Biomolecules

ELEC-E3260

Microscopy (of Biomolecules)

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What Will You Learn Today?

- **Microscopy**

  Microscopy techniques and their applications to (bio)molecules
  - Scanning Probe Microscopy (AFM, STM)
  - Electron Microscopy (EM)

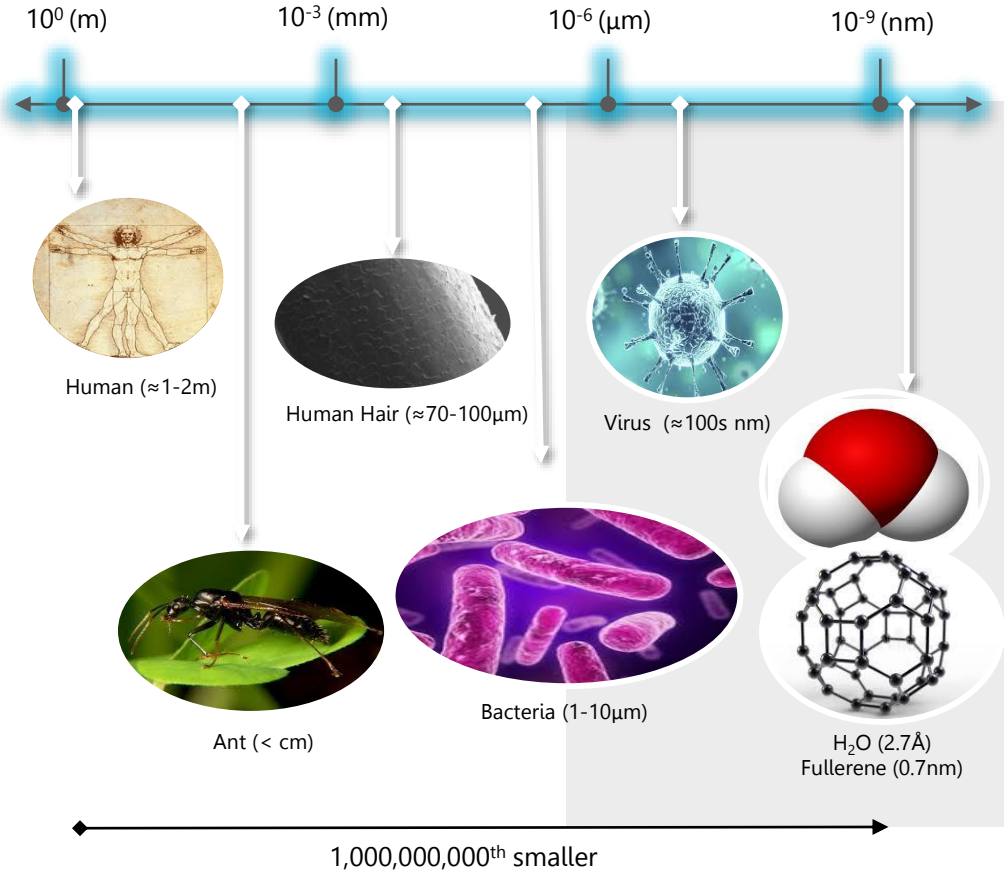
  - Let´s try some microscopy!

Microscopy: Beyond Human Eye

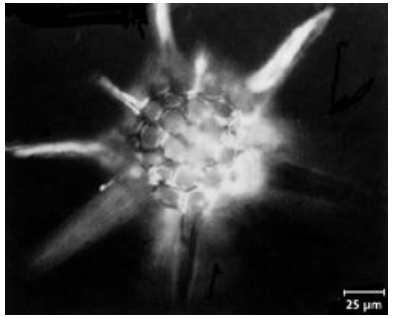
observe structures too small for the naked eye to observe

optical microscope is not able to produce an image of an object that is smaller than the wavelength (<1µm)

→ objects smaller than λ/2 cannot be resolved (diffraction limit)



(optical image)



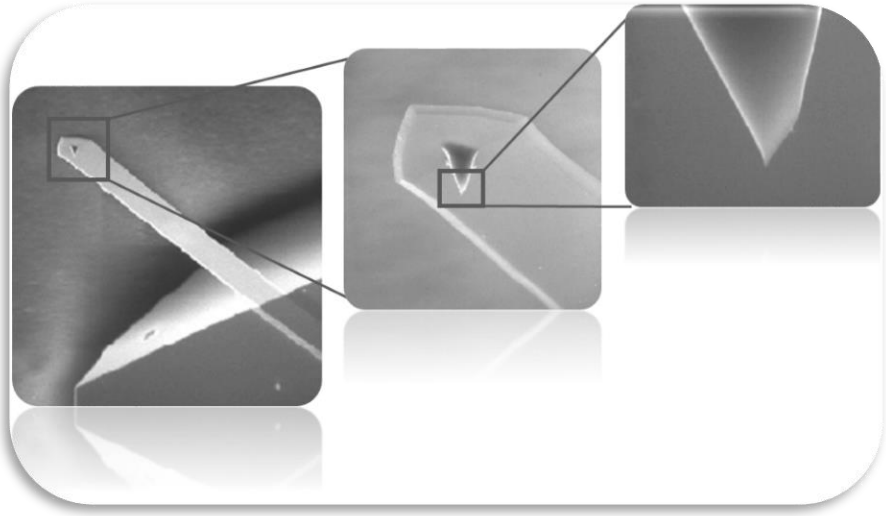
using probes other than visible light

(electron microscope)

