


Richard P. Feynman:
“There is plenty of room at the bottom”
1959. december 29.

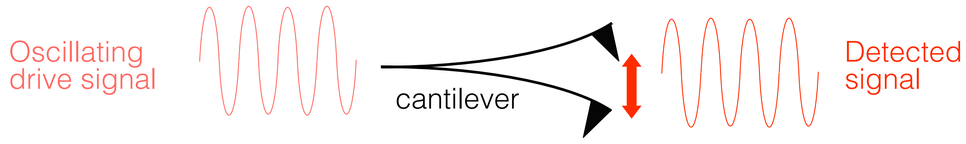
Gerd Binnig

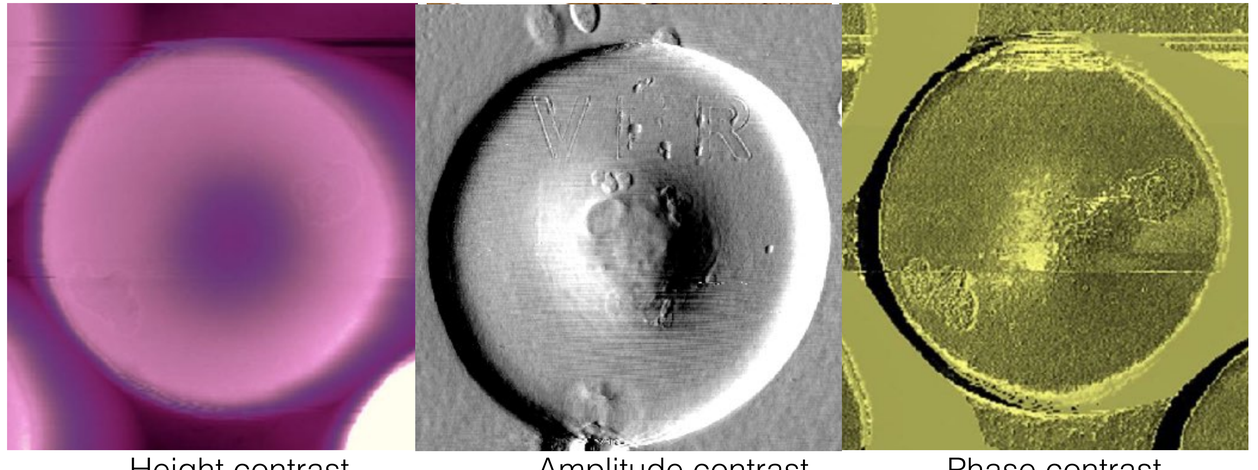


Heinrich Rohrer



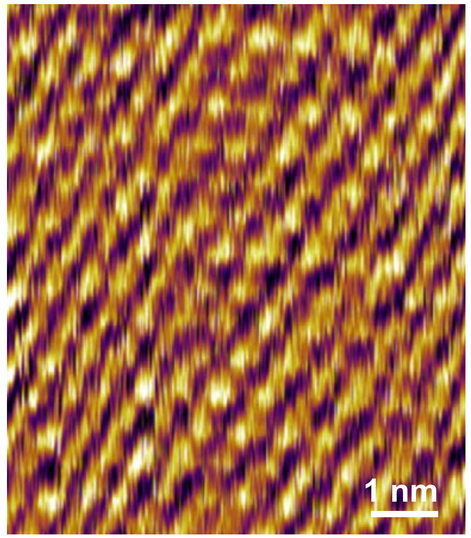
Oxygen atoms on the surface of a rhodium crystal



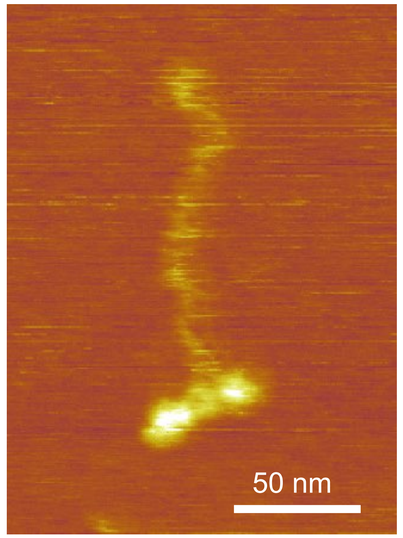


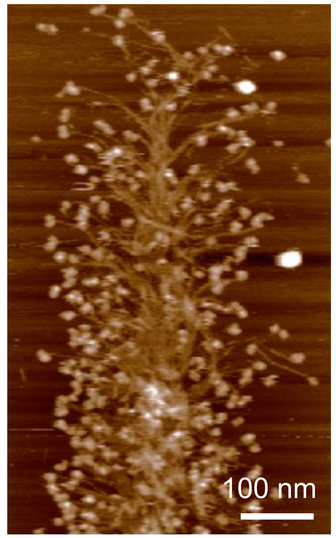
Height contrast | Amplitude contrast | Phase contrast

