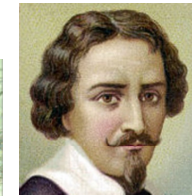
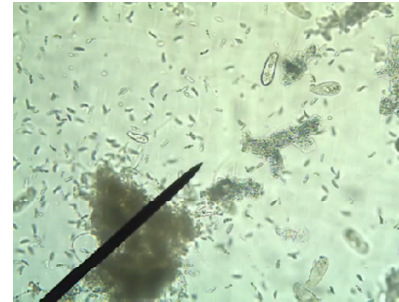


MICROSCOPY OF THE CELL AND ITS COMPONENTS

MIKLÓS KELLERMAYER

Light microscopy



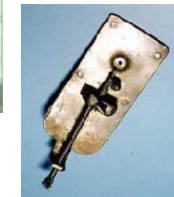
Zacharias Jensen
(1580-1638)



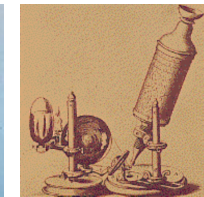
Anton van Leeuwenhoek
(1632-1723)



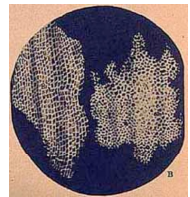
Robert Hooke
(1635-1703)



Leeuwenhoek's simple
microscope



Robert Hooke's
microscope

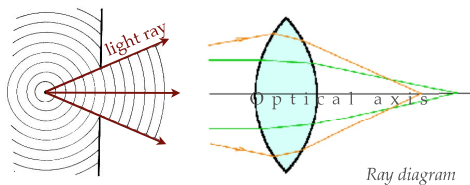


Cells in cork
(Robert Hooke)

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").

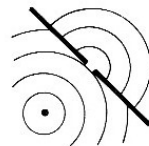


- Optical (light) ray ("light beam"): abstraction, mathematical line.
- Arrows represent the direction of energy propagation.
- Optical axis: line connecting the midpoint of optical components (e.g., lenses).
- Principle of reversibility: the direction of energy propagation (arrows) may be reversed.

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

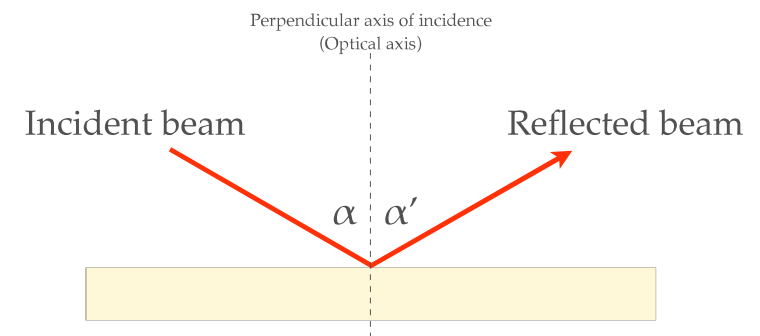
Wave optics

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



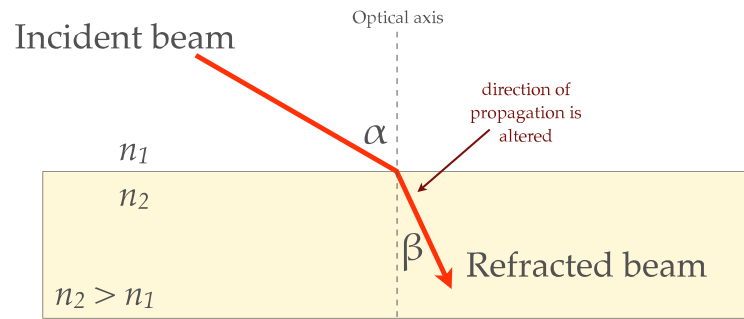
$$n_1 = \frac{c}{c_1}$$

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 ($\alpha = \alpha'$).

REFRACTION

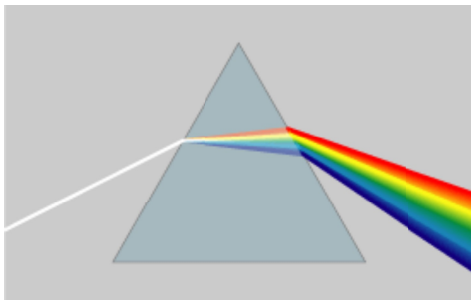


- α = angle of incidence; β = angle of refraction.
- Incident and refracted beams and axis of incidence are in the same plane.
- Snell's law:

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

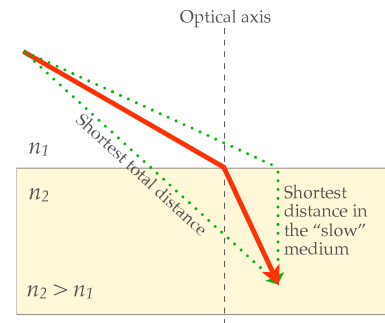
DISPERSION

Index of refraction depends on wavelength!



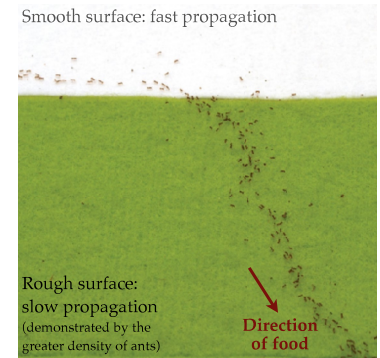
- The greater the frequency of light - the greater the refractive index.
- A prism decomposes white light according to wavelength (physical color).

EXPLANATION OF REFRACTION: FERMAT'S PRINCIPLE OF LEAST TIMES



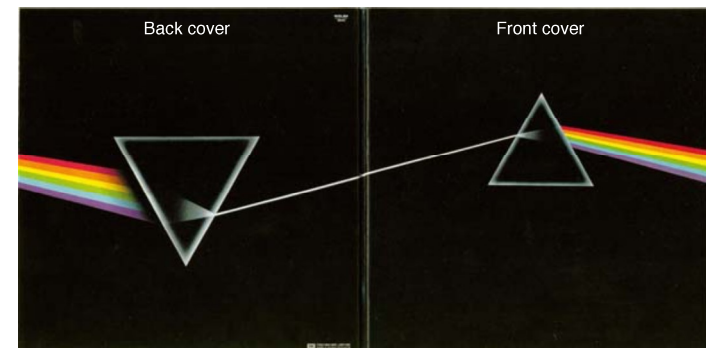
Light "chooses" the path that can be covered in the least time (i.e., fastest).

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



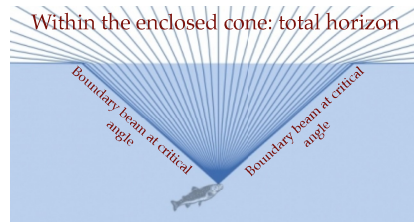
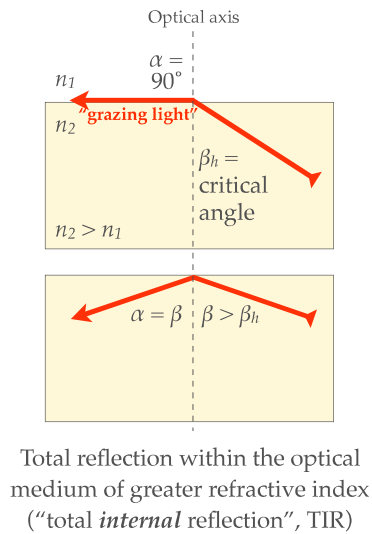
Path "selection" by ants (*Wasmannia auropunctata*) at the boundary of media with different "resistances".