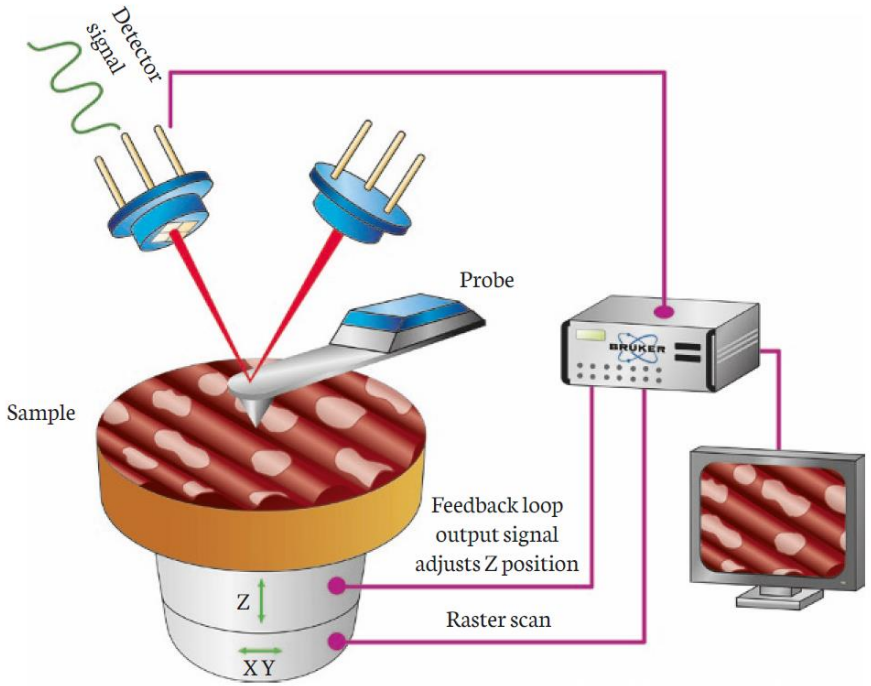
Scanning Probe Microscopy: Interaction with Surface

Atomic Force Microscopy (AFM): Working Principle

Atomic force microscopy helps study the surface properties of materials, based on the ***interaction between surface and the AFM tip***



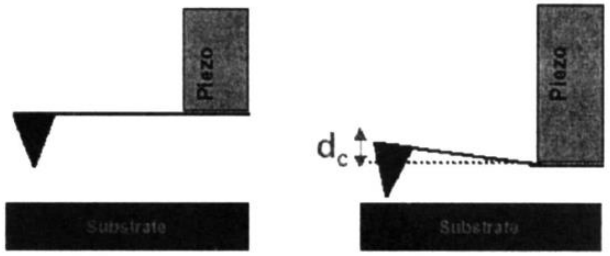
materials available @Bruker.com



AFM: Working Principle (II)

AFM cantilever obeys Hook´s law
(constant compliance region)
→ cantilever can be treated like a spring

$$F = -k_c \times d_c$$



force can be due to *electrical*, *magnetic*, *chemical* or *electrochemical interaction*
→ different AFM-based techniques

Visualization

structure: molecules, microphase separation, crystallites, blends/composites, surface topography, chemical composition

processes: crystallization/melting, wetting/dewetting, diffusion, reaction, adsorption, self-assembly

Properties

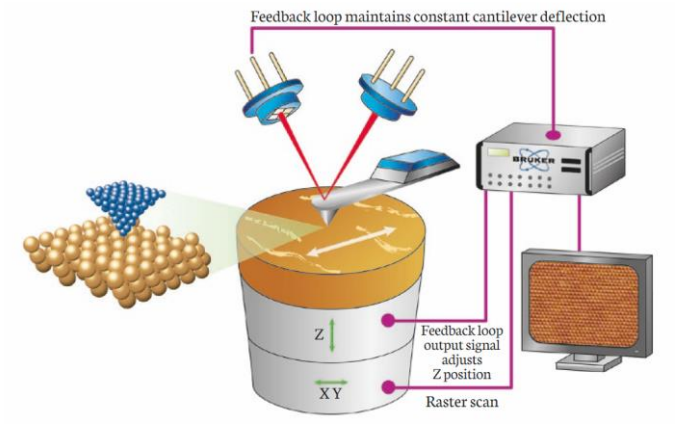
adhesion, friction/wear, indentation, viscoelasticity,

thermal, electronic

Lithography

deposition/writing, indentation/scraping, local reactions, resistive heating, manipulating particles, extending molecules

