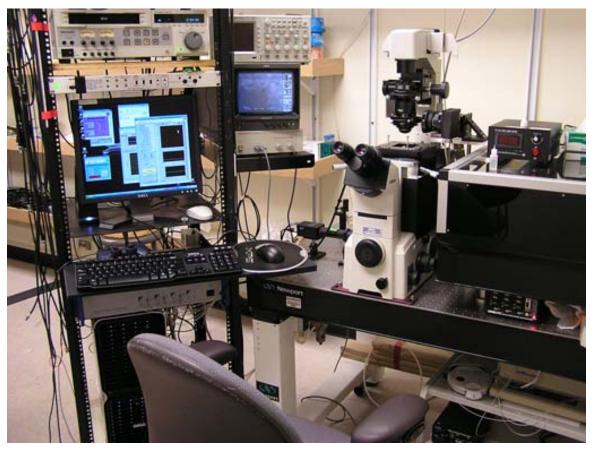# World of the Cell
## *Chapter Appendix: Visualizing Cells and Molecules*

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# Introduction

- Microscopes are <u>indispensable tools for cell biologist</u> because structures to be examined are too small to be seen by eye
- **First useful light microscope** was invented in **1590** by **Janssen & Janssen**
- Later improvements done by **Robert Hooke** to **see cells for the first time** and by **Antonie Leeuwenhoek** to **visualize internal cell structures**
- Development of the **electron microscope** in **1930s** to see structures in the **range of few nanometers** (virus, ribosomes, DNA etc.)
- In the past century many efforts have been made to visualize small molecules, cells or living organisms <u>in their natural environment</u> (**non-destructive microscopy**)

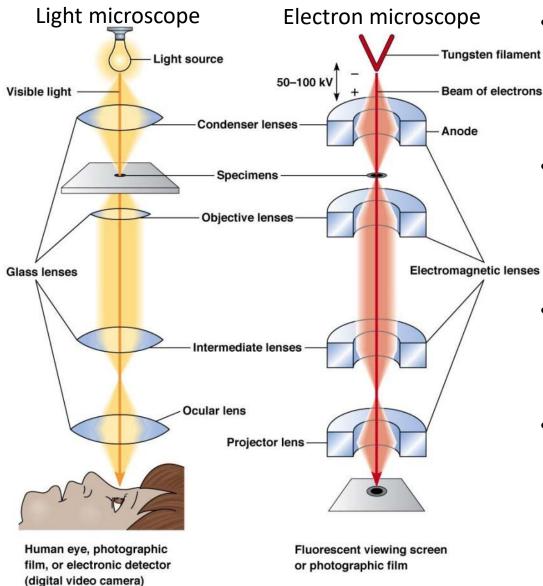Some modern microscopes actually don't really look like microscopes that we use to know:
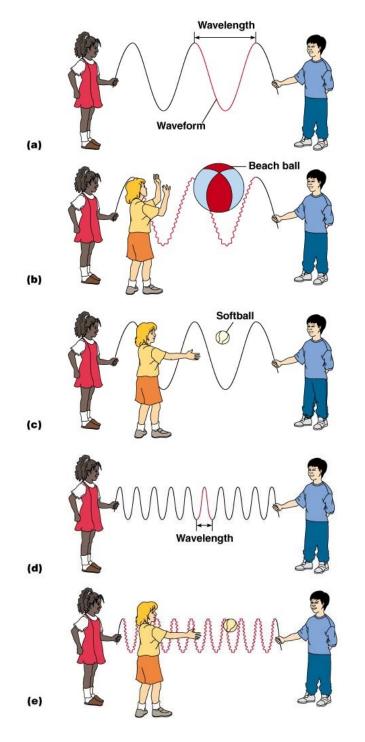
**Optical tweezers setup**

**Scanning tunneling microscope**

# Basic components of a microscope

## Light microscope

- Light source
- Visible light
- Condenser lenses
- Specimens
- Objective lenses
- Glass lenses
- Intermediate lenses
- Ocular lens

Human eye, photographic film, or electronic detector (digital video camera)

## Electron microscope

- Tungsten filament
- 50–100 kV
- Beam of electrons
- Anode
- Specimens
- Electromagnetic lenses
- Projector lens

Fluorescent viewing screen or photographic film

- 3 important components are required for a microscope: **illumination source**, **specimen** and **lenses** which focus the illumination on the specimen and **form the image**
- In the light microscope the illumination source is **visible light** and in the electron microscope it is a tungsten filament to create a **beam of electrons**
- **Glass lenses** are used in light microscopy while in electron microscopy **electromagnetic lenses** are used
- The image can be **viewed by eye** in light microscopy or on a **photographic film** or **on a monitor** via a digital camera (that can also take a digital image)

(a) Wavelength / Waveform

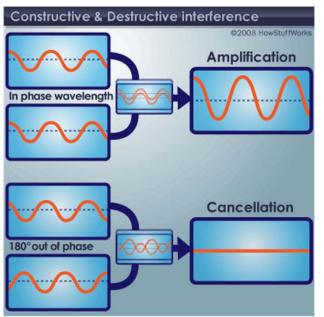(b) Beach ball

(c) Softball

(d) Wavelength

(e)

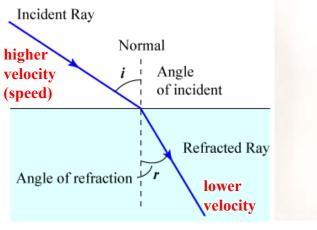# Importance of the wavelength in microscope resolution

- Important for microscopy is **how (or if) a specimen interacts with the wave of light**
- Because the **beach ball** (here our specimen) is of the same size as the wavelength of the rope it will **possibly hit the rope** and disturb/interfere its motion
- A **softball** thrown to this rope is more unlikely to hit the rope because it is **too small**
- If the rope is moved faster up and down the **wavelength will be shorter**. A **softball** thrown to the rope is now **likely to hit it** and to disturb (**interfere**) the motion.
- **Only if an object interferes with the illuminated light it can be detected.** If there is no disturbance of the wave, nothing will be detected.
- Thus, the wavelength determines how small the object can be to be detected.
- The wavelength of light is about 400-700 nm. Hence, objects in this range can be detected.
- Electrons have much smaller wavelength and objects of a few nanometers can be seen (viruses etc.)

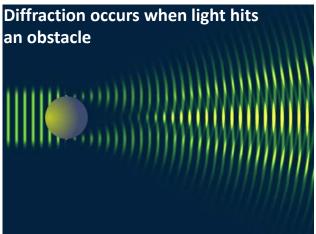# Interference, diffraction and refraction of light waves

- Two types of <u>wave interference</u> can occur: **constructive interference** (waves add up and are amplified) or **destructive interference** (waves are cancelled out)
- If an <u>obstacles is brought into a ray of light</u>, a typical **light diffraction pattern** is visible
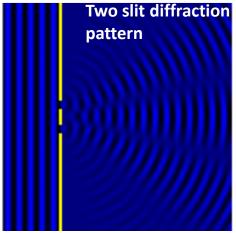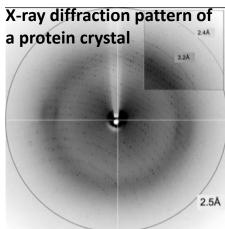- Diffraction can occur with all kind of waves such as light rays, sound waves, water, x-rays…

- **Refraction** <u>is the bending of light</u> that occurs <u>at the interface of two media</u> of different density. **Refraction index** is the **change of velocity** of light in the media.
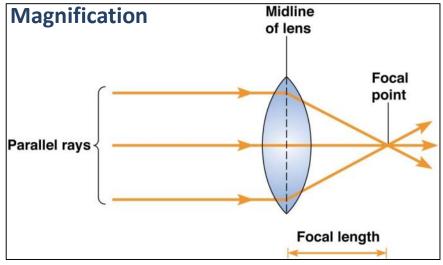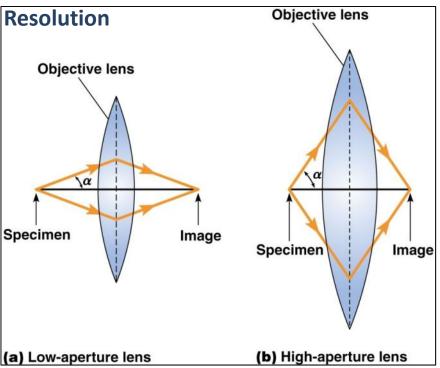


Constructive & Destructive interference
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Amplification

In phase wavelength

Cancellation

180° out of phase



Incident Ray

higher velocity (speed)

Normal

*i* Angle of incident

Refracted Ray

Angle of refraction *r*

lower velocity



**Diffraction occurs when light hits an obstacle**

**Two slit diffraction pattern**

**X-ray diffraction pattern of a protein crystal**

2.4Å

3.2Å

2.5Å

# Focal length and angular aperture



**Magnification** — Midline of lens, Parallel rays, Focal point, Focal length

**Resolution** — Objective lens, Specimen, Image, α; (a) Low-aperture lens, (b) High-aperture lens
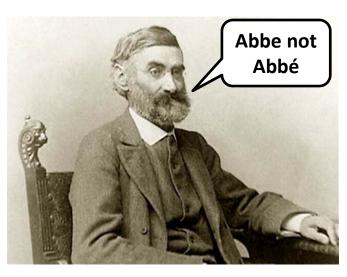
- The **focal length** is the distance between the midline of the lens and the **focal point** (rays passing thru a lens converge to a focus)
- The focal length depends on the **refraction index** of the lens, the **medium** the lens is **immersed** (oil, water, air) and the **geometry** of the lens
- The **inverse** of the **focal length** (1/f) is the magnification strength of a lens (**diopter** [m]). The *shorter* the *focal length* (and the higher the diopter) the *higher* the magnification.
- **Angular aperture** is the *"half angle α of the cone of light entering the objective lens"* in a microscope
- Angular aperture refers to how much light *reflected* **from the specimen** passes thru the lens. Thus, the higher the aperture the more information about the specimen can be acquired (image resolution).
- A high quality lens in a modern microscope has an angular aperture of about 70°.
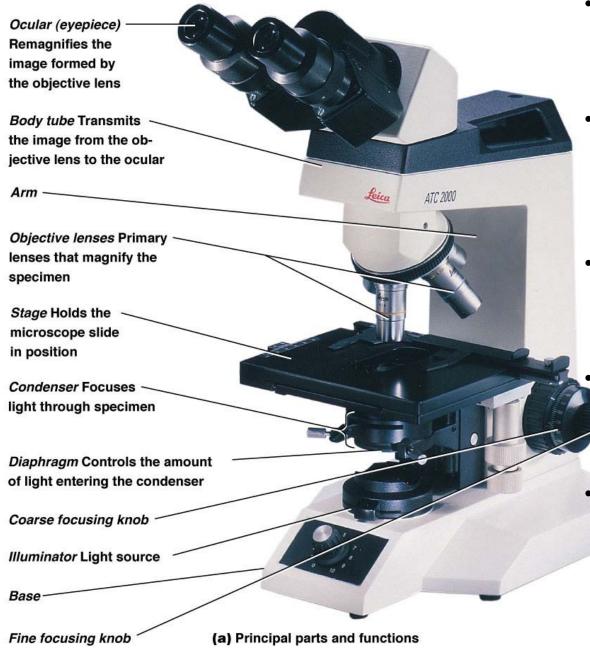
# Resolution and Abbe equation