DISPERSION APPEARS IN INTERESTING PLACES...

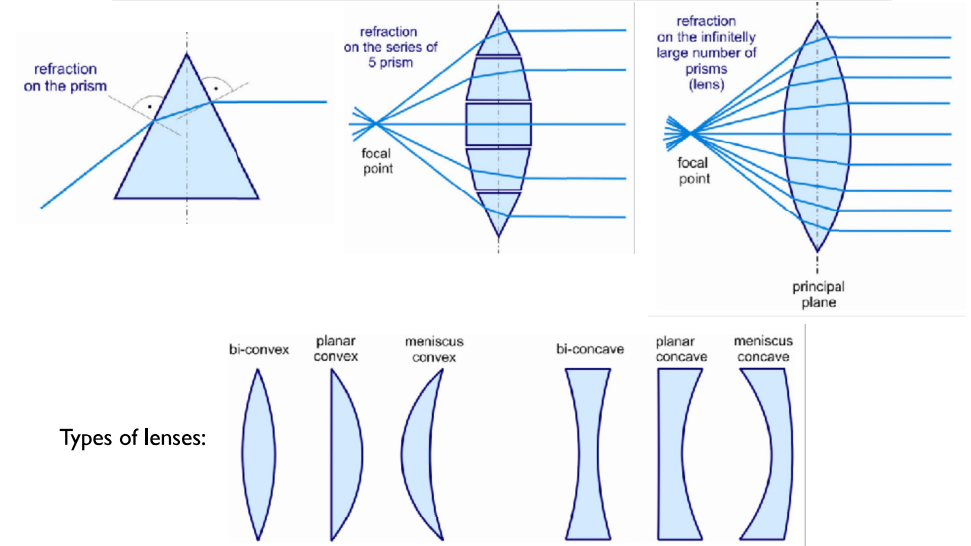


Pink Floyd: The Dark Side of the Moon

TOTAL INTERNAL REFLECTION



REFRACTION ON CURVED SURFACE



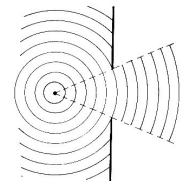
Wave phenomena I.

Diffraction

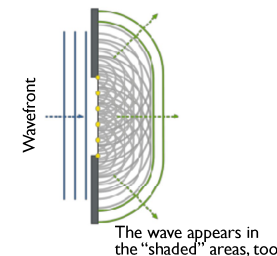
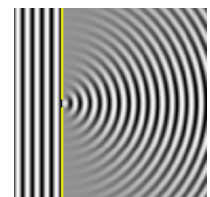
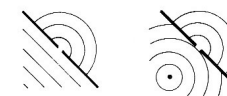
Huygens-Fresnel principle:
every point of a wavefront is the source of further waves



Slit much greater than the wavelength (λ)

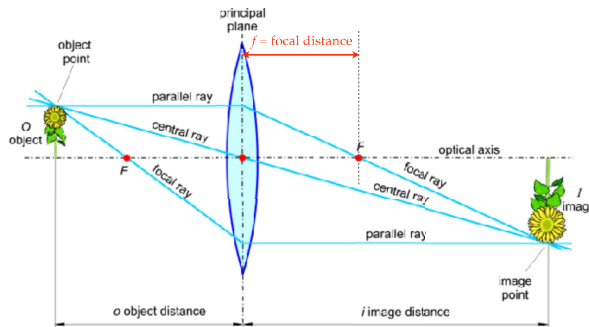


Slit much smaller than wavelength (λ)



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 $2f$ distance

Magnification

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

Lens equation

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

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

Optical power of refractile surface

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

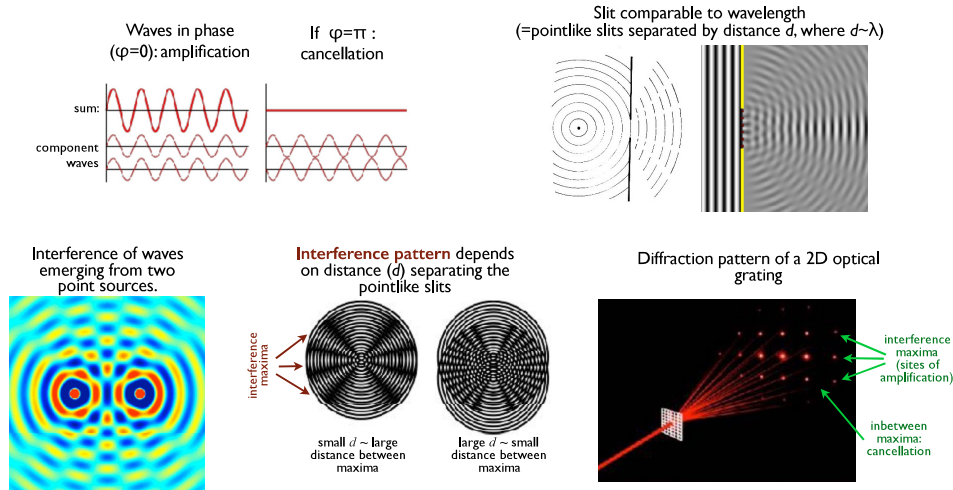
$n - n' = \text{difference between the refractive indices of optical media}$

$r = \text{radius of curvature of refractile surface}$

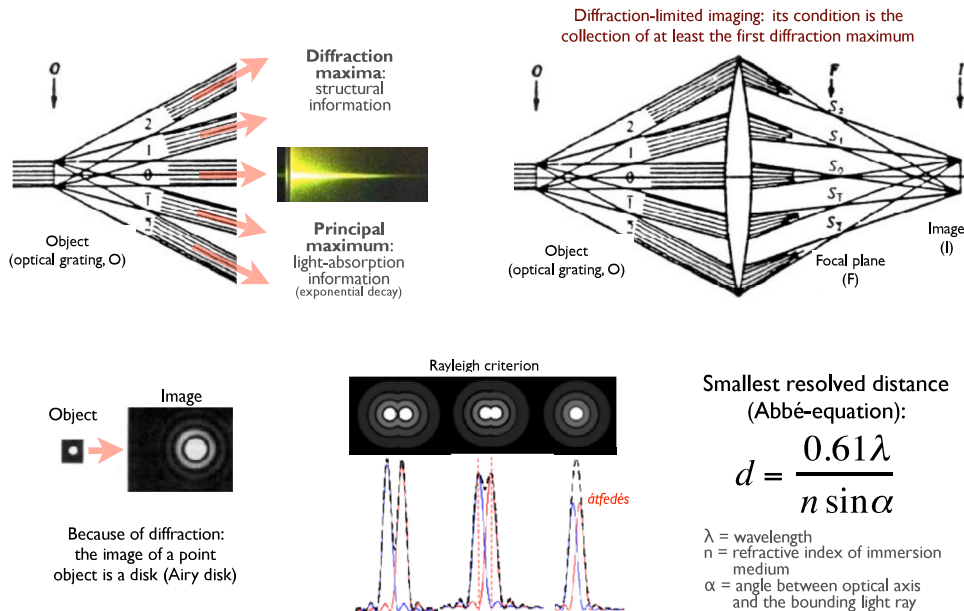
Wave phenomena II.

interference

Principle of superposition



Resolving power of the light microscope is limited by wave optics



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.