AFM: Operation Modes



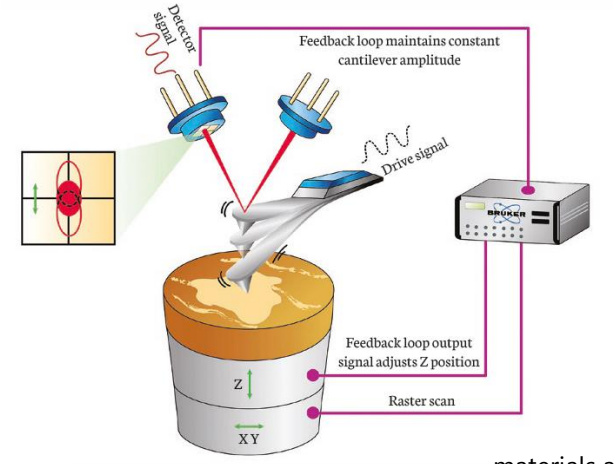
constant force

contact **mode**
(<0.5nm probe-surface distance)

probe is in ***permanent physical contact*** with the sample surface as it scans, with changes in topography causing the cantilever to bend up and down

non-contact **mode**
(0.1-10nm probe-surface distance)

cantilever is set to vibrate at its resonance frequency. When tip is brought close to sample surface, the forces between the tip and the surface affect its resonance frequency



constant amplitude

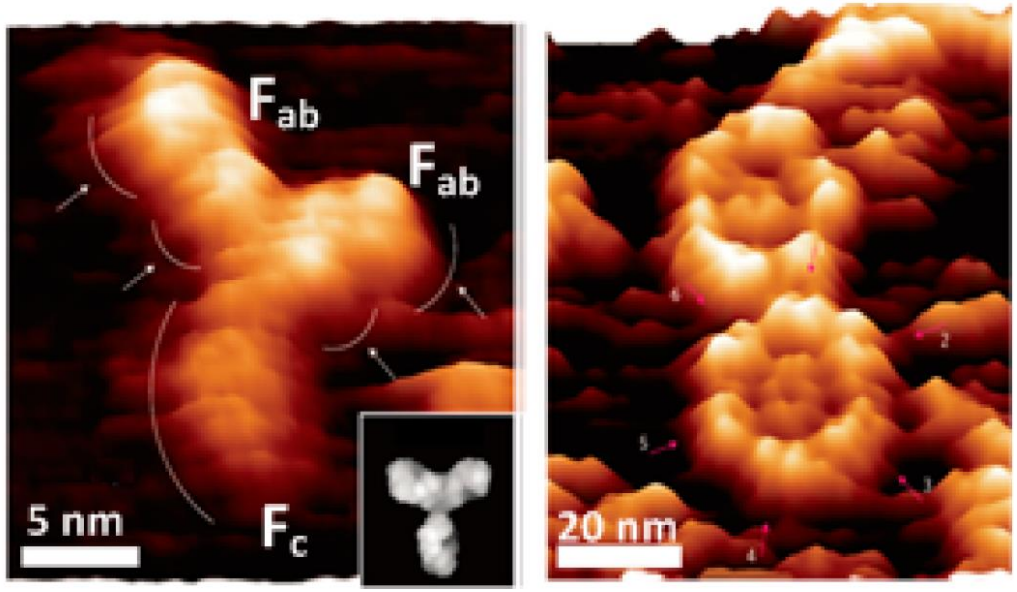
tapping **mode**
(0.5-2nm probe-surface distance)

cantilever is set to lightly tapping on the surface, thus eliminating lateral forces that can damage soft sample. Tapping mode can be performed both in air and fluid environment (*relevant for biological samples*)