AFM of atoms, molecules, complexes

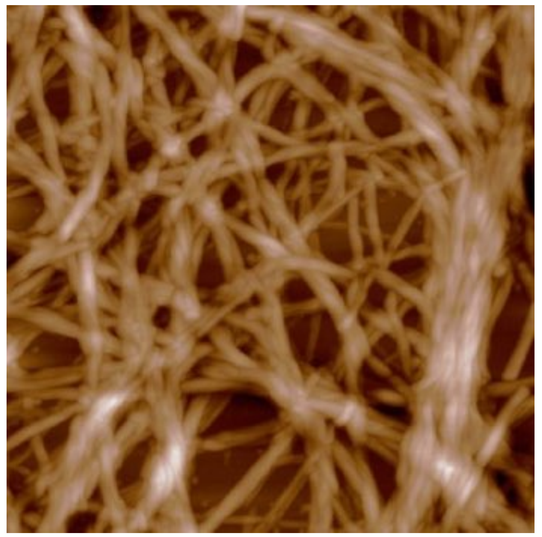


Mica

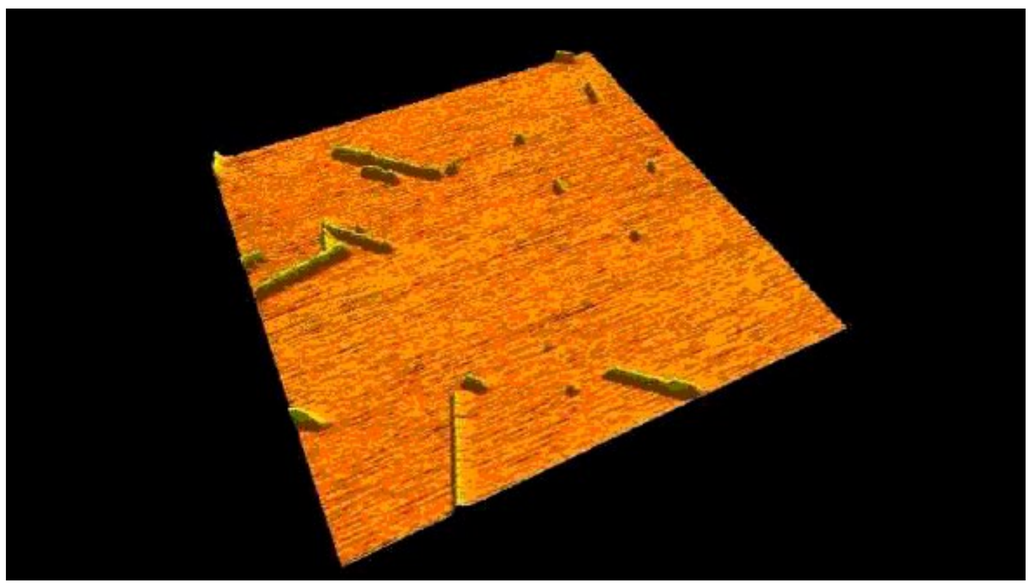




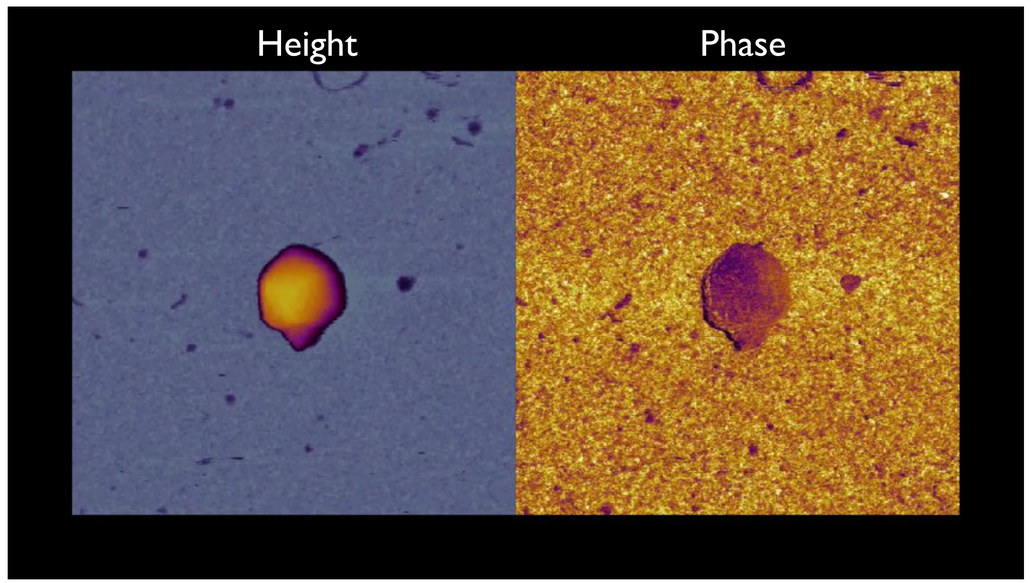
Myosin molecule and filament



Amyloid ß1-40 fibrils



Amyloid fibril growth

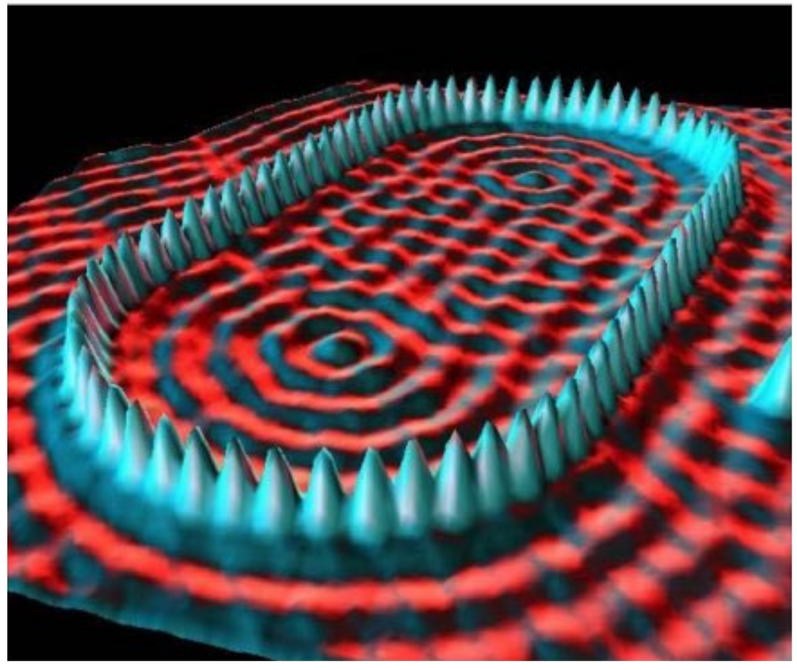


Viral DNA ejection

Scanning Probe Microscopies (SPM) II.

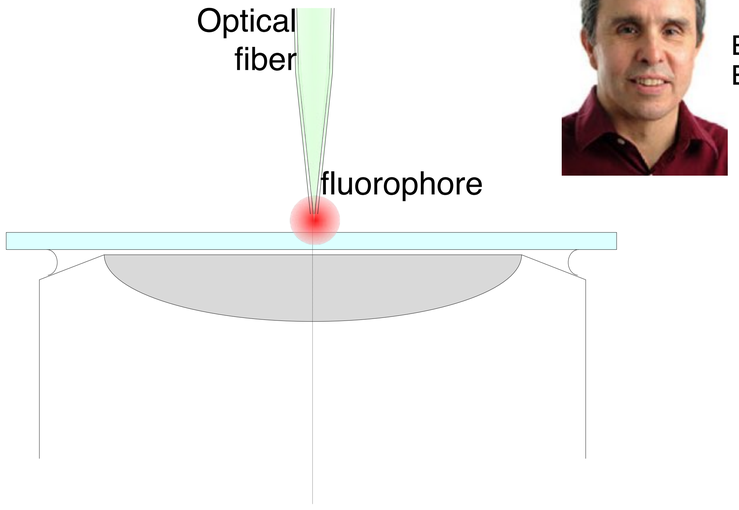
Scanning Tunneling Microscopy (STM)

Image is formed according to the tunneling current emerging in a distance-dependent manner between the atoms of the sample and the tip



STM image of iron atoms organized on a surface

Nier-Field Scanning Optical Microscopy (NSOM)



Eric Betzig

Optical fiber-end diameter: smaller than the wavelength of light

Distance between fiber end and the sample: smaller than the wavelength of light

Resolution: depends on the diameter of the optical fiber (as low as few 10 nm)

Correlative microscopy:
AFM and fluorescence combination