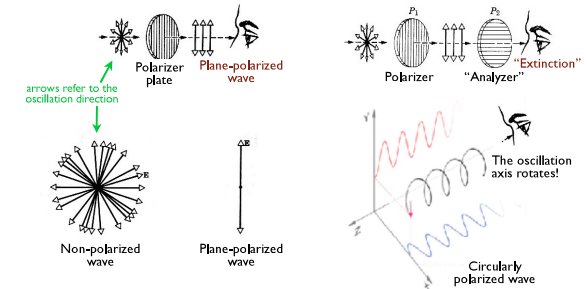


Polarization of Mechanical waves



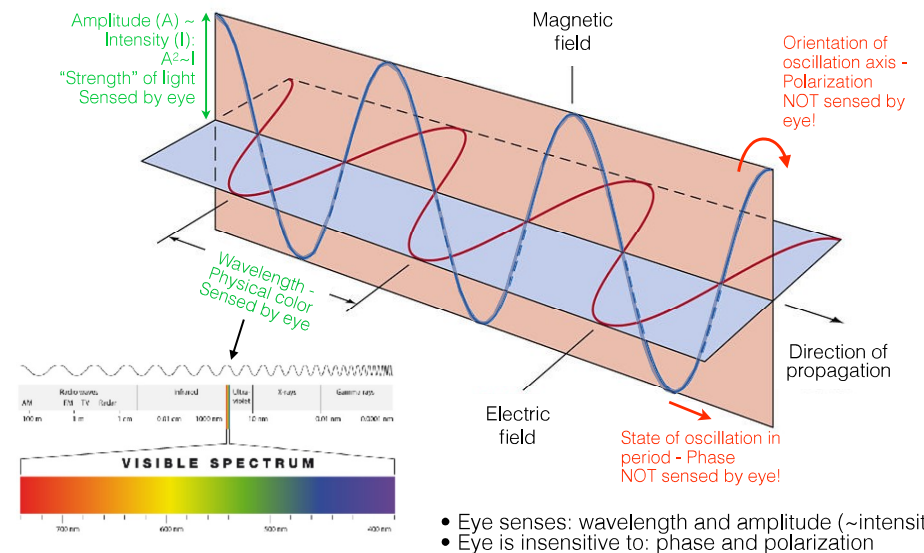
Polarization of Electromagnetic waves

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



Detectable parameters of light

Electromagnetic (transverse) wave



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

Schematics of the microscope

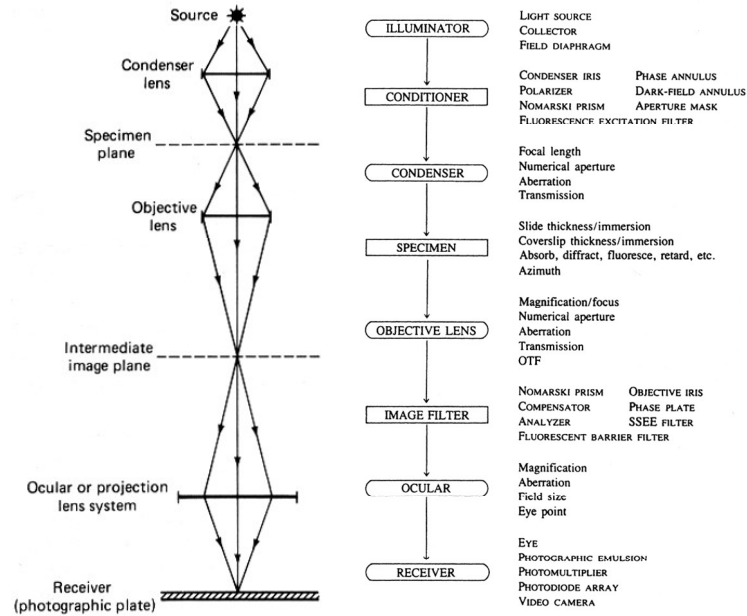
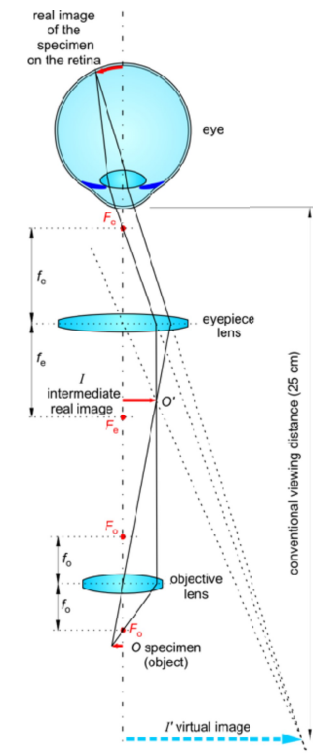
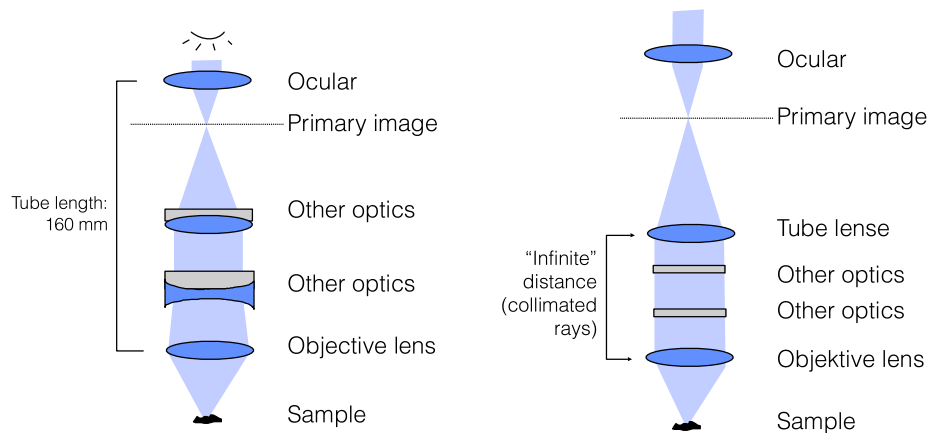


Image formation in the compound microscope

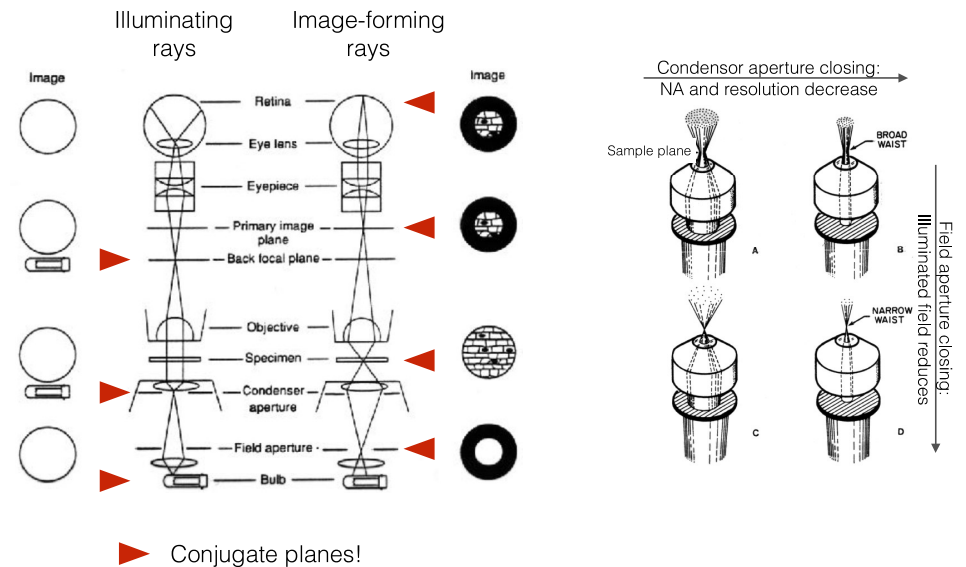
- Magnified, up-side-down, virtual image
- Condition of the formation of projected image: an accessory lens (eye lens) needs to be positioned in the optical path.
- Projection screen: retina