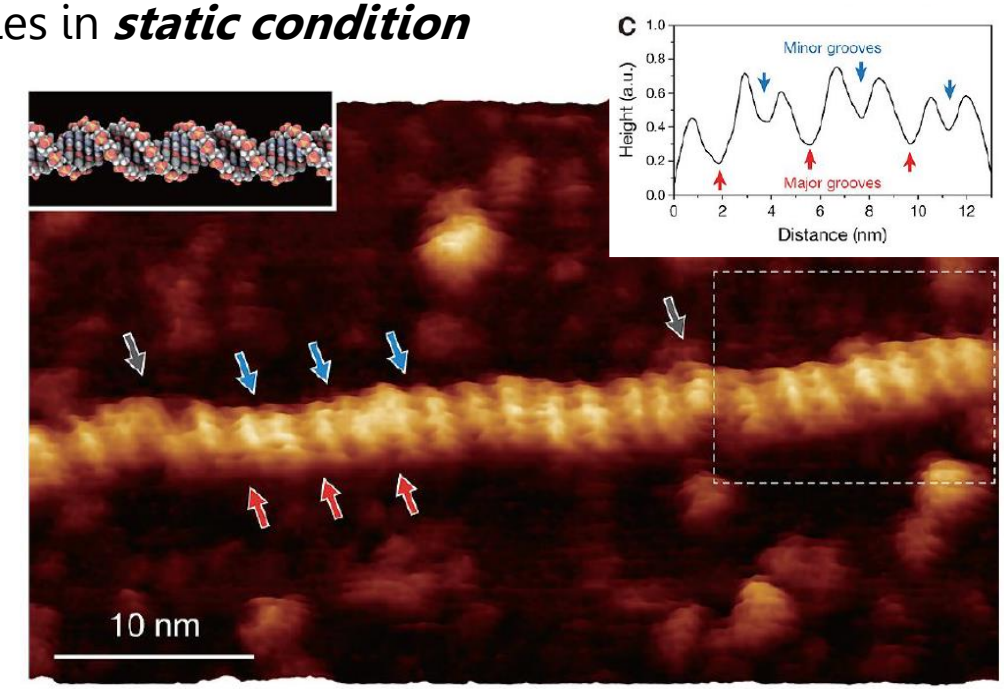
materials available @Bruker.com

AFM: Single Molecule Static Imaging

Single biological molecules in ***static condition***



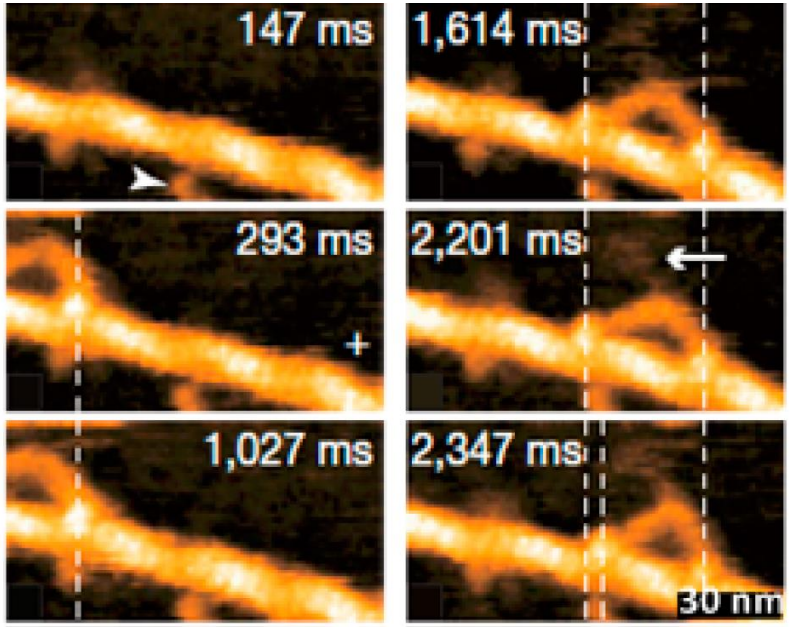
IgG antibody molecule: high-resolution AFM image of anti-HSA mouse monoclonal antibody (IgG) adsorbed on a mica and self-assembled antibody hexamers composed of six IgG molecules [Nat. Mater. **13**, 264 (2014)].



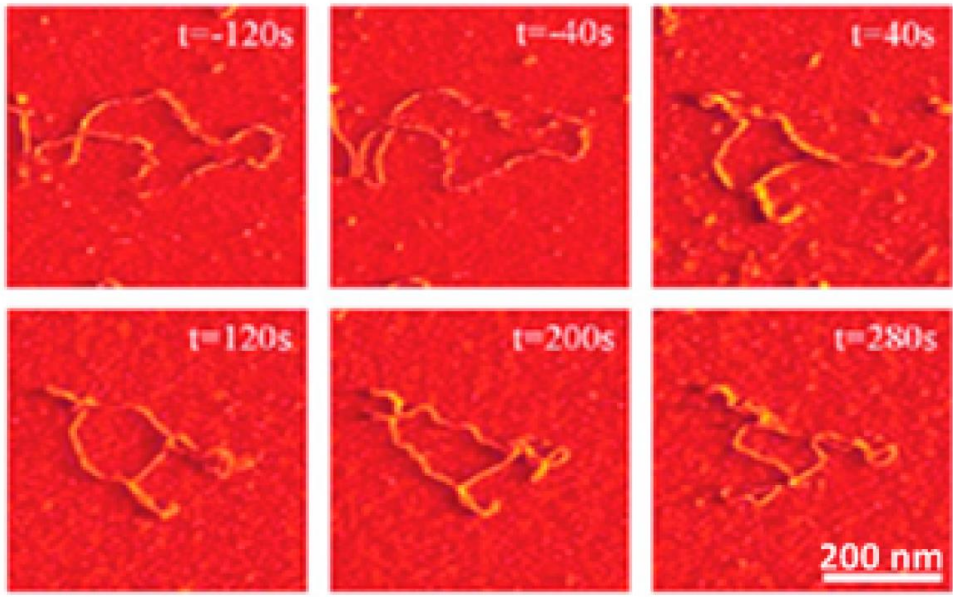
DNA: red and blue arrows indicate the positions of major and minor grooves of B-DNA, respectively, in aqueous solution. Gray arrows indicate the local melting regions of the plasmid DNA [ACS Nano **7**, 1817 (2013)].