- **Resolution** is how close two points can be to still be distinguished from each other under the microscope and it is *fundamentally limited by the diffraction of light* (e.g. 450 nm).
- The **angular aperture** is an factor that *influences* a microscope's resolution
- Other factors are the **wavelength of light** (of the illuminating source) and the **refractive index of the medium** the specimen is embedded
- In **1873** German physicist **Ernst Karl Abbe** has identified this relation and postulated the *Abbe equation*:

$$r = \frac{0.612\,\lambda}{n \sin \alpha}$$  r = resolution (or D = **diffraction limit**), $\lambda$ = wave length, n = refractive index of the medium, $\alpha$ = angular aperture

$$r = \frac{0.612\,\lambda}{NA}$$  NA = **numerical aperture** (alternative way to express n sin $\alpha$)
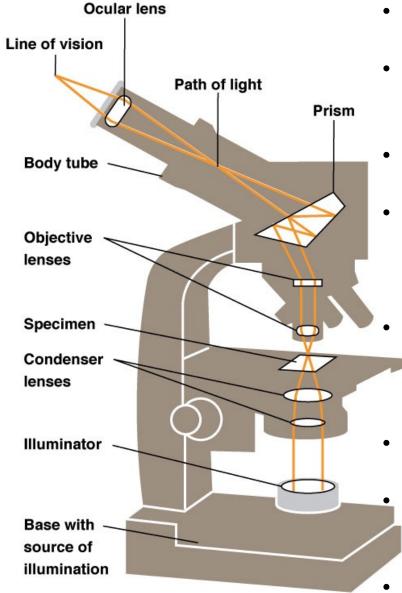
- Having a high quality lens ($\alpha = 70°$) and using low frequent visible light (450 nm blue) the resolution limit of a modern light microscope is about **300 nm** (n = 1 for air)
- Using **immersion oil** instead of air (n = 1.5) the resolution would be around **200 nm** (with UV light even 100 nm)
- Because electrons have a much shorter wavelength, the resolution of electron microscopes is also much better (**2 nm** for biological samples)

**Abbe not Abbé**

# Components of a principal light microscope

**Ocular (eyepiece)** Remagnifies the image formed by the objective lens

**Body tube** Transmits the image from the objective lens to the ocular

**Arm**

**Objective lenses** Primary lenses that magnify the specimen

**Stage** Holds the microscope slide in position

**Condenser** Focuses light through specimen

**Diaphragm** Controls the amount of light entering the condenser

**Coarse focusing knob**

**Illuminator** Light source

**Base**

**Fine focusing knob**

**(a) Principal parts and functions**

- Because a regular light microscope uses several lenses in combination it is also called **compound microscope**
- The arm of a microscope holds the **objective revolver** that can be turned to use different objectives of different magnification (100x, 60x, 10x...)
- It is important to read the labels of the lenses as they might be optimized for different media (air, water immersion oil)
- Advanced microscopes have automated revolver and the magnification can be changed by software.
- The importance of the **microscope arm** also lies in the reduction of vibration and mechanical drifts that may shift the sample on the stage

# Light path of a principal light microscope



Ocular lens
Line of vision
Path of light
Prism
Body tube
Objective lenses
Specimen
Condenser lenses
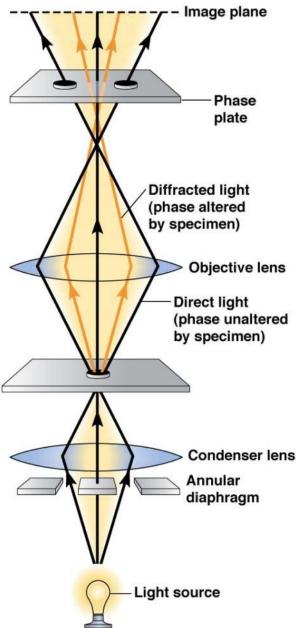Illuminator
Base with source of illumination
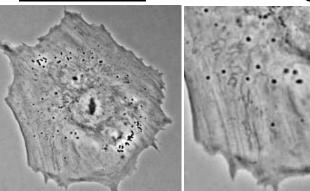
(b) The path of light (bottom to top)

- The optical path begins with a **source of illumination**
- The illumination is placed <u>in the base of the microscope</u>. Advanced microscopes use **mercury/xenon** lamps or **lasers**.
- The **condenser** <u>collects and focuses the light</u> onto the specimen
- The specimen is often <u>mounted on a glass slide</u> with a thin <u>cover glass</u> on top. Advanced microscopes have movable stages in X/Y directions that is **motorized** and can be controlled by software.
- The **objective lens** is the primary lens for magnification of the *reflected light from the specimen.* The light from the objective lens is then <u>directed by a prism to the ocular lens</u>.
- The **ocular lens** adds <u>some additional magnification</u> and is typically 5x or 10x (usually exchangeable)
- Multiplying the magnification of the ocular lens and the objective lens leads to the <u>final magnification</u> (if no intermediate lens present)
- Such a standard (and inexpensive) microscope is also called **brightfield microscope**

# Phase contrast microscope amplifies variations in refractive indices of the specimen



- Image plane
- Phase plate
- Diffracted light (phase altered by specimen)
- Objective lens
- Direct light (phase unaltered by specimen)
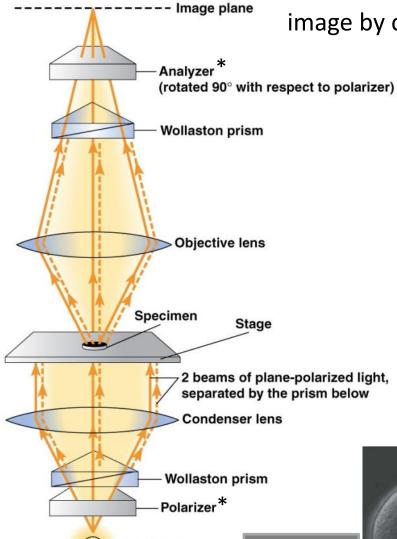- Condenser lens
- Annular diaphragm
- Light source

- The importance of the phase contrast microscope lies in the ability to observe specific structures inside the cell that cannot be seen in bright field microscope
- The phase contrast microscope is able to visualize cellular structures that vary in thickness and refractive index
- Non-invasive method: Cells **do not need to be stained** (no use of dyes) and fixed (killed due to staining process)
- More **thicker structures slow down the velocity of light rays changing the phase of these waves**
- The phase contrast microscope is able to convert phase changes into differences in the brightness of the light (thicker structures then appear darker)
- Important is the **phase plate** that **accelerates direct light** (unaffected by specimen) and **slows down** the light that comes from the specimen (**diffracted light**). These two types of light **interfere** causing **stronger contrast**.

## DIC contrast microscopes deliver a 3D impression of an image by creating shadow-forming illusions

**Image plane**

**Analyzer*** (rotated 90° with respect to polarizer)

**Wollaston prism**

**Objective lens**

**Specimen** — **Stage**

2 beams of plane-polarized light, separated by the prism below

**Condenser lens**

**Wollaston prism**

**Polarizer***

**Light source**

* Polarizers are needed for this technique to work

Chromosomes in DIC

- **Differential interference contrast microscopy** (DIC) or ***Nomarski*** microscopy (named by its inventor) provides a 3D type of impression of structures with high refractive indices
- A **prism splits** *polarized rays* of the light source into **two separate beams**
- When they pass thru the specimen, any changes caused by different thickness will **cause** these **two polarized beams** **to interfere**
- The 3D impression arises from a **shadow-forming illusion** as **one edge** of the thick structures **appears brighter** than the other edge
- The **second prism** combines the two light beams again into one

# Fluorescence microscopy detects singles molecules in cells
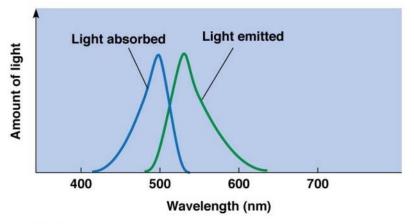
- To **detect specific molecules**, proteins, enzymes etc. in cells, a technique named **fluorescence microscopy** was developed
- Fluorescence happens when <u>high frequent light shines</u> on an **atom** from which an **electron** <u>jumps to an higher energy state</u>. <u>When it falls back</u> to its ground state it eventually <u>emits</u> a **photon** (that is of *longer* wavelength than the excitation light).



(a) Energy diagram

- Advanced (and commercially available) fluorescent molecules have characteristic **absorption and emission spectra** important <u>to understand the use of specific filters</u> in the microscope
- The <u>excitation</u> peak of **GFP** is around **488** (blue) and <u>emission</u> is around **509** (green)
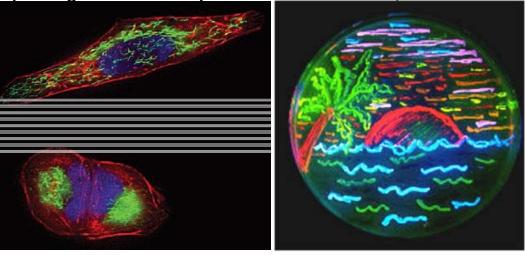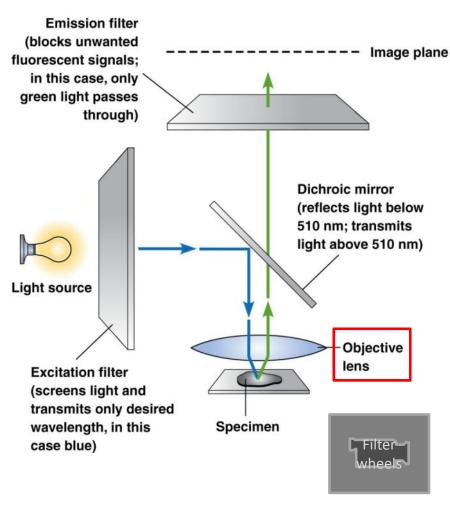


(b) Absorption and emission spectra

**Interphase and dividing cell expressing different fluorophores**



**Colonies of bacteria expressing different fluorophores**
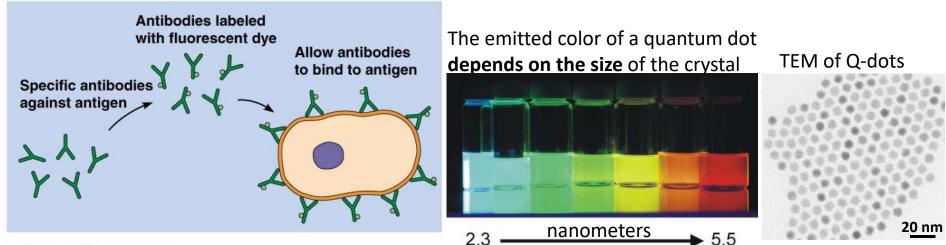
# Optics of the fluorescence microscope



Emission filter (blocks unwanted fluorescent signals; in this case, only green light passes through)

Image plane

Dichroic mirror (reflects light below 510 nm; transmits light above 510 nm)

Light source

Objective lens

Excitation filter (screens light and transmits only desired wavelength, in this case blue)

Specimen

Filter wheels
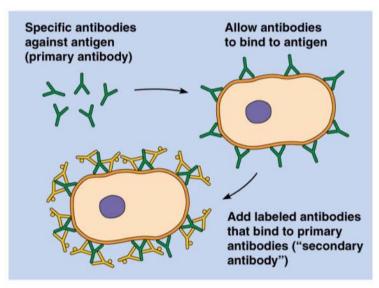
*This is the scheme of an upright fluorescence microscope. Many are actually inverted which is more convenient for cell culture observation.*
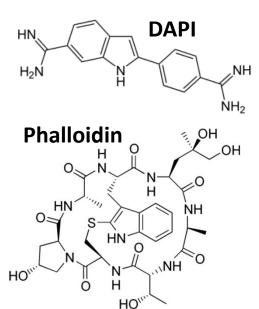
- The light source of a standard fluorescence microscope is usually a (powerful) **mercury, xenon** or **LED** lamp.
- **Excitation filter** lets only a certain wavelength pass (e.g., blue for GFP excitation)
- Some microscopes have a filter set to excite at different wavelengths
- **Dichroic mirrors** reflect and transmit light only below or above a certain wavelength
- Here, the blue light hits the dichroic mirror that reflects the light onto the specimen *through the objective lens*. Specimen then emits fluorescent light (green).
- Fluorescent light passes thru the objective lens and the dichroic mirror and is later filtered by an **emission filter**.
- Because the light passes first thru the objective lens and then shines "upon" the specimen this type technique is called **epifluorescence** microscopy (epi- = Greek = upon; also epidermis = "upon the dermis")

# The use of fluorescent probes

- Besides GFP, so called **fluorescent probes** (dyes) can be used as indicators to visualize molecules and even ions (e.g., calcium) in cells (green **fluorescein** (FITC) or red **rhodamine**).
- **Immunostaining** is a popular method that employs **fluorescent antibodies**
- An antibody (or immunoglobulin) is a large Y-shaped protein (produced by B-cells) to identify and neutralize foreign objects such as unwanted proteins, bacteria and viruses (= **antigens**). The tip of the "Y" has two antigen binding sites.
- Antibodies are generated by injecting a foreign protein (antigen) into an animal (rabbit, mouse etc.) and to collect the blood serum (with the **antibodies**) afterwards.
- Techniques exist to link a fluorescent molecule to an antibody so the targeted antigen can be visualized.
- Novel fluorescent labels are **quantum dots** which are nanoscale crystals with semiconductor properties that emit light after excitation (e.g., with UV light).