Ordinary vs. infinity-corrected microscope optics



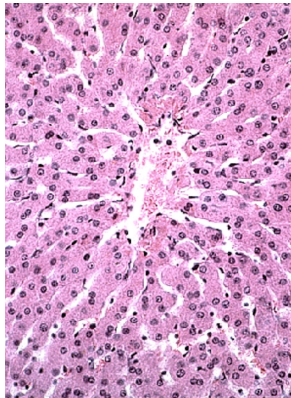
Köhler Illumination



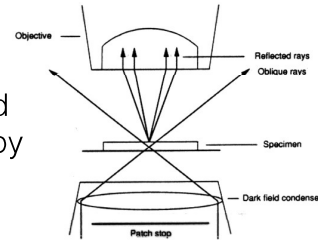
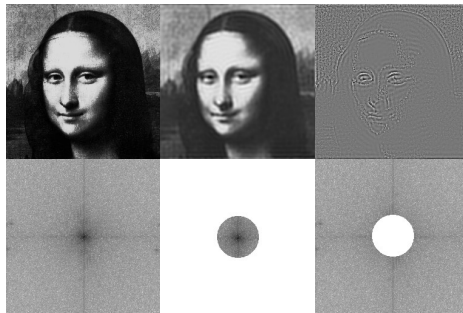
Contrast mechanisms I.

Absorption contrast

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



Dark field microscopy



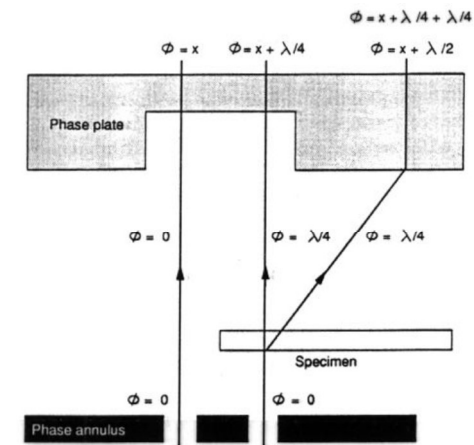
Contrast mechanisms II.

Phase contrast microscopy

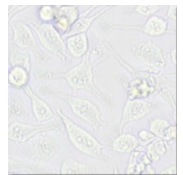


Frits Zernike
(1894-1993)
Nobel-prize

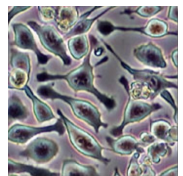
- **Phase:** shows the state of vibration within the entire period (2π).
- Expressed with the **phase angle** (ϕ).
- Phase difference between waves: **phase shift** (retardation or acceleration)



Live (unstained) cells



Bright-field microscopic image

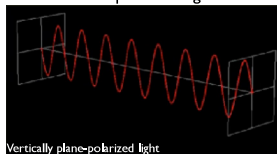


Phase-contrast microscopic image

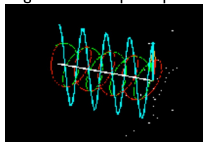
Contrast mechanisms III.

Polarization microscopy

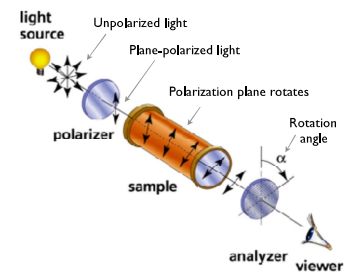
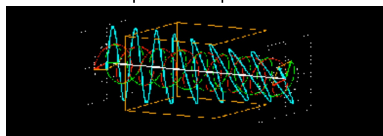
Plane-polarized light



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:



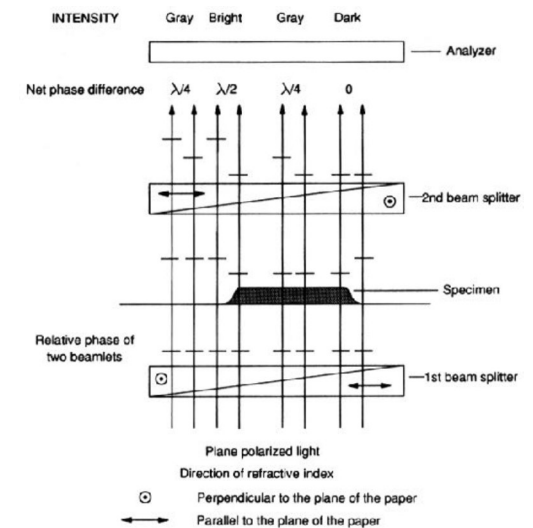
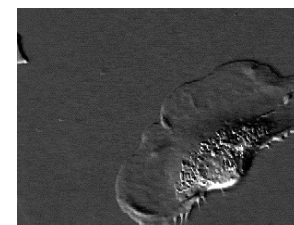
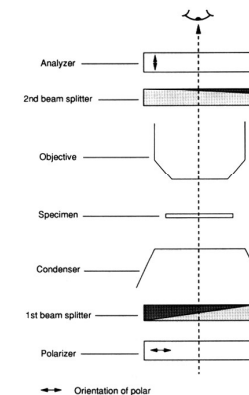
Striated muscle fiber in the polarization microscope



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

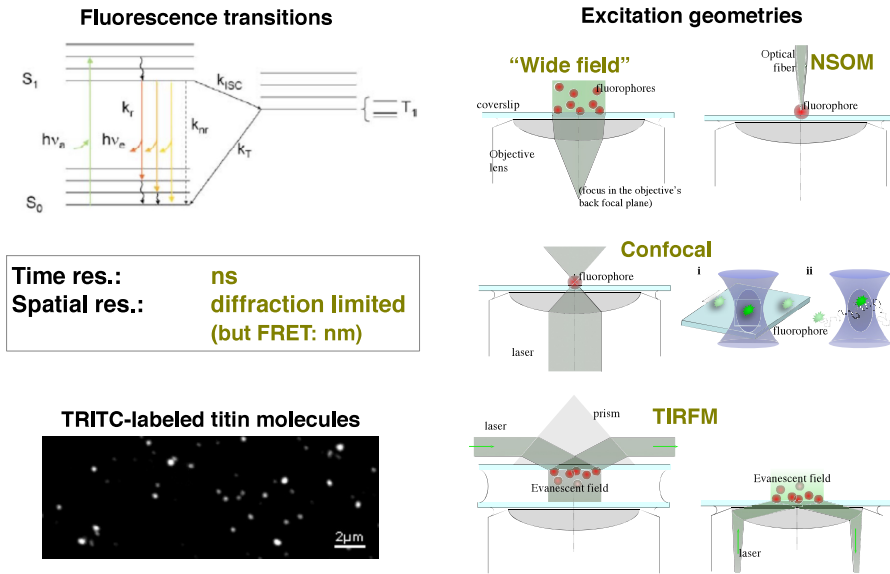
Contrast mechanisms IV.