AFM: Single Molecule Dynamic Imaging

Single biological molecules in ***dynamic condition***



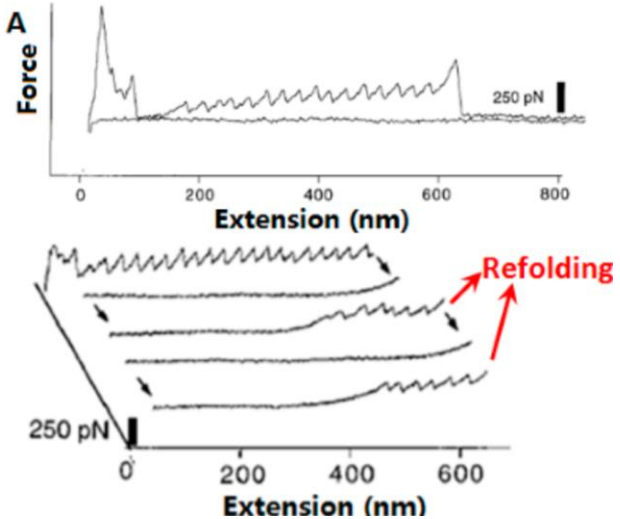
"Walking" **myosin molecule:** successive AFM images showing the processive movement M5-HMM. Arrows indicate coiled-coil tail of M5-HMM tilted towards the minus end of actin [Nature **468**, 72 (2010)].



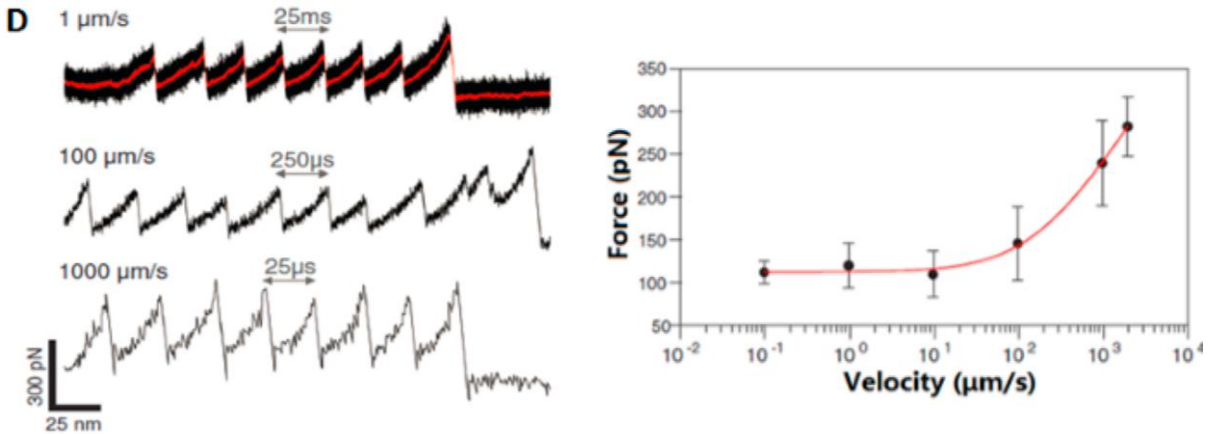
DNA after drug stimulation: AFM images of the conformational changes of single DNA induced by the injection of daunorubicin (Dau) [Nano Lett. **13**(11), 5679 (2013)].

AFM: Molecular Mechanics

AFM can reveal dynamic information in the unfolding process of single protein



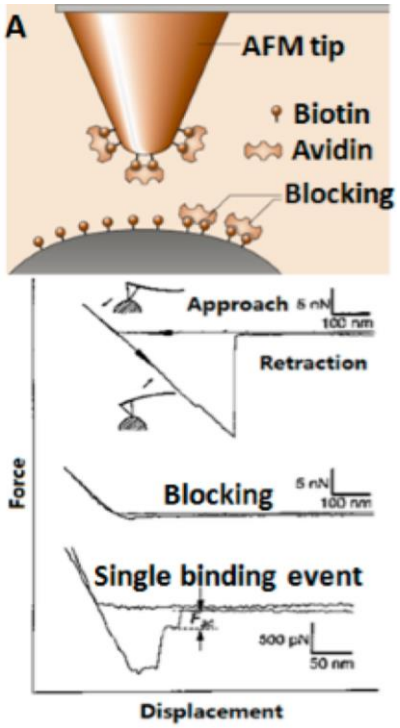
Titin molecule: force extension curves obtained by stretching titin proteins show periodic features consistent with their modular construction. Repeated stretch-relaxation cycles of single titin fragments demonstrate refolding [Science **276**, 1109 (1997)].