(a) Immunofluorescence

The emitted color of a quantum dot **depends on the size** of the crystal

2.3 ⟶ nanometers ⟶ 5.5

TEM of Q-dots

20 nm

# The use of fluorescent probes



Specific antibodies against antigen (primary antibody)

Allow antibodies to bind to antigen

Add labeled antibodies that bind to primary antibodies ("secondary antibody")
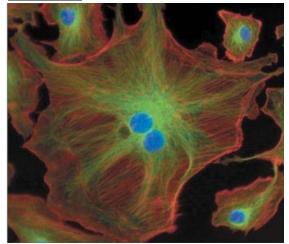
**(b)** Indirect immunofluorescence
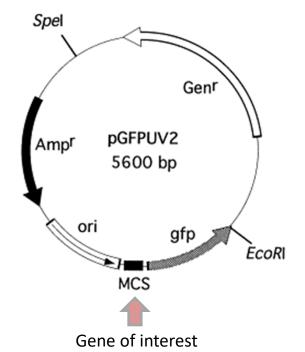
- **Indirect immunofluorescence** means that the cell is incubated with an **primary antibody** (non-fluorescent) and a **secondary antibody** (fluorescent) is used against the first antibody.
- In this case the primary antibody acts as the antigen for the secondary antibody.
- Because more than one fluorescent antibody can bind to the primary antibody, the signal will be enhanced making this technique more efficient than direct immunofluorescence

Other important fluorescent probes are **Fura-2** that changes its color based on Ca$^{++}$ gradients, **DAPI** that binds to A-T-rich regions in DNA or **phalloidin** (a mushroom toxin) that binds to actin and can be **linked to a fluorescent dye**



**DAPI**

**Phalloidin**
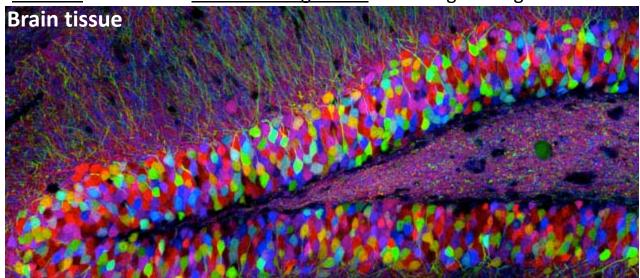


- **Green**: anti-tubulin (primary antibody) with secondary green fluorescent antibody
- **Blue**: DAPI
- **Red**: Texas red phalloidin

# The use of fluorescent probes

- The advantage of using **GFP** is that it <u>does not rely on available antibodies</u> (which are also <u>expensive</u>). Also <u>immunocytochemistry</u> often requires <u>fixing</u> (killing) <u>of cells</u>.
- For **GFP expression** a <u>gene of interest</u> is <u>inserted</u> (cloned) <u>into a DNA</u> (ring) <u>plasmid</u> that is introduced into cells.
- Cloning requires several steps to <u>open the DNA ring</u> with **restriction enzymes** (at the multiple cloning site, **MCS**), to <u>insert the gene of interest</u> and to <u>close the ring</u> (**ligation**).
- These steps are <u>carried out in *E. coli*</u> bacteria so the vector contains <u>antibiotic resistance genes</u> (*amp*icillin or *gen*tamicin) for the selection of successful clones
- To introduce the DNA into cells, **microinjection**, **transfection** (with <u>liposome vehicles</u>) or **transduction** (with <u>SV40 virus infection</u>) are the primary methods.



*SpeI*
Gen^r
Amp^r
pGFPUV2
5600 bp
ori
gfp
*EcoRI*
MCS
Gene of interest
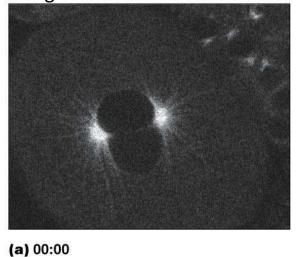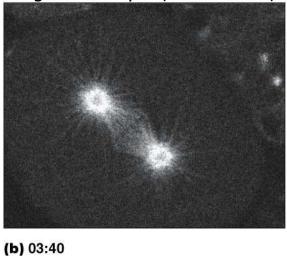
Brainbow ("brain and rainbow"): *randomly* <u>expressing different ratios</u> of red, green, and blue fluorescent protein in neurons. <u>Individual neurons</u> in the brain <u>can be distinguished</u> from neighboring neurons.



**Brain tissue**

# The use of fluorescent probes
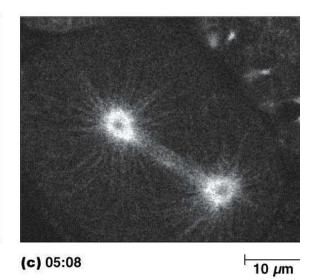
The importance of GFP also lies in the possibility of live cell imaging

Image series of mitotic events in *C. elegans* embryos (tubulin-GFP)



(a) 00:00          (b) 03:40          (c) 05:08          10 μm

Mitosis in breast cancer cells
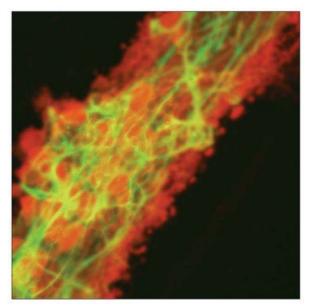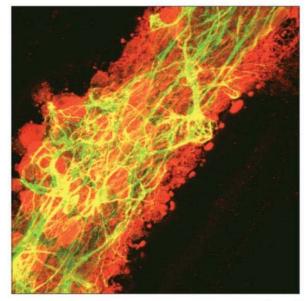
# Laser scanning confocal microscopy (LSCM)



(a) Traditional fluorescence microscopy
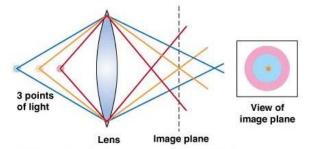


(b) Confocal fluorescence microscopy
25 µm

- In <u>traditional fluorescence microscopy</u> the image seen is a result of the emitted <u>fluorescence from all layers</u> within the cell: The **image tends to blur**.
- In confocal microscopy <u>only one focal layer (plane) is imaged</u> (*confocal = "having the same focus"*) excluding the fluorescence from layers above and below: The image becomes **sharper and more crisp**.
- To accomplish <u>visualizing only one image plane</u>, a **laser is employed** (laser scanning confocal microscopy, LSCM).
- A laser has also the advantage that it can <u>image several focal layers</u> (**Z-plane**) that can be combined into a **3D image**

Slicing thru a cell

# LSCM: Focal plane principle and pinhole add-on
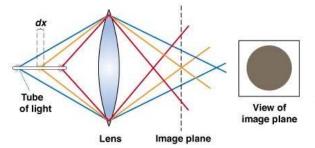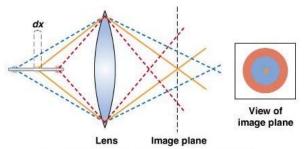


(a) Formation of an image of a single point of light by a lens



(b) Formation of an image of a point of light in the presence of two other points



(c) Formation of an image of a section of an equally bright tube of light
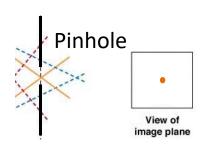


(d) Formation of an image of a brightened section of a tube of light

- Imaging a <u>pointed light source</u> normally results in a <u>sharp point</u> that is <u>surrounded by a halo</u> (blur of light)
- <u>Imaging three different objects</u> located *in different focal planes* (blue, orange, red) results in visualizing the object that is in the image plane and other objects as halos

  <u>Relations between light source, lens and the seen image:</u>
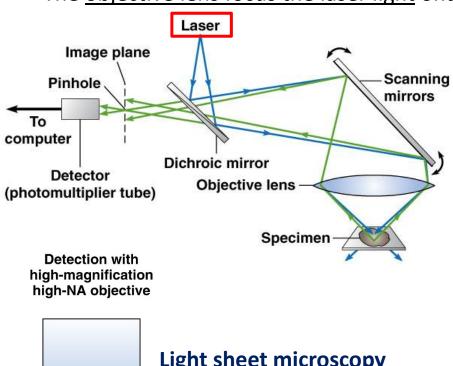
  $$\frac{1}{f} = \frac{1}{o} + \frac{1}{i}$$

  f = focal length
  o = distance object (light) to the lens
  i = distance image to the lens

- A cell <u>expressing countless GFPs</u> (in different focal planes) is more like "imaging a tube of light". The result is a <u>completely blurred image</u> (individual structures such as represented by the ideal "3 points of light" are difficult to be resolved)
- Even looking at only one section (dx) of the tube results in the same blurred image due to the <u>many merged light points</u>
- However, illuminating a **defined focal plane** with a strong **laser** would result in an <u>contrast rich</u> image of the desired object *due the exclusion of other layers*
- Using an additional **pinhole** (<u>aperture</u>) would even eliminate more blur (out-of-focus rays)



Pinhole

# Components of the LSCM

- In the LSCM the <u>laser light</u> (e.g., blue excitation) is <u>reflected by a dichroic mirror</u> **to a scanning mirror** that guides the light <u>to the objective lens</u>
- The <u>objective lens focus the laser light</u> onto a defined (diffraction-limited) spot in the cell



Laser

Image plane

Pinhole

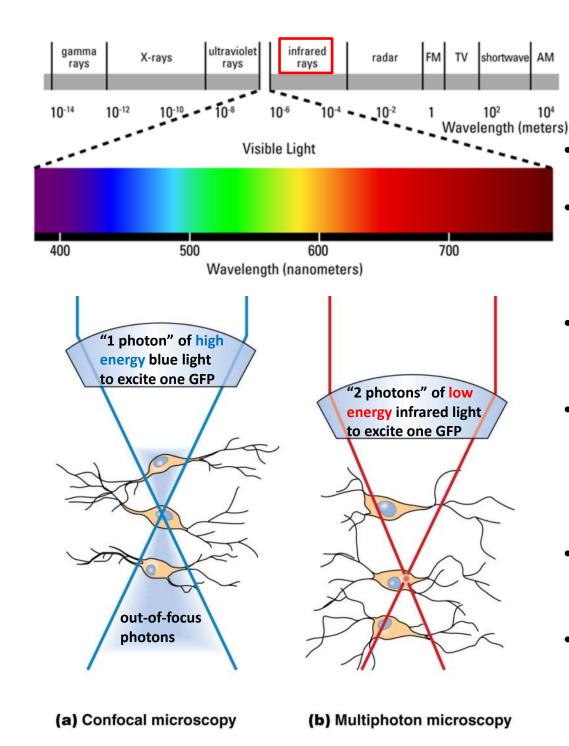To computer

Detector (photomultiplier tube)