Differential Interference Contrast (DIC, Nomarski) Microscopy



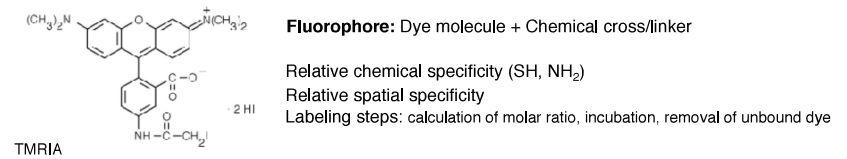
Cotrust mechanisms V.

Fluorescence



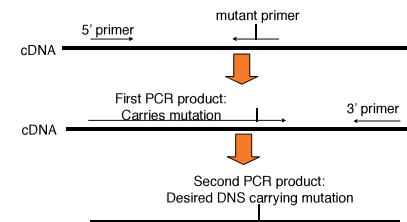
Source of fluorescence I.

1. Native side-chain labeling

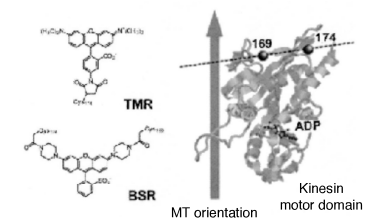


2. Targeted point mutagenesis

Targeted placement of cysteine residues



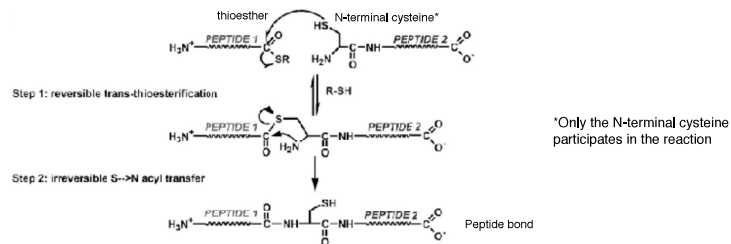
Special case: Bifunctional fluorophore



Source of fluorescence II.

3. Peptide ligation

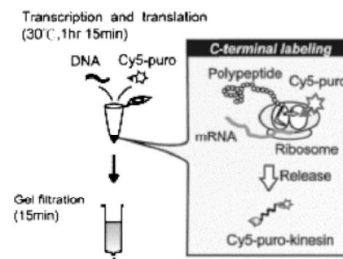
Construction of protein from synthetic, fluorescently labeled peptides



4. C-terminal labeling with puromycin derivatives

Puromycin:

- antibiotic
- binds to ribosome A site, instead of aminoacyl tRNA
- blocks protein synthesis
- binds covalently to the C-terminus of the already synthesized protein
- its fluorescent conjugates may be used for protein labeling



Source of fluorescence III.

5. Point mutagenesis of non-natural amino acids

1. Direct: intrinsic fluorophore derivatives (e.g., 7-aza-tryptophan)
2. Indirect: amino acids with non-proteinogenic reactive groups (e.g., keto)

6. Reconstitution of protein complex from labeled subunits

Applicable only in the case of multi-subunit proteins

Source of fluorescence IV.

7. Conjugation with fluorescent proteins

a. Green Fluorescent Protein, GFP

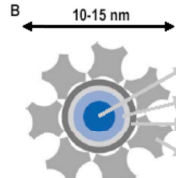
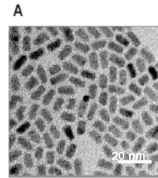


Size, structure: ~27 kDa, 238 aa, 11-strand β -barrel
Chromophore: Ser65-Tyr66-Gly67 side chains of central β -strand
Fluorescence depends on intact 3D structure
Tandem fusion constructs: genes of GFP and protein of interest
Advantages: *in vivo* measurements, spectral variants of GFP mutants.
Disadvantages: blinking, terminal labeling, interference with target protein.

b. Photoactivated GFP analogue

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

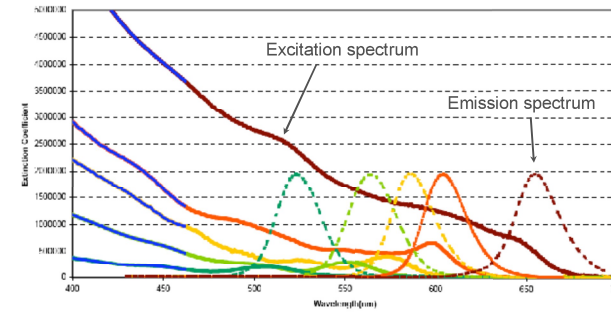
8. Quantum dots



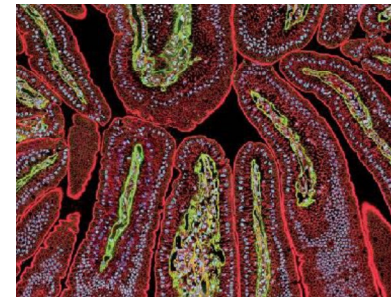
Semiconductor nanocrystals
 Emission spectrum depends on particle size

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

Quantum dot labeling



Advantages:
 Wide excitation spectrum
 Tunable emission spectrum
 Resistance to photobleaching

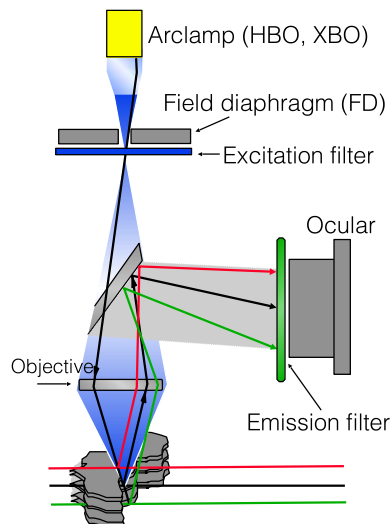


Red: actin
 Green: Laminin
 Blue: nucleus

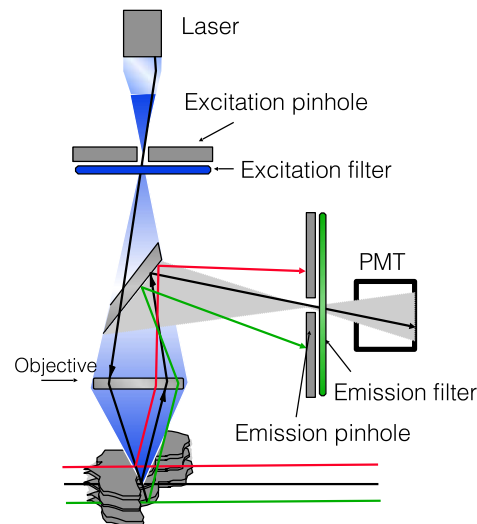
A mouse intestinal section visualized using fluorescent Qdot nanocrystal conjugates. Actin was labeled with a mouse anti-actin monoclonal antibody and visualized using red-fluorescent Qdot 655 goat F(ab')₂ anti-mouse IgG. Laminin was labeled with a rabbit anti-laminin polyclonal antibody and visualized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