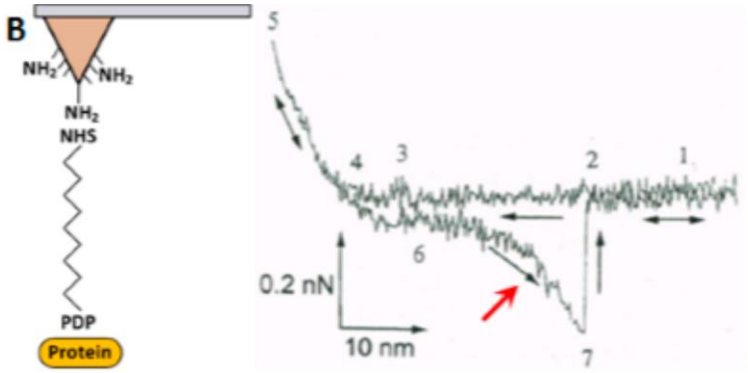


High-speed unfolding of titin molecule: typical force extension curves are recorded at different retraction velocities (1, 100, 1000μm/s) and dynamic force spectrum of the intermediate unfolding state [Science **342**, 741 (2013)].

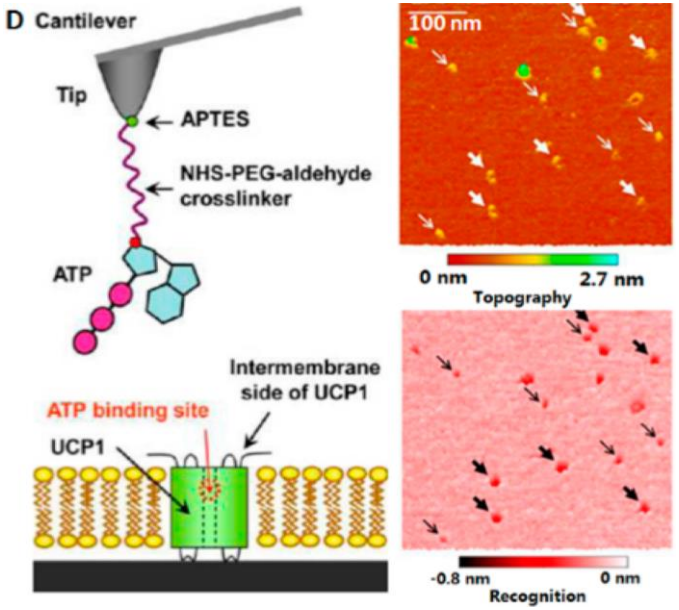
AFM: Molecular Recognition

Detecting and recognizing individual receptor-ligand events





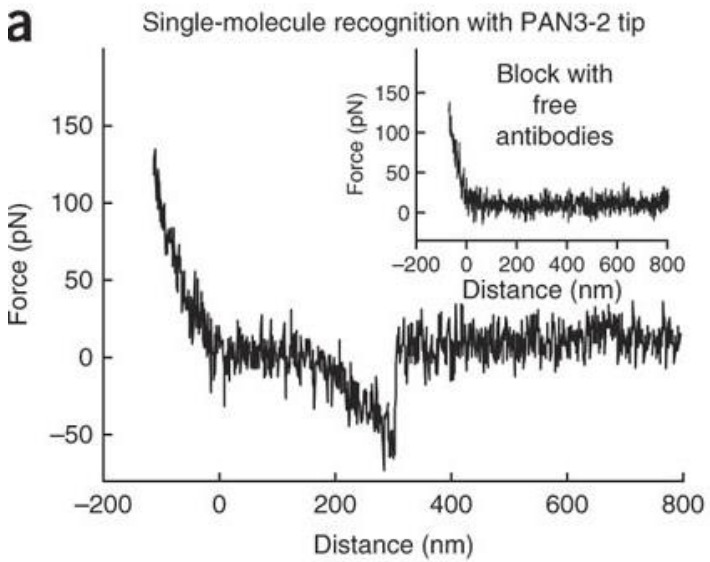
Antibody-antigen: antibody is linked to AFM tip via PEG molecule. Force curve recorded on antigen-coated substrate exhibits a significant molecular unbinding peak [Proc. Natl. Acad. Sci. USA **93**, 3477(1996)].



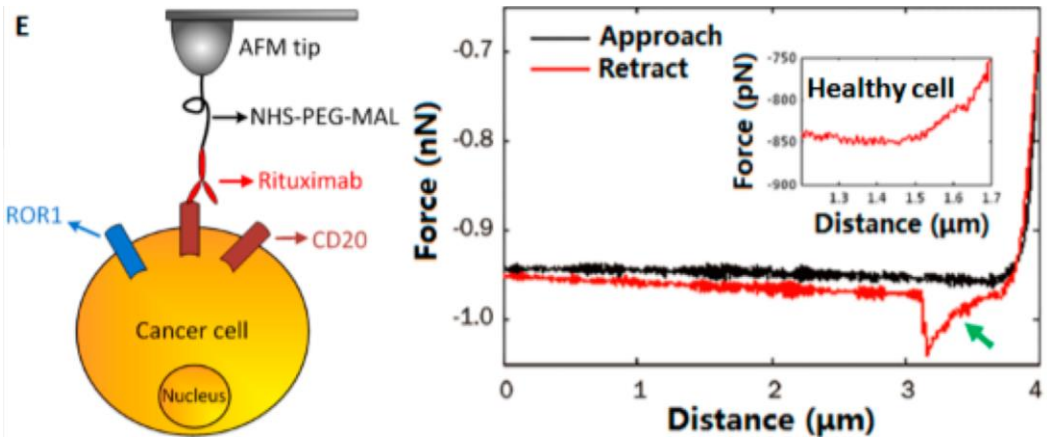
Proteins reconstituted in lipid bilayer. Imaging on UCP1-reconstituted lipid bilayer is performed using ATP-functionalized tip [J. Am. Chem. Soc. **135**, 3640 (2013)].