Scanning mirrors

Dichroic mirror

Objective lens

Specimen

Detection with high-magnification high-NA objective

**Light sheet microscopy**

Light sheet

Illumination with low-magnification objective

- The <u>scanning mirrors</u> can <u>move the laser light in a precise pattern</u> within the desired focal plane
- The **emitted light** (e.g., green) <u>goes back thru the same path</u>, however, it can **pass** thru the **dichroic mirrors**
- A **pinhole** in the image plane <u>reduces blur</u> from out-of-focus rays
- <u>Signal detection</u> occurs via a photomultiplier or (more often) via a **CCD camera**
- The **image** can be **processed** by a computer (<u>image enhancement</u>)
- In **light sheet microscopy** the specimen is illuminated from the side (and not from top) resulting in <u>less "photodamage"</u> since only a <u>thin plane</u> (and not the whole sample) of the specimen is illuminated

Spinning Disk Microscope Configuration

Spinning disk
Wagner-Lab

# Spinning disc microscope

- <u>Disadvantage of LSCM</u> is that the strong laser can cause **GFP bleaching**
- GFP bleaching means that <u>single GFP proteins are **destroyed** by the laser</u> reducing and **fading** GFP signals
- To circumvent this problem the (Nipkow) <u>spinning disc microscope was developed</u>
- Two fast spinning discs contain <u>arrays of microlenses and pinholes</u>, respectively
- Thus, the specimen is only exposed to very short "laser pulses"
- Though only single laser spots are illumi-nating the image, a <u>continuous image is seen</u> as the <u>discs are rotating very fast</u>
- **Image acquisition is very fast** (laser does not need to scan across the whole image) and <u>useful for imaging of rapid and dynamic events in cells</u> (vesicle transport)
- Disadvantage is that it <u>cannot produce slices as thin as produced by an LSCM</u> (and the resolution is not as good as in LSCM based on the quality of microlenses )
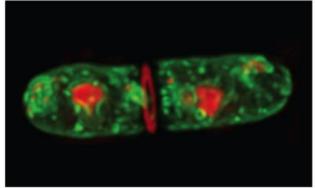
# Two-photon and multi-photon excitation microscopy



(a) Confocal microscopy

(b) Multiphoton microscopy

- To excite a GFP, the high energy of shortwave blue light is needed
- However, it is also possible to excite a GFP when using the lower energies of two photons from **longwave infrared light**
- Each infrared light photon carries half of the energy needed to excite the GFP
- The advantage of infrared light is that it is able to penetrate deep into the tissue. Thus, two-photon microscopy is the choice when **examining thick tissues**
- Because such fluorescence events occur only very near the focal plane **no pinhole is needed**
- Also photobleaching is reduced because of the very few out-of-focus photons

# Deconvolution microscopy and digital image processing

**Fission yeast cell (red: DNA)**



(a) Images series before…



(b) … and after processing

4 μm

- A deconvolution ("deblurring") microscope is a <u>standard fluorescence microscope</u> with a **special software**
- In deconvolution microscopy a <u>series of images throughout the thickness</u> (Z-plane) of the specimen <u>is acquired</u>
- A microscope with a <u>motorized stage</u> allows for the automation of this process
- A computer software <u>mathematically removes the blur</u> caused by <u>out-of-focus rays</u> from each plane and assembles all slices into a single (3D) image
- **Digital image processing** becomes an important tool to <u>enhance images</u> and to <u>reduce background</u>
- <u>High speed and high resolution</u> **EMCCD cameras** allow for the recording of fast processes in cells (<u>time-lapse imaging</u>)
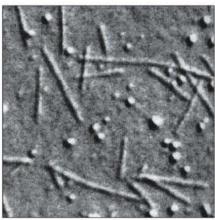


(a) **Contrast enhancement**

(b) **Image background**

(c) **Background subtraction**
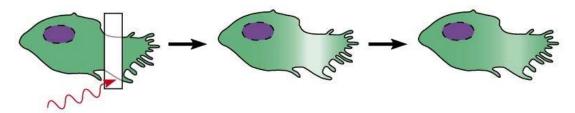
(d) **Image averaging**
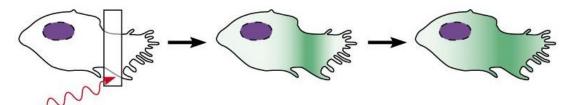
2.5 μm

# FRAP (fluorescence recovery after photobleaching), photoactivation and photoconversion

- **Photobleaching** is <u>not only</u> an unwanted phenomenon in fluorescence microscopy
- It can be used to <u>measure the dynamic behavior of molecules</u> in cells
- A specific <u>area can be bleached</u> with a **strong laser** (GFPs "destroyed") and the **speed of diffusion** of neighboring GFP-labeled proteins into the bleached area **determined**
- This method is called **FRAP** (<u>fluorescence recovery after photobleaching</u>)
- **Photoconversion** is in principle the <u>opposite method</u>: When light (usually UV light) is focused on a specific area, "special" <u>GFP molecules can be activated</u> (**photoactivation**)
- This is possible with **modified GFP** that <u>only emits green light when exposed to UV light</u>
- Some fluorescent molecules are enveloped by a shielded cage (**caged compounds**): UV light induces a <u>cleavage process</u> to **uncage** (**free**) the compound (e.g., caged ATP)
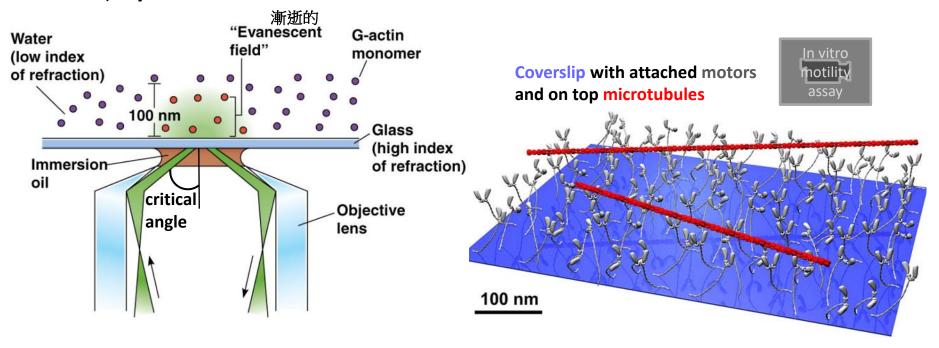


**(a) Photobleaching**
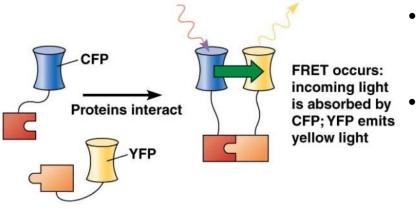


**(b) Photoactivation**
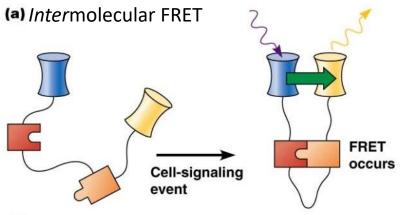
# Total internal reflection microscopy (TIRF)

- Total internal reflection microscopy (**TIRF**) is a microscopy technique <u>useful to investigate objects which are located</u> **very close to the coverslip**
- Examples are, focal adhesions, basal lamina, vesicle secretion in neurons, membrane dynamics, *in vitro* actin polymerization or *in vitro* motility assay
- When <u>light moves</u> from a <u>medium with a high refractive index</u> (glass) to a <u>medium with a much lower reflective index</u> (water/cell) then the light is (**totally internal**) **reflected**
- Though the light appears to completely go back into the objective lens <u>a small light field very near to the surface of the glass is generated</u> (**evanescent wave field**)
- Thus, <u>fluorescence only occurs at a 100 nm thin layer</u> and **excludes all other** (more upper located) **layers in the cell**

# Fluorescence resonance energy transfer (FRET)



(a) *Inter*molecular FRET



(b) *Intra*molecular FRET



(c) FRET when Ras is active
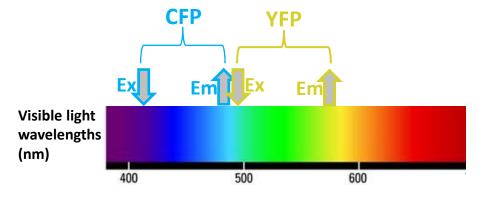
- If two fluorophores (with matching properties) are close enough (about 10 nm) a phenomenon known as **resonance energy transfer** occurs.
- If **CFP** (donor) is excited at the **blue-violet range** it will emit **cyan fluorescence**. This fluorescence is the right excitation fluorescence for YFP (acceptor). Thus, **yellow light** will be emitted.
- If the FRET donor and acceptor are NOT close enough the cell will emit **cyan light**
- Though no photon exchange occurs in FRET a photon will be emitted by the acceptor
- FRET can also be used to investigate **intramolecular folding of proteins** (acceptor and donor fused to the protein's side chains)
- FRET is further useful to **determine protein activities** in cells

# Optical tweezers (optical trap)
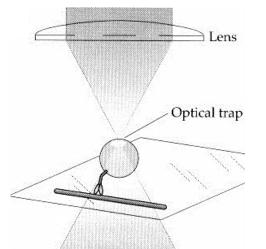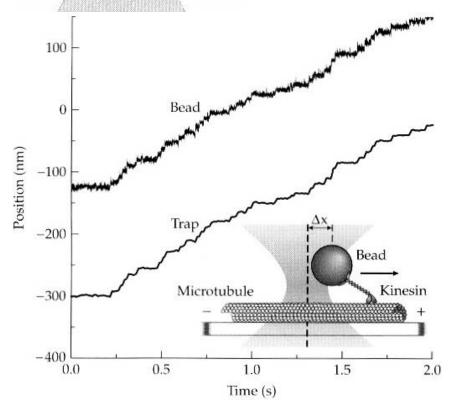
- In a **highly focused** (divergent) **laser beam** a <u>force termed as</u> **light pressure** happens
- These "differential forces" are <u>able to hold</u> **small plastic beads** <u>in position</u>
- If a myosin (or kinesin) is <u>attached to the bead</u> the **step size** of the motor on the polymer (actin or microtubule) can be **determined**
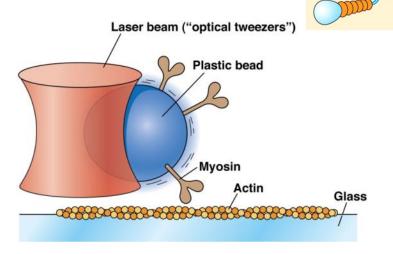
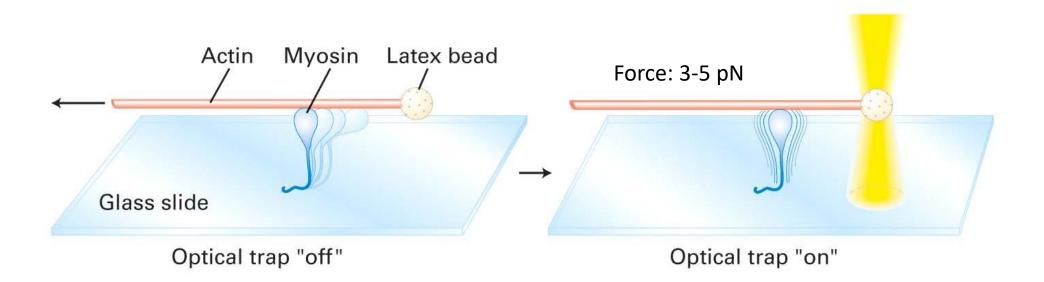Step size kinesin: **8 nm** (length of one tubulin)
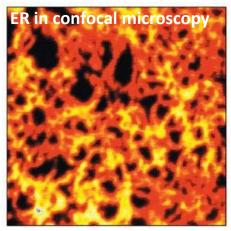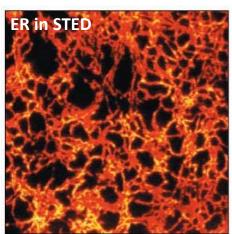Step size myosin II = 5-10 nm
Step size myosin V = 36 nm

myo II

myo V

# Optical tweezers (optical trap)

- Vice versa, a <u>bead can be also attached to a cytoskeletal filament</u>
- When the <u>motor pulls on the filament</u> the <u>force exerted on the filament can be measured</u> in relation to the bead displacement
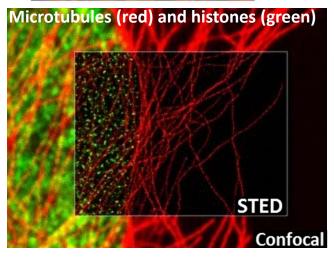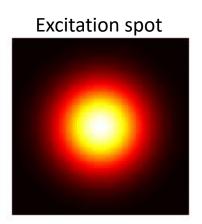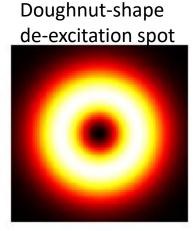