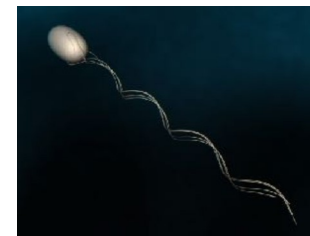
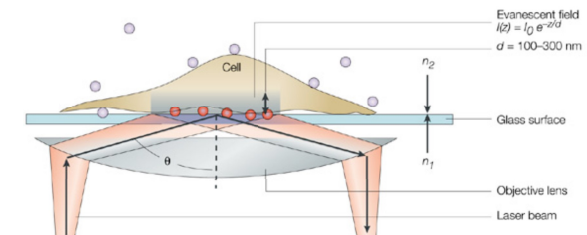
“Wide field”



Confocal

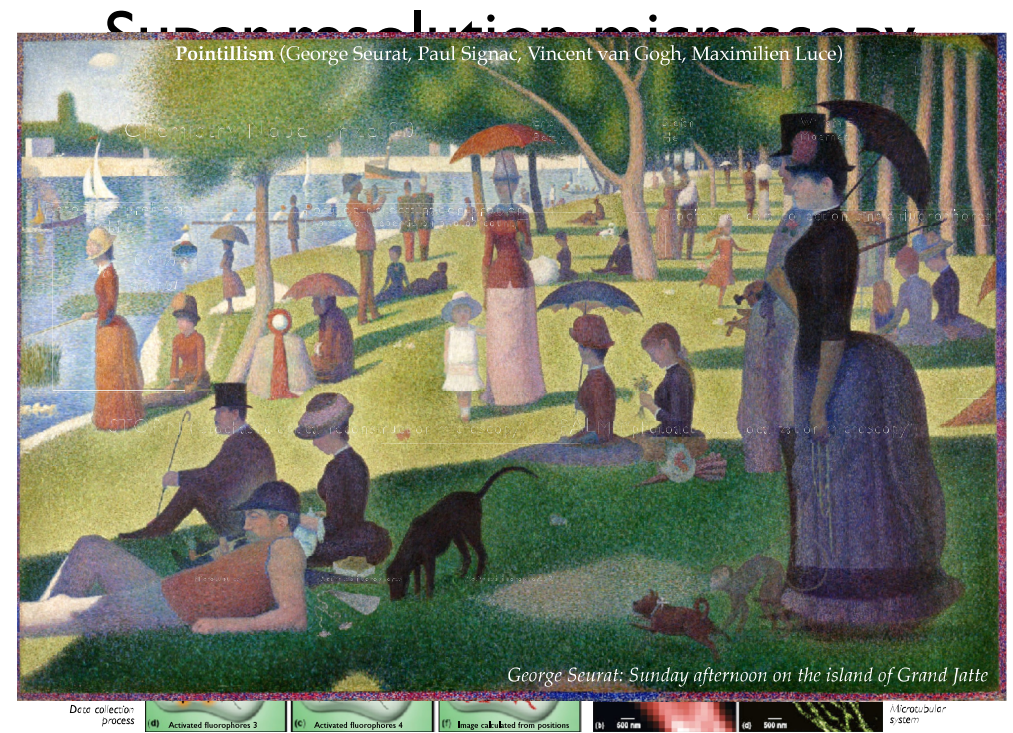
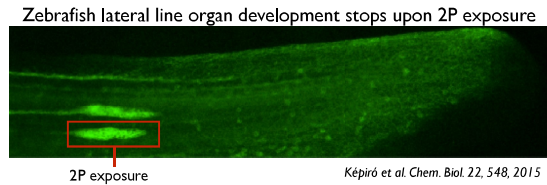
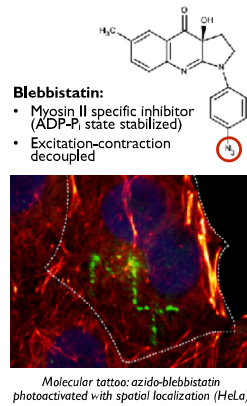
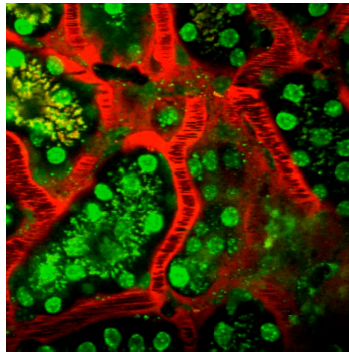
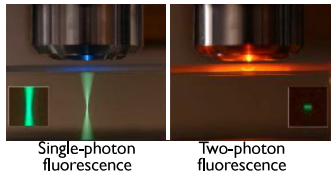
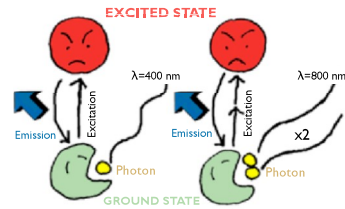


Total Internal Reflection Fluorescence Microscopy (TIRFM)



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

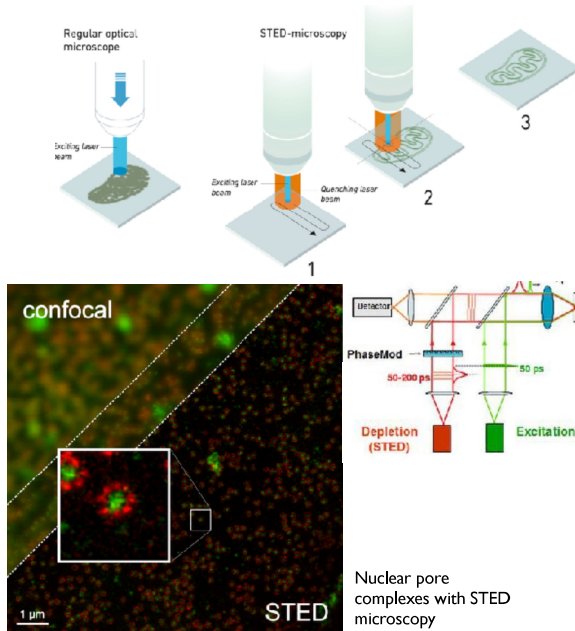
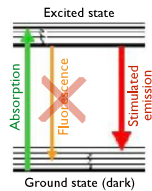


STED microscopy (STimulated Emission Depletion)