AFM: Molecular Activities on Cell Surface

AFM can detect individual receptors on cell surface



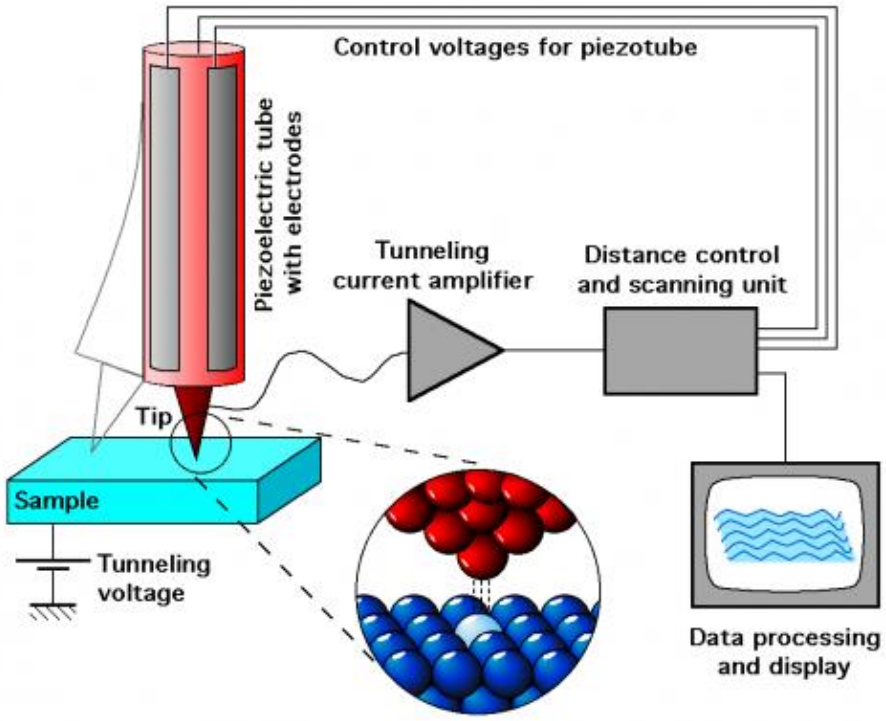
Recognition of SGLT1 (protein) on cell surface by AFM tip coated with specific antibodies: force curve showing specific interaction between the antibody and SGLT1 upon tip-surface retraction. The interaction is blocked by adding free antibodies to the solution (inset) [Nat. Protoc. **6**, 1443 (2011)].



Molecular recognition on primary tumor cell from clinical lymphoma patients. Tumor cells from bone marrow sample are recognized by ROR1 fluorescence labeling. A specific unbinding peak (green arrow) is in the force curve recorded on tumor cell but not in the one for the healthy cell. [Exp. Cell Res. **319**, 2812 (2013)].

Scanning Tunneling Microscopy (STM)

Study the surface properties of materials with high resolution and it is based on the ***charge tunneling between surface and the tip***



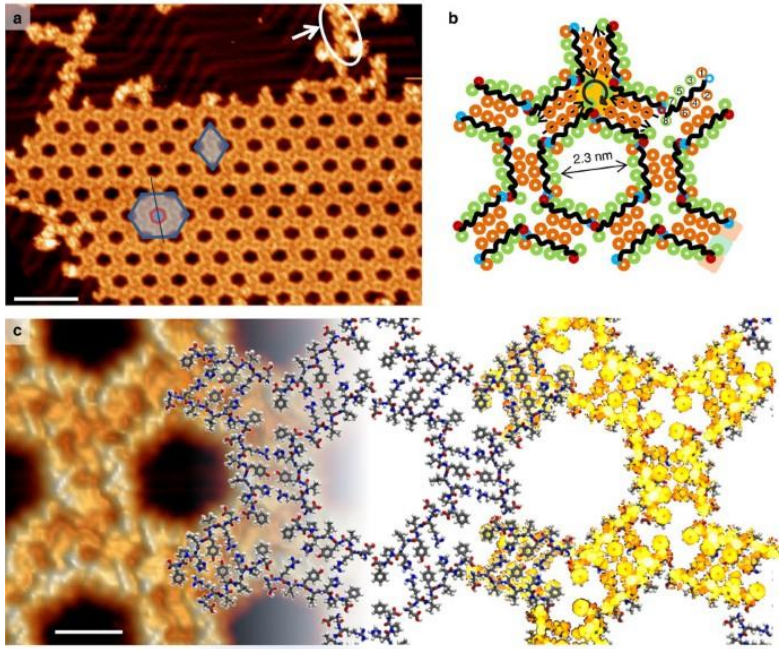
sharp tip approaches a ***conducting surface*** at a very close distance (1nm) → tunneling current starts to flow. The tip is mounted on a piezoelectric tube, which allows tiny movements by applying a bias to its electrodes