Actin   Myosin   Latex bead

Glass slide

Optical trap "off"

Force: 3-5 pN

Optical trap "on"

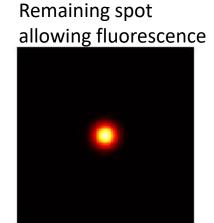ER in confocal microscopy


ER in STED

# Superresolution microscopes: STED, STORM and PALM

- Recently, attempts have been made to <u>push the limit of light microscopes</u>
- Especially <u>to overcome the problem of diffraction</u> in the Abbe equation
- These microscope techniques are collectively called **superresolution microscopes**
- In **STED** (**stimulated emission microscopy**) <u>short laser pulses</u> are used to stimulate emission of photons.
- However, <u>after the first pulse a second **ring-shaped depletion pulse** follows that moves the excited electron to a lower energy state</u> (**de-excitation**)
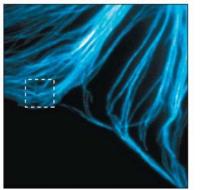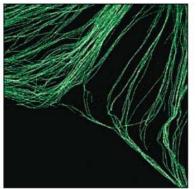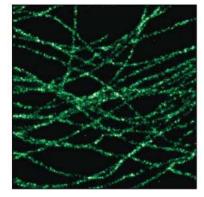- The result is a <u>very small spot for emission stimulation</u>


Microtubules (red) and histones (green)
STED
Confocal


Excitation spot


Doughnut-shape de-excitation spot


Remaining spot allowing fluorescence

# Superresolution microscopes: STED, STORM and PALM
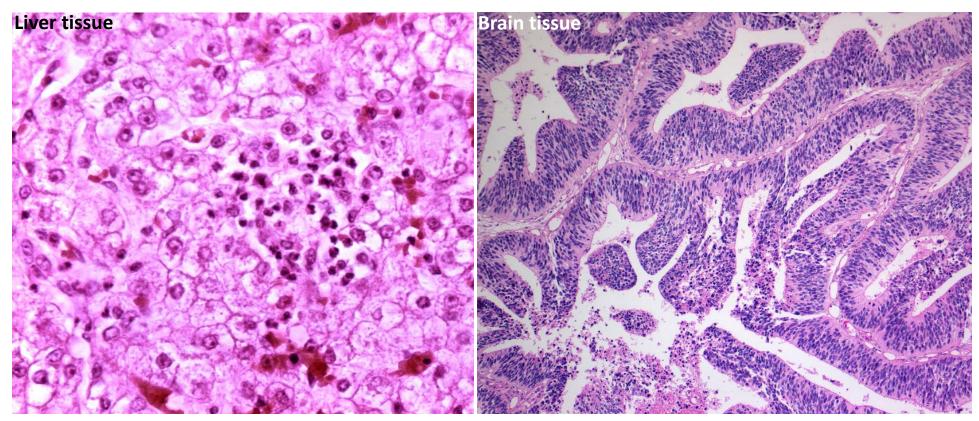

Anti-tubulin in epifluorescence




Anti-tubulin in STORM



Superresolution MTs

Superresolution kinesins

- **STORM** (<u>st</u>ochastic <u>o</u>ptical <u>r</u>econstruction <u>m</u>icroscopy) and **PALM** (<u>p</u>hoto<u>a</u>ctivated <u>l</u>ocalization <u>m</u>icroscopy) use **photoactivatable** (or photoswitchable) **fluorophores** (that can be easily and quickly turned on and off)
- <u>Sequential activation</u> of photoswitchable fluorophores <u>create high resolution images</u> due to the reduction of blur
- After a fluorophore subset is **activated**, **deactivation follows** (and the next fluorophore subset is activated and imaged)
- Super-resolution images are **constructed via software** (<u>postprocessing of image data</u>)
- PALM is also called **fPALM** or **FPALM** meaning <u>f</u>luorescence <u>p</u>hoto<u>a</u>ctivated <u>l</u>ocalization <u>m</u>icroscopy
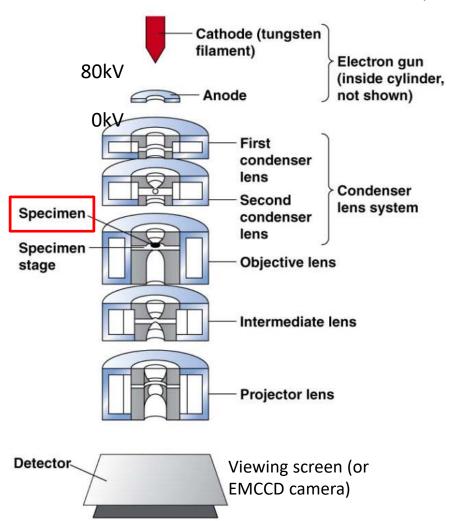
# Sample preparation in light microscopy

- "No good microscopy without good sample preparation."
- In **histology**, thin slices of tissues are stained with specific dyes that make particular cell types and cellular structures visible (also serving as **contrast enhancers**)
- To dye (stain) cells or tissues they often have to be treated with fixatives first to **preserve cell components**
- Most common fixatives are **formaldehyde** or **glutaraldehyde** which are crosslinking fixatives as they **create covalent chemical bonds** between proteins in tissues
- **Perfusion** is an fixation approach in which the fixative is injected into the bloodstream of the animal

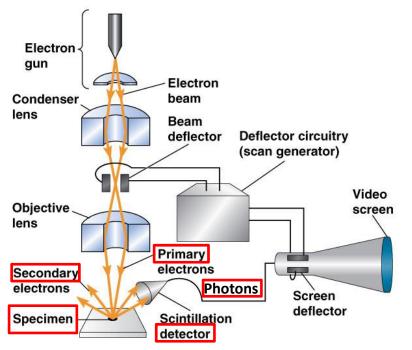Liver tissue

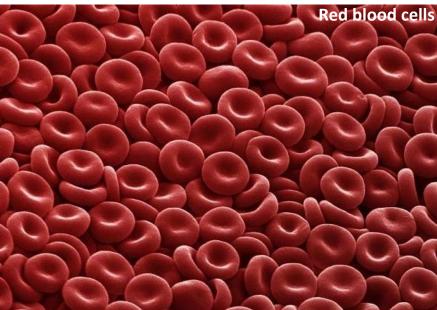Brain tissue

# Differences between TEM and SEM

- The TEM is a popular electron microscope that allows scientists to image <u>cellular components</u> in the **nanometer range** (double membranes, rER, viruses etc.)
- **TEM** means <u>transmission electron microscope</u>, because **electrons are transmitted "through the specimen"** (located within the objective lens)
- Because **electrons travel not far in air**, the <span style="color:orange">**whole TEM is under vacuum**</span>

Cathode (tungsten filament)

80kV

Anode

Electron gun (inside cylinder, not shown)

0kV

First condenser lens

Second condenser lens

Condenser lens system

Specimen

Specimen stage

Objective lens

Intermediate lens

Projector lens

Detector

Viewing screen (or EMCCD camera)
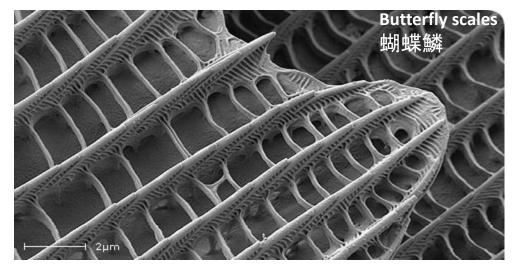
- As the <u>cathode</u> is kept at about <u>80kV</u> and the <u>anode at 0V</u> **electrons accelerate**
- Since **electrons are negatively charged** their <u>movement can be controlled by</u> **magnetic forces**
- Just like a glass lens, **electromagnetic lenses** can **bend electron rays**
- After the electron beams have passed the lens system, the <u>image is projected on</u> a **viewing screen** that <u>fluoresces if hit by electrons</u>
- **Electrons are scattered** <u>depending on how electron-dense the material is</u> (gold = high scattering, cytosol = low or no electron density)
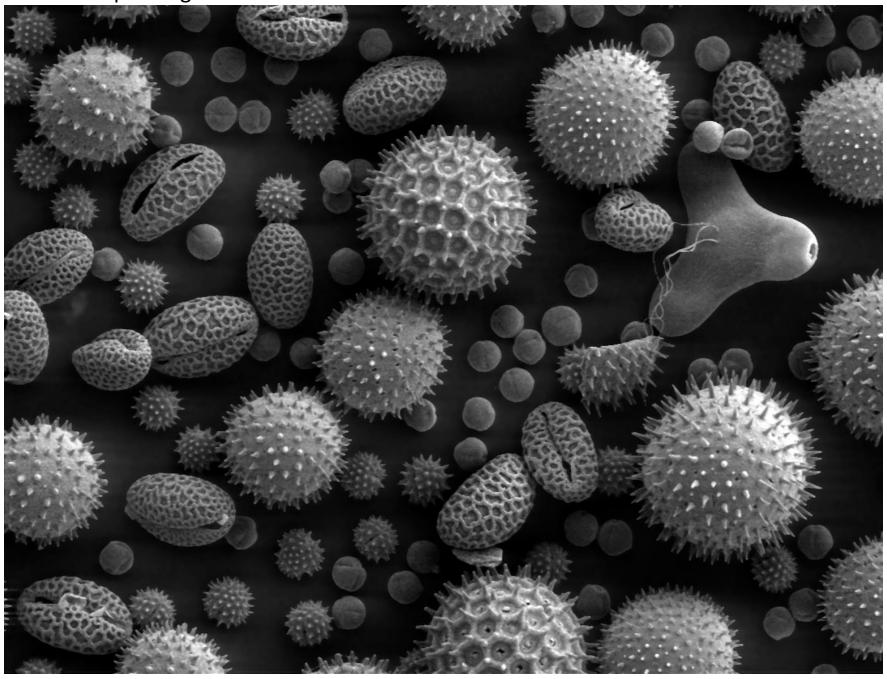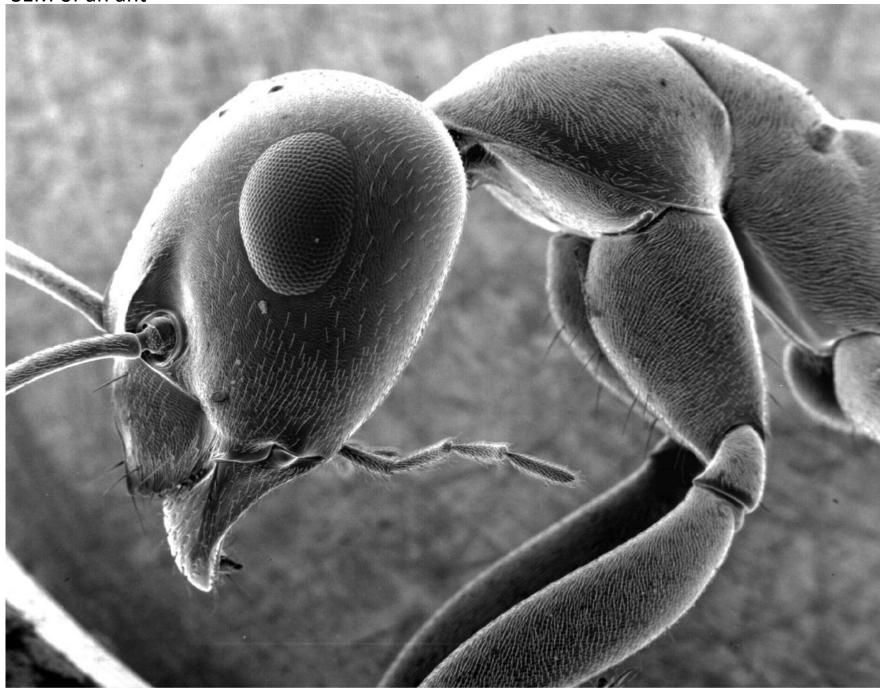
# Scanning electron microscope