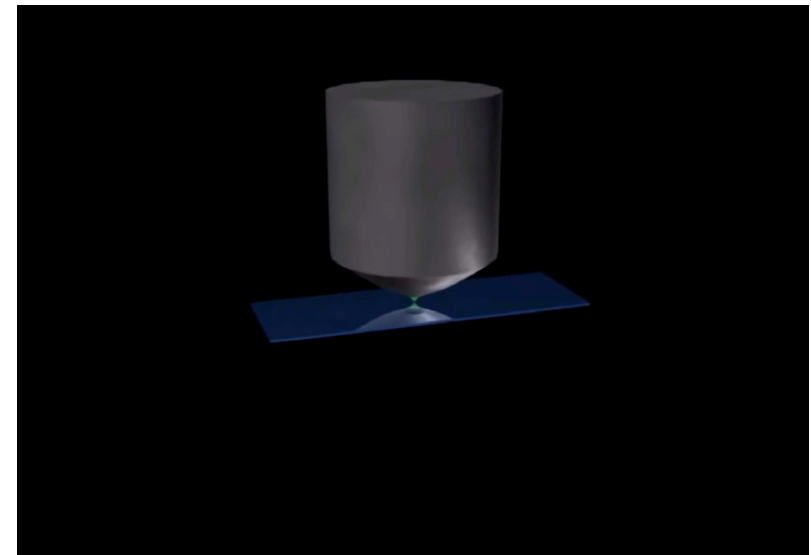


Hell: $d = \frac{\lambda}{2 \cdot NA \sqrt{1 + I/I_s}}$

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



STED microscopy



Jakub Chojnacki

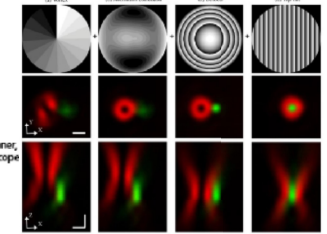
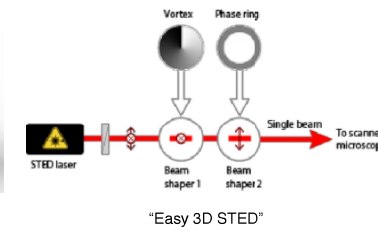
RESCue STED

Problem:
excitation intensity on
the fluorophore is
enormous (~MW/cm²)

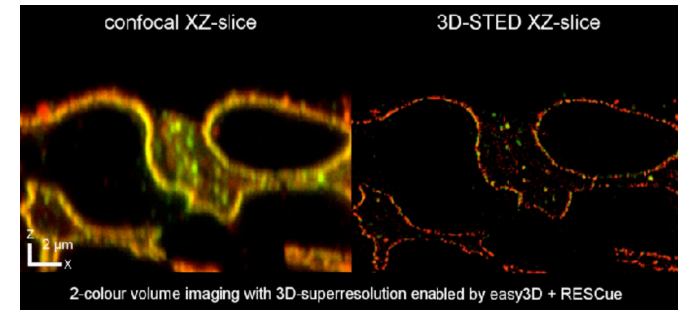
3D STED



Programmable SLM (Spatial Light Modulator)



Aberration correction



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

(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 $c = \lambda f$



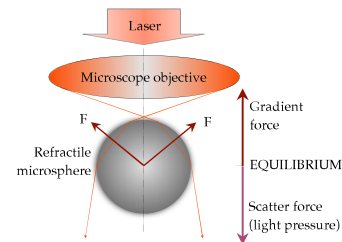
Louis-Victor-Pierre-Raymond, 7th duc de Broglie (1892-1987)

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

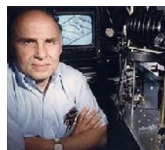
Momentum of the photon:

$$P = \frac{h}{\lambda}$$

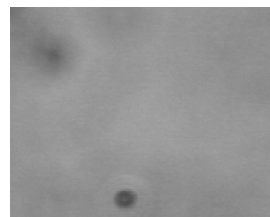
Refractive particles may be **captured** with photonic forces:



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

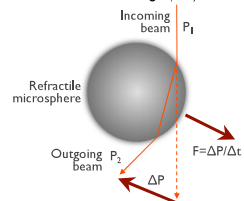


Arthur Ashkin (1970) Nobel-prize 2018



3 μm latex (polystyrene) microspheres in the optical trap

Refraction is accompanied by photonic momentum change (ΔP):



Biomolecule manipulation with light



"Tractor beam", Star Trek

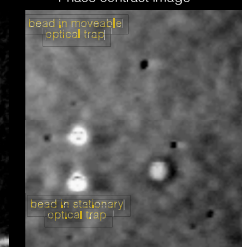
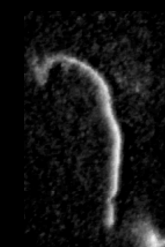


E. coli bacterium

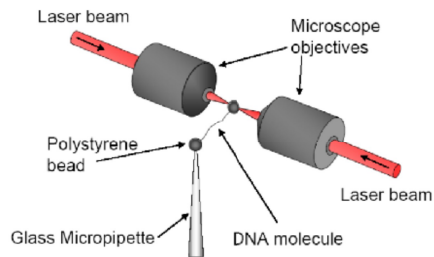
Actin filament

DNA

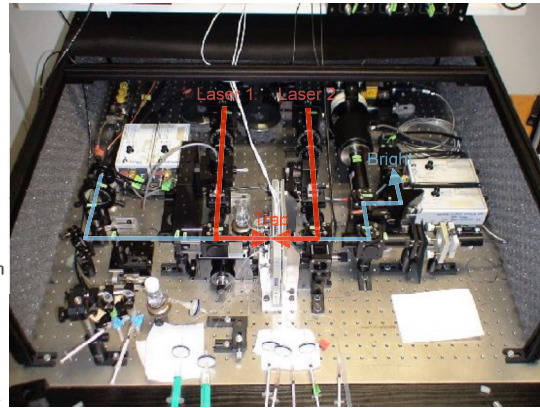
Fluorescence image



Force measurement with optical tweezers



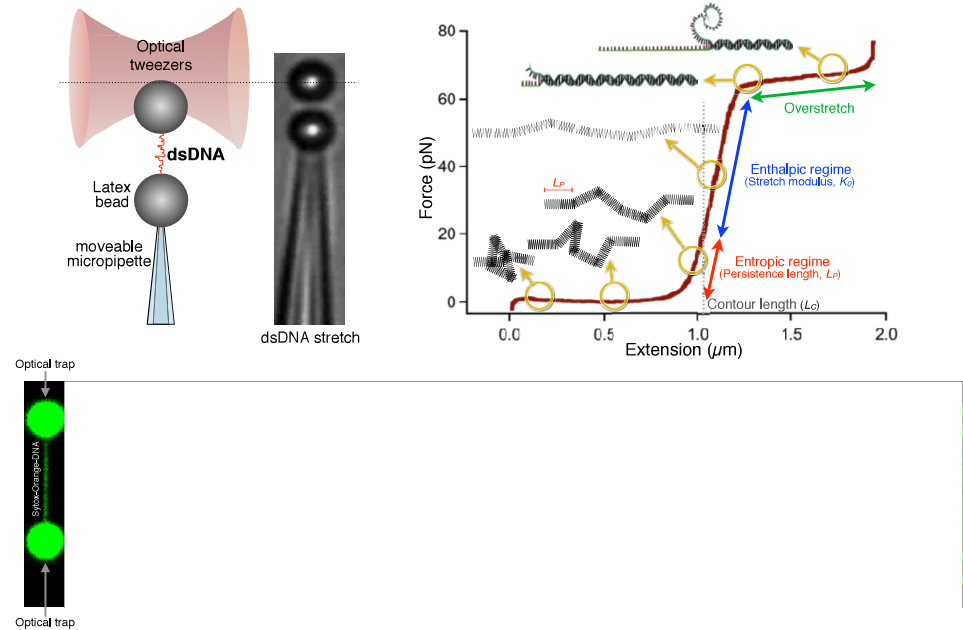
Két lézergusaras optikai csipesz berendezés



Force calibration

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

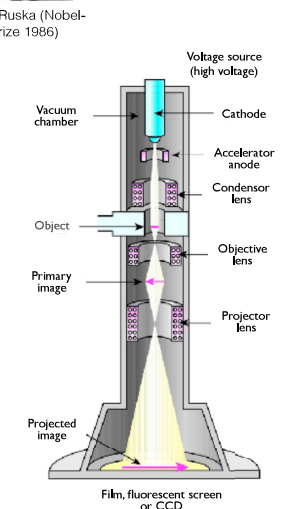
Mechanical extension of dsDNA



Ernst Ruska (Nobel-prize 1986)