- The <u>difference</u> between **SEM** (scanning electron microscope) and **TEM** is that in SEM <u>electrons are detected</u> that **deflect from the outer surface** of the specimen (and not those that went through the specimen)
- The surface of the specimen is **scanned by a beam of electrons** giving a <u>3D impression</u>
- Scanning is provided by **beam deflectors**
- **Primary electrons** hit the surface of the specimen and <u>force emission of</u> **secondary electrons** which are <u>finally detected by a</u> **scintillator**
- A **scintillator** <u>emits a</u> **photon** <u>when hit by an</u> **electron**. These <u>photons</u> are then <u>used to</u> **generate an image** on a **video screen**.



Red blood cells



Butterfly scales
蝴蝶鱗

2µm
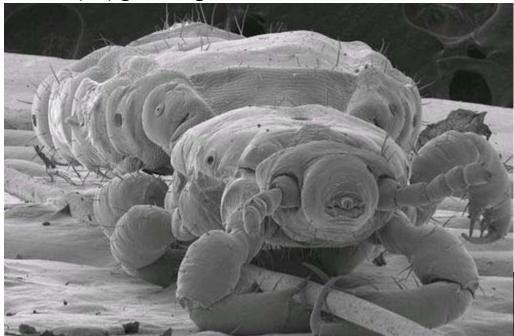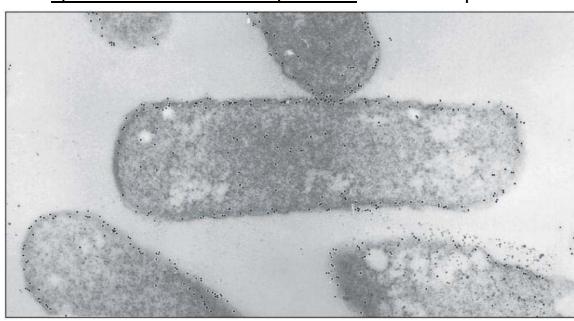
SEM of pollen grains

SEM of an ant

Louse (虱) grabbing a human hair



Hydrothermal worm (found recently near deep sea hydrothermal vents)



*C. elegans*
SEM

# Sample preparation for electron microscopy

- Specifically for TEM, <u>sample preparation is critical</u> as <u>cellular structures</u> are usually of <u>low electron density</u>
- Thin sections (50-100 nm) are done with an ***ultra*microtome** that uses a **diamond knife**
- Electron density of cellular structures is increased by the <u>treatment with heavy metals</u> such as **osmium tetroxide** or **uranyl acetate** (which are very toxic and care must be taken)
- **Cryofixation** is a <u>less aggressive</u> method and it <u>does not cause as many artifacts</u> as heavy metal staining
- Cryofixation is a **rapid freezing method** done under high pressure (**high pressure freezing**)
- A **freeze substitution step** is often necessary to <u>replace the water by an organic solvent</u> (acetone) and that <u>allows for subsequent embedding</u> and sectioning
- **Immunoelectron microscopy** is an important technique that allows for the <u>detection of specific molecules and proteins</u> in the sample
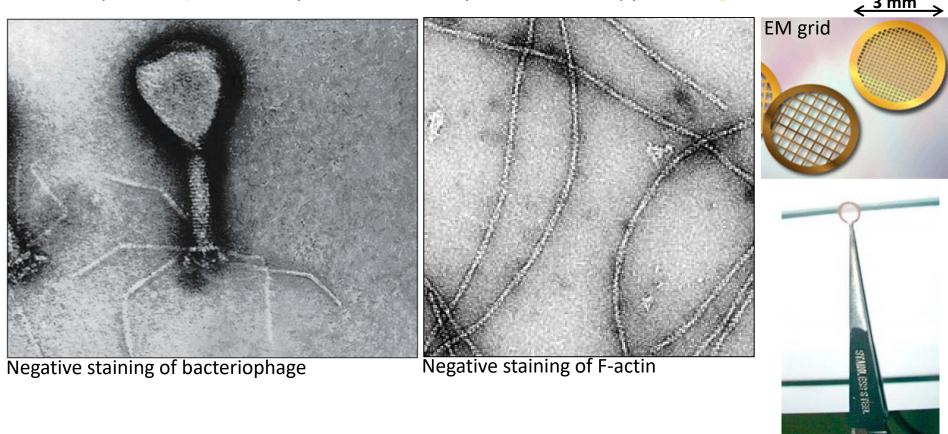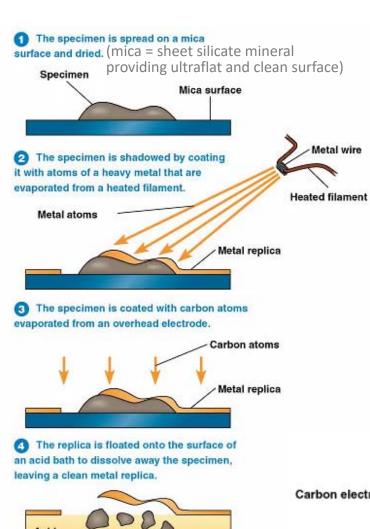


Antibody detection of a specific membrane protein in the bacterial cell wall

- In **immunoEM** primary antibodies are detected by <u>secondary antibodies that are labeled with</u> **small gold beads**
- The <u>very electron dense gold beads</u> can be easily seen under the TEM

# Sample preparation for electron microscopy

- **Negative staining** is a method that allows for the visualization of <u>isolated organelles and purified proteins</u> in buffer
- The <u>solution is simply treated with uranyl acetate</u> and then placed on a **copper grid** that is covered with an **ultrathin** (pioloform) **plastic film** (ultrathin sections do not need this film and can be directly placed on the grid)
- In TEM these specimens **appear in negative contrast**: background and <u>edges</u> (borders of specimen) are **heavily stained** so the specimen itself appears **bright**
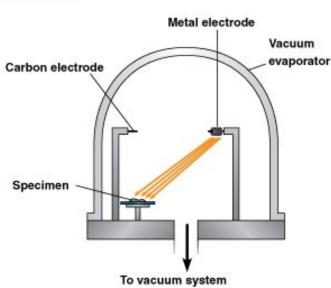


Negative staining of bacteriophage



Negative staining of F-actin



3 mm

EM grid

# Sample preparation for electron microscopy

**① The specimen is spread on a mica surface and dried.** (mica = sheet silicate mineral providing ultraflat and clean surface)

Specimen
Mica surface

**② The specimen is shadowed by coating it with atoms of a heavy metal that are evaporated from a heated filament.**

Metal wire
Heated filament
Metal atoms
Metal replica

**③ The specimen is coated with carbon atoms evaporated from an overhead electrode.**

Carbon atoms
Metal replica

**④ The replica is floated onto the surface of an acid bath to dissolve away the specimen, leaving a clean metal replica.**

Acid bath
Specimen dissolving away

**⑤ The replica is washed and picked up on a copper grid for examination in the TEM.**
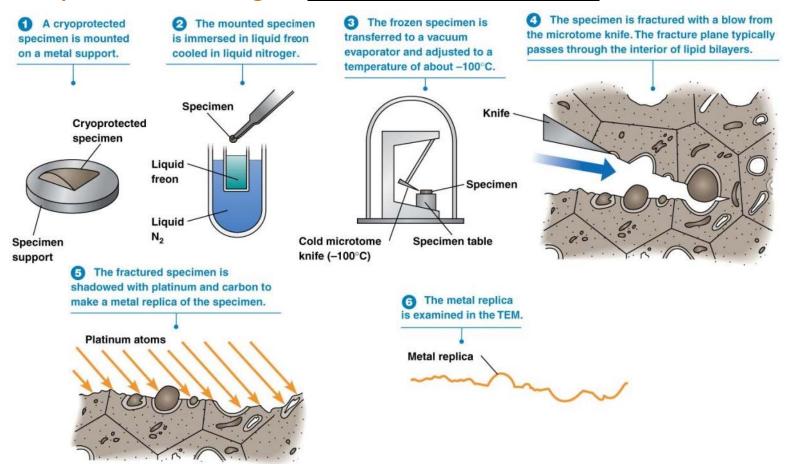
Metal replica
Copper grid

- In the **platinum shadowing technique** a **metal replica** of the specimen is made
- Shadowing is a very delicate method allowing for the visualization of **single proteins** and **DNA coils**
- Specimen is shadowed by coating it with atoms of a heavy metal (usually **platinum**) that **evaporates** from a **heated filament** in vacuum
- Coating occurs at an angle of 10-45°
- Additional coating with **carbon atoms** from top strengthens and supports the metal replica
- The **specimen** is then **eliminated** via an acid bath
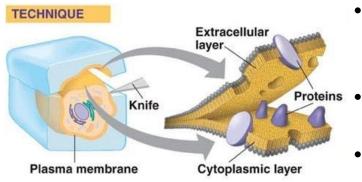- The replica is placed on a copper grid for examination

Metal electrode
Vacuum evaporator
Carbon electrode
Specimen
To vacuum system
**(b) Vacuum evaporator**

Tobacco mosaic virus

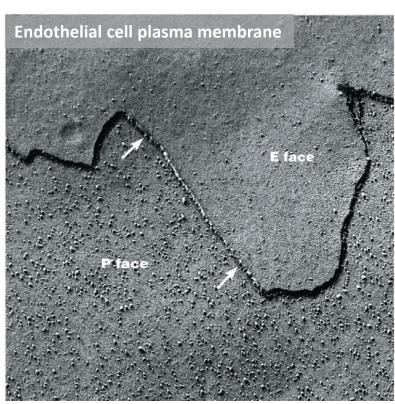# Sample preparation for electron microscopy

- **Freeze fracturing method:** **Frozen specimens** are extremely hard and if cut with a knife it fractures (opens) at weak points (for example **along lipid bilayers**)
- A first step involves **cryoprotection** to prevent the formation of ice crystals
- To cryoprotect a specimen it is fixed first and then treated with glycerol
- Specimen is immersed in cooling liquid (freon, 代甲烷) that is kept cold in liquid nitrogen
- Under vacuum, a **ice-cold microtome knife** then hits the specimen
- After **platinum shadowing** the metal replica can be observed with the TEM
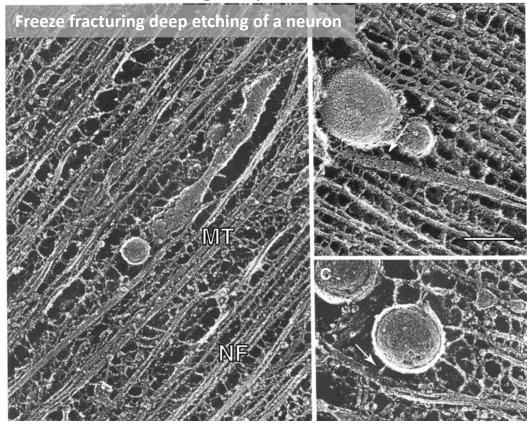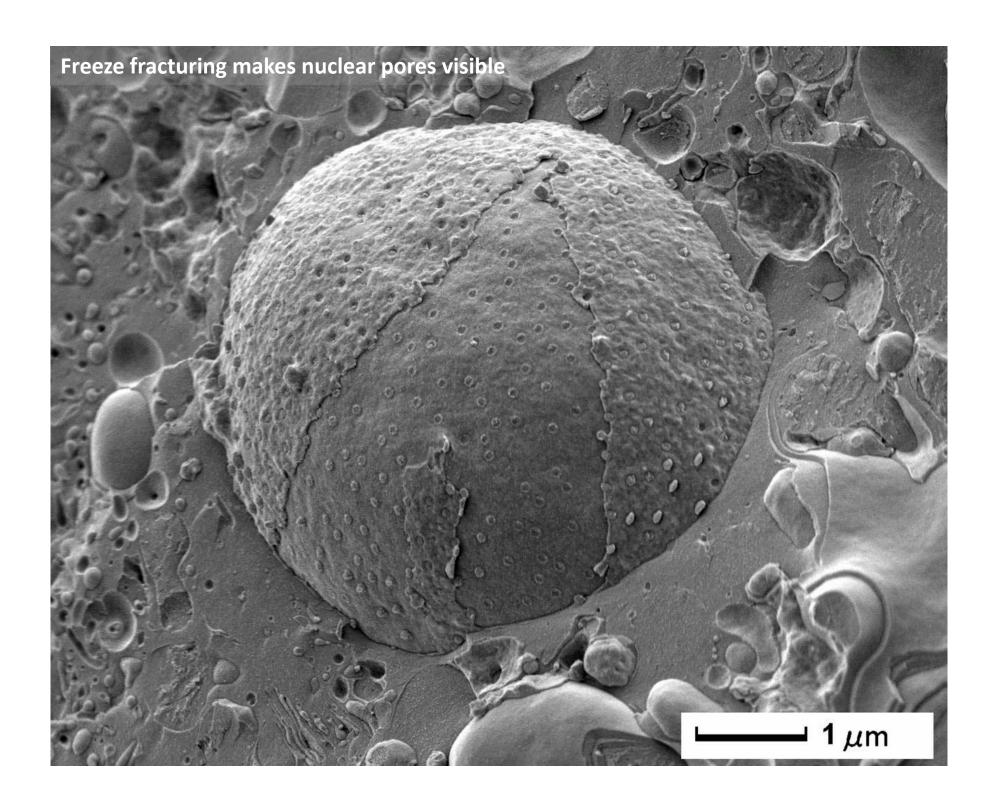
# Sample preparation for electron microscopy

- In **freeze fracturing**, intramembranous particles (**IMP**s) can be seen that stayed with one monolayer. Usually it is the monolayer at the P face (*p*rotoplasmic side).



TECHNIQUE

Extracellular layer

Knife

Proteins

Plasma membrane

Cytoplasmic layer

- The **P face** is the interior face of the monolayer that faces to the cytosol. The **E face** is the interior face of the monolayer that is on the *e*xterior side of the cell.
- In the **deep etching method** the knife is placed for a *certain longer time* over the fractured specimen
- This allows for etching deeper into the cell



Endothelial cell plasma membrane

E face

P face



Freeze fracturing deep etching of a neuron

MT

NF

C

Freeze fracturing makes nuclear pores visible

1 μm

# Sample preparation for electron microscopy

- In **stereo electron microscopy** a special **specimen stage** is used that **can be tilted**
- The specimen is <u>imaged from two different views</u>. The resulting **stereo pair** can be observed with a **stereoscopic viewer** (or by letting the eyes slightly crossed)
- In **3D electron tomography** the **ultrathin slices** of the specimen are rotated and **imaged at several angles**
- A <u>computer assists to assemble these images</u> into a 3D model of the specimen

Drosophila polytene chromosome

Golgi and lysosomes in a dendritic cell



**(a)** (needs stereoscopic viewer)  0.5 μm
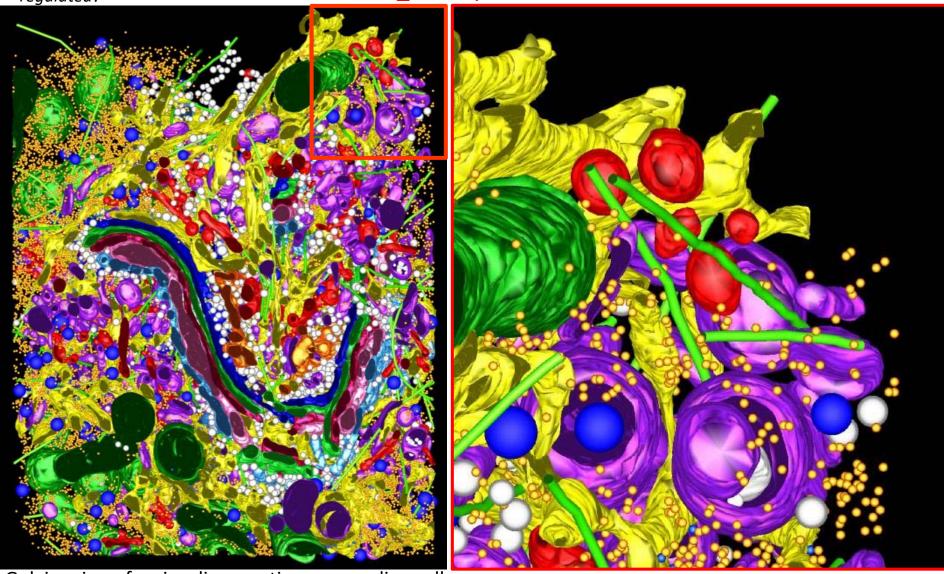
**(b)** Single ultrathin slice  200 nm

**(c)** 3D tomography  200 nm

# 3D electron tomography

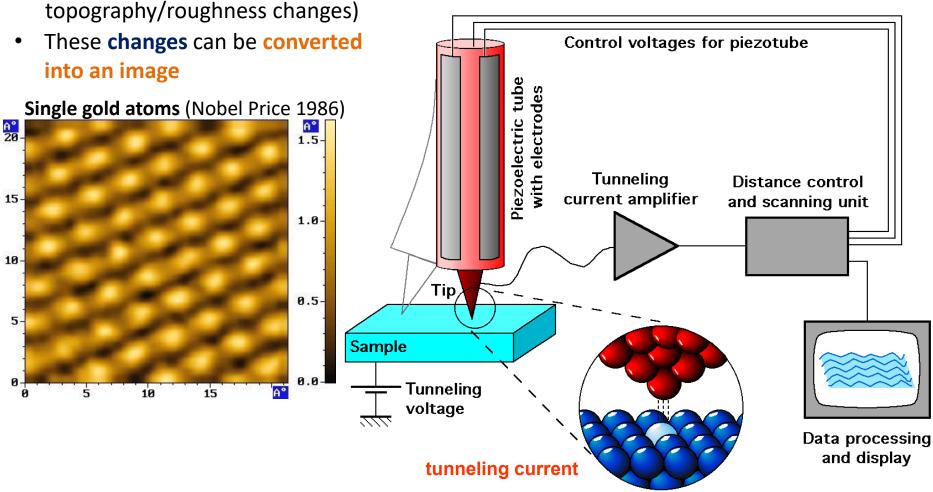*"How can such a crowded cell be properly regulated?"*



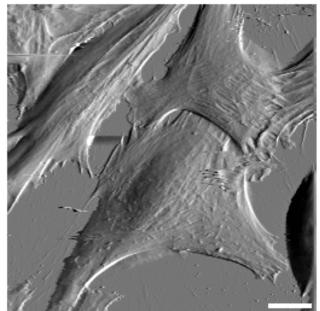Golgi region of an insulin secreting mammalian cell

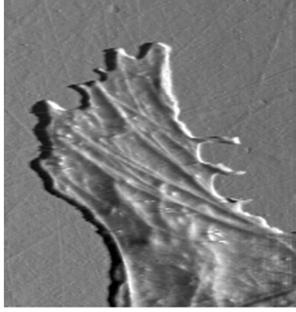# Non-optical imaging techniques: STM (scanning tunneling microscopy)

- STM measures the **topography** of a surface using a **tunneling current**
- When a fine (conductive) tip is brought close a sample (with an applied voltage), **electrons begin to tunnel through the gap**
- The **tunneling current** **changes during** the **scanning** process (when the sample's topography/roughness changes)
- These **changes** can be **converted into an image**

**Single gold atoms** (Nobel Price 1986)



Control voltages for piezotube

Piezoelectric tube with electrodes

Tip

Sample

Tunneling voltage

tunneling current

Tunneling current amplifier

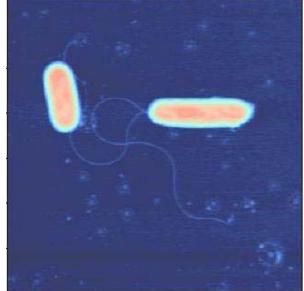Distance control and scanning unit

Data processing and display

# Atomic Force Microscope (AFM): "The STM for Biologists"
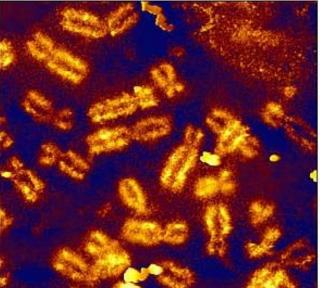


- AFM is a non-optical microscope that employs a **tiny needle that scans along the surface** of a specimen.
- The method is **nearly non-destructive** and is ideal for biological material as cells and tissues.
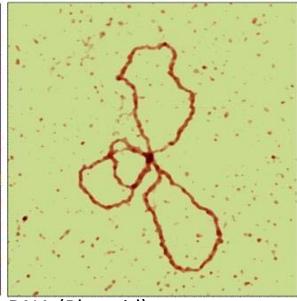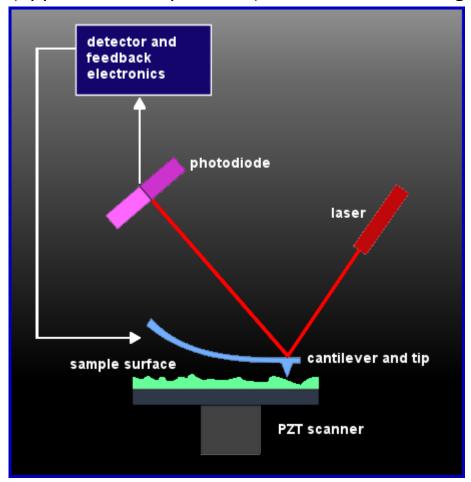
Real-time AFM myosin walking



Bacteria with visible flagella

Chromosomes

DNA (Plasmid)

# Atomic Force Microscope (AFM): "The STM for Biologists"

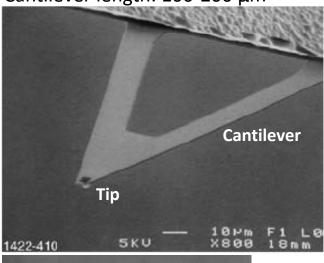Atomic force means: the **force** of the cantilever (applied to the specimen) is in the **atomic** range