Electron microscopy

Uses the electron wave as a matter wave



Transmission electron microscope (TEM)

Ray source:
electron gun

Focusing:
diverting the electron
with magnetic lens

$$F = eBV_e \sin \alpha$$

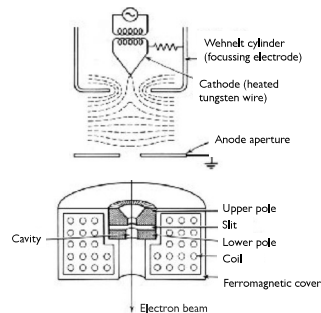
Resolution:

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

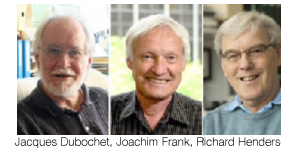
Based on the de Broglie wavelength the theoretical resolution is: $d \sim 0,005 \text{ nm}$ ($\approx 5 \text{ pm}$).

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

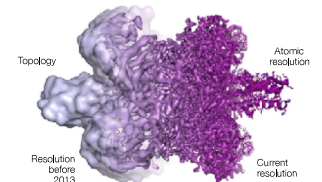
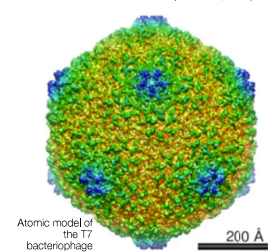
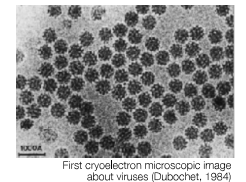
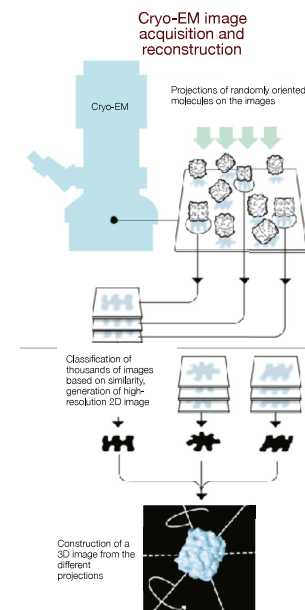
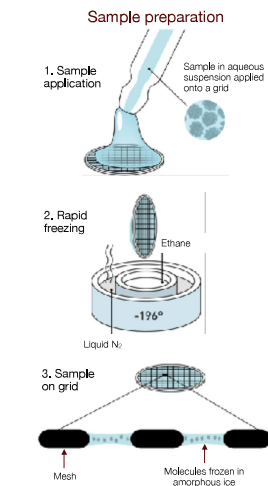
d =smallest resolved distance
 λ =de Broglie wavelength
 α =angle between the optical axis and
the direction of the magnetic field



Nobel-prize in chemistry 2017: cryoelectron microscopy

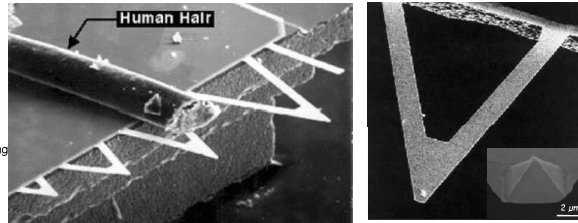
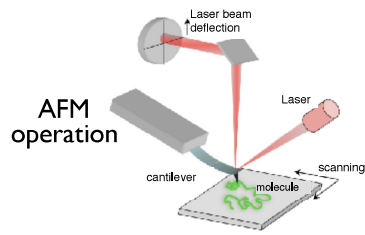


Jacques Dubochet, Joachim Frank, Richard Henderson

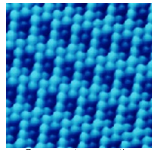


Scanning Probe Microscopies (SPM) I.

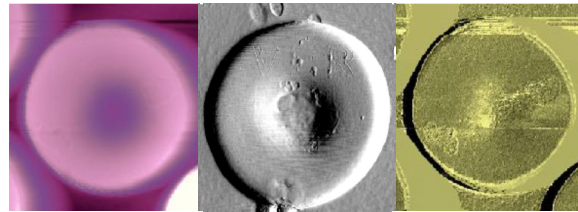
Atomic Force Microscope (AFM)



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



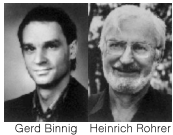
Oxygen atoms on the surface of a rhodium crystal



Height contrast

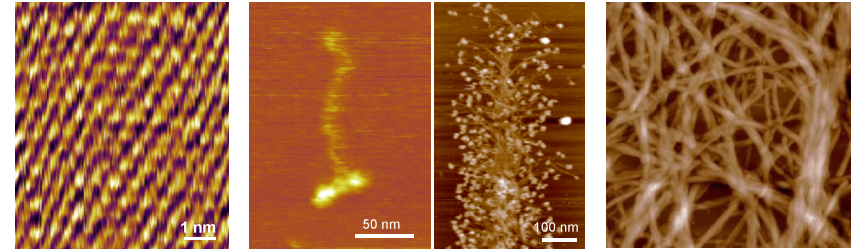
Amplitude contrast

Phase contrast



Gerd Binnig Heinrich Rohrer

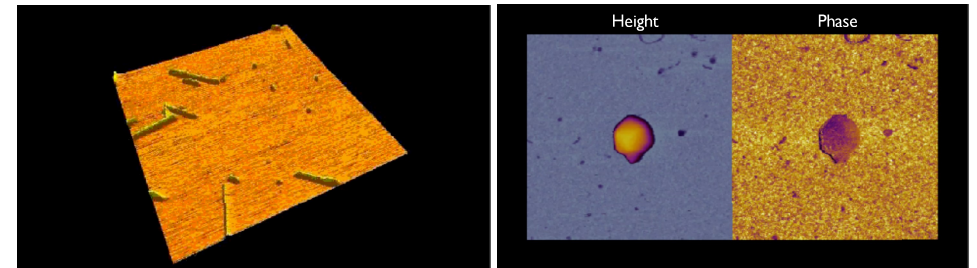
AFM of atoms, molecules, complexes



Mica

Myosin molecule and filament

Amyloid β1-40 fibrils



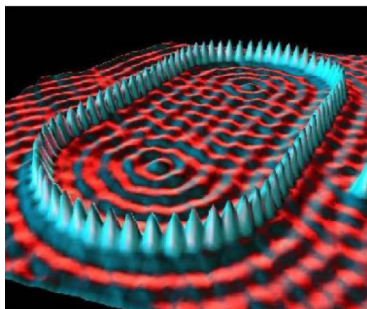
Amyloid fibril growth

Viral DNA ejection

Scanning Probe Microscopies (SPM) II.

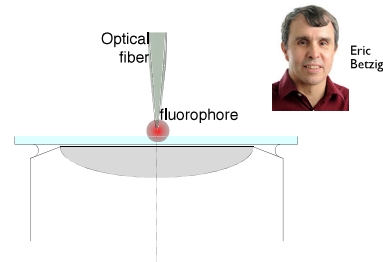
Scanning Tunneling Microscopy (STM)

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)



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