Spring constant (k): 0.6-0.06 N/m
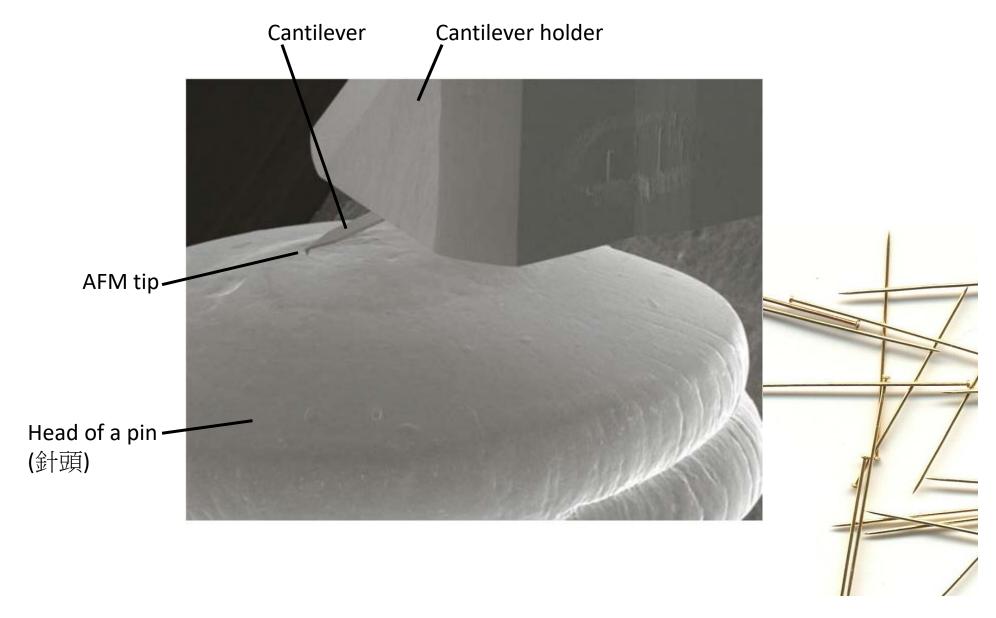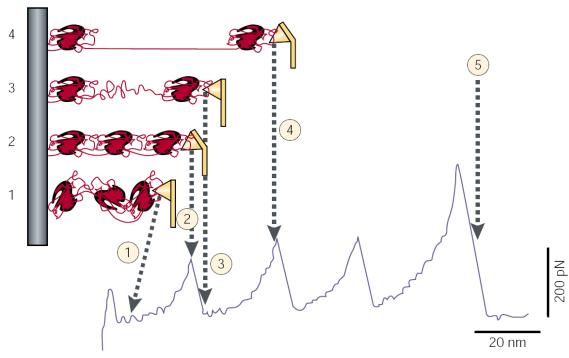Tip radius: 20-60 nm
Cantilever length: 100-200 μm



detector and feedback electronics

photodiode

laser

sample surface

cantilever and tip

PZT scanner

AFM model



Cantilever

Tip

1422-410  5KU  10μm  F1 L0
X800  18mm



Cantilever

Tip

5KU  10μm  F1 L
X850  18mm

SEM images of AFM tips

# How large is the AFM tip?

AFM tip compared to a pin (針):

Cantilever

Cantilever holder

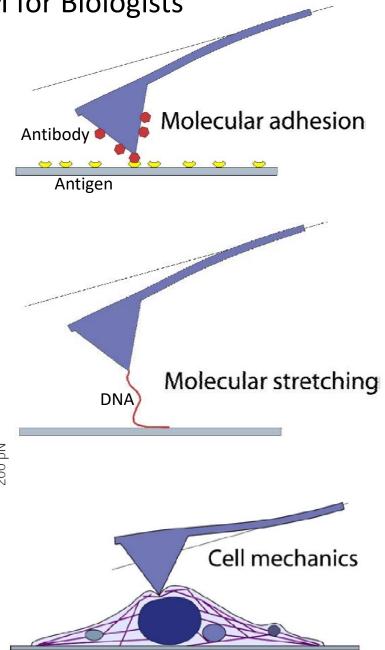AFM tip

Head of a pin
(針頭)

# Atomic Force Microscope (AFM): "The STM for Biologists"

- **Protein unfolding**: AFM tip grabs the end of a protein and <u>protein</u> **unfolds** <u>into its several domains</u> **with increasing force**
- A series of <u>snap-back points</u> results each <u>representing</u> **breaking of chemical bonds**
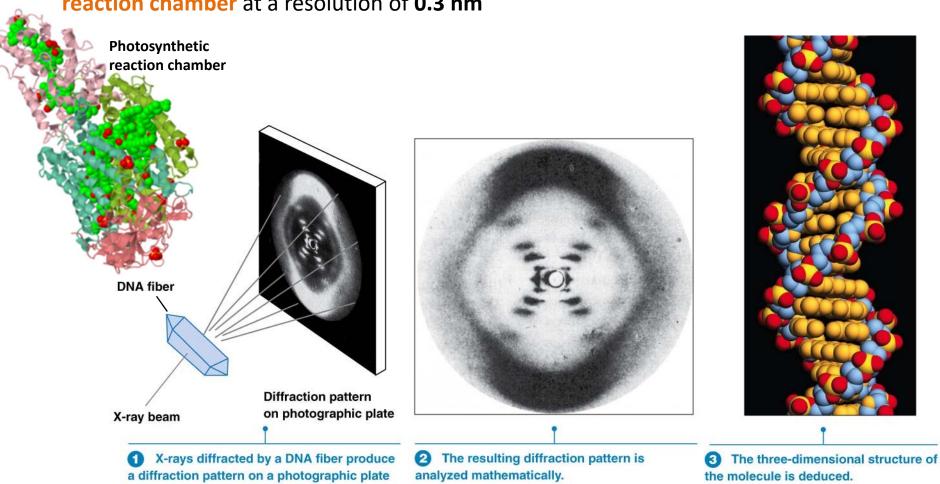


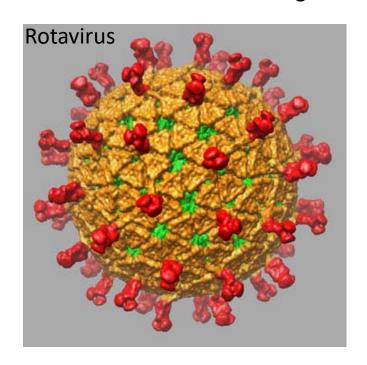Domain unfolding of repeating immunoglobulin-like domains



Molecular adhesion

Antibody

Antigen

Molecular stretching

DNA

Cell mechanics

200 pN

20 nm

# X-Ray crystallography

- **X-ray crystallography** allows to "visualize" 3D structures of **single molecules**
- This techniques usually requires a **protein crystal** that is **exposed to X-rays**
- The beams are reflected by the specific arrangements of the atoms in the crystal
- As a result a **specific diffraction pattern** is received which are **analyzed mathematically**
- **1985**: **Nobel Prize** for describing the molecular organization of the **photosynthetic reaction chamber** at a resolution of **0.3 nm**



**Photosynthetic reaction chamber**

DNA fiber

X-ray beam

Diffraction pattern on photographic plate

❶ X-rays diffracted by a DNA fiber produce a diffraction pattern on a photographic plate or other detector.

❷ The resulting diffraction pattern is analyzed mathematically.

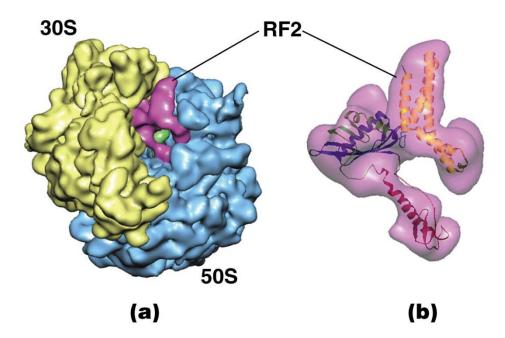❸ The three-dimensional structure of the molecule is deduced.

# CryoEM, 3D EM and X-Ray crystallography work hand in hand

- In **cryoEM** not only whole cells or tissues can be observed but also **molecules in solution**
- The molecules (or macromolecules) are **rapidly frozen** (cryofixation)
- Imaging of the sample also occurs at very deep temperatures (-170°C)
- **3D electron tomography** is often used for generating a 3D view of the molecules
- More detailed information from **X-ray crystallography** can be then added to receive an even better understanding of the molecule



Rotavirus

CryoEM clathrin cage

CryoEM nuclear pore



Combination of an **cryoEM** image of the 30S and 50S subunit of a **ribosome** (left) with its **releasing factor RF2** (generated by **X-ray crystallography**)

# The Nobel Prize in Chemistry 2017
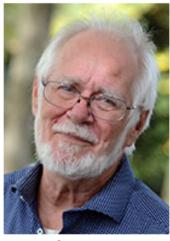
2017 Nobel Prize in cryo-EM

Photo: Félix Imhof © UNIL [CC BY-SA 4.0]
**Jacques Dubochet**
Prize share: 1/3

Photo: B. Winkowski © Columbia University Medical Center
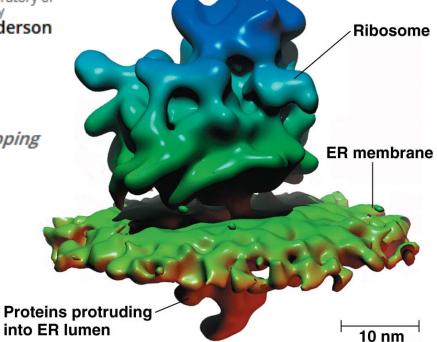**Joachim Frank**
Prize share: 1/3

Photo: MRC Laboratory of Molecular Biology
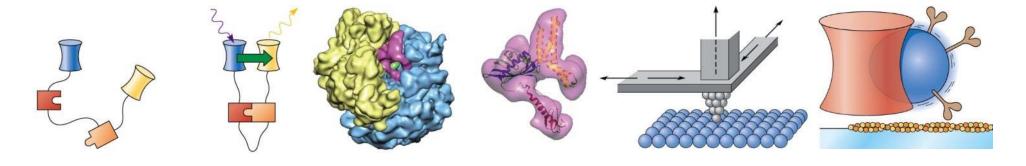**Richard Henderson**
Prize share: 1/3

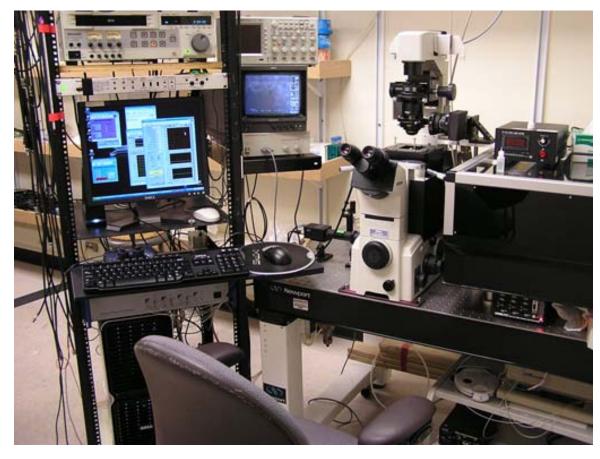The Nobel Prize in Chemistry 2017 was awarded to Jacques Dubochet, Joachim Frank and Richard Henderson *"for developing cryo-electron microscopy for the high-resolution structure determination of biomolecules in solution"*.

Ribosome

ER membrane

Proteins protruding into ER lumen

10 nm

# World of the Cell

*The end of chapter appendix*

*Thank you!*