STM electronics allows to control the tip position such that tunneling current (thus tip-surface distance) is kept constant, while scanning a small area of the sample surface

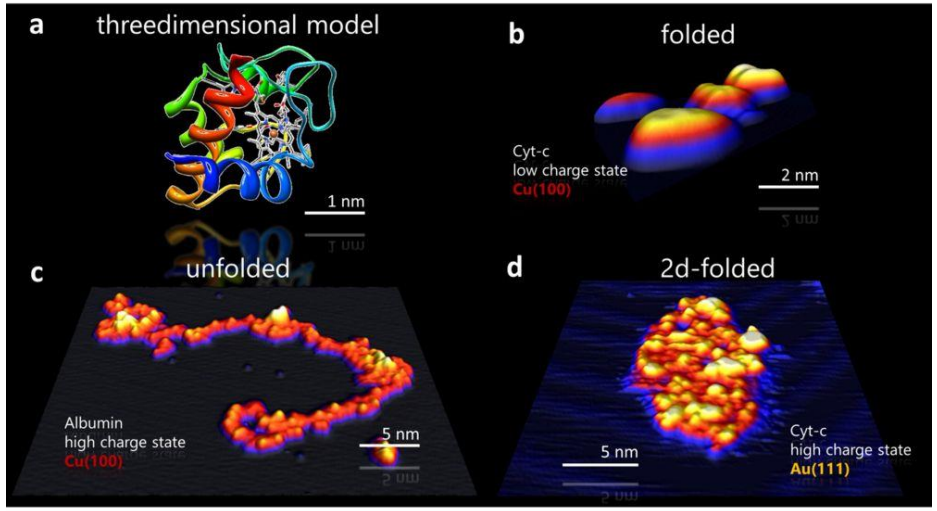
movement is recorded, resulting in surface topography image.
In ideal conditions, individual atoms on the surface can be resolved

STM images shows the *geometric structure* of the surface, while depending on the electronic density of states of the sample, as well as on *tip-sample interaction*

STM: Towards Single Atom Resolution



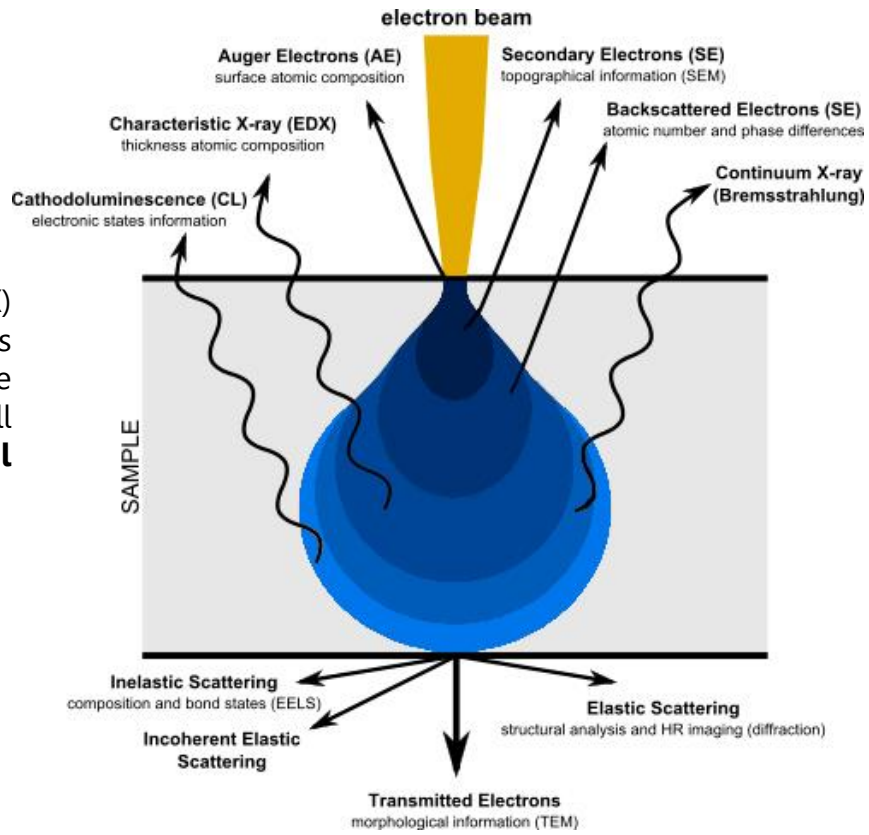
Peptides on gold: long range, highly ordered network formed by the peptide Angiotensin II on the Au(111) surface. Three dimers of two peptides each intersect at each node. [Nature Comm. **7**, 10335 (2016)].



Protein structures on surfaces as observed in STM. Depending on the deposition condition folded, unfolded and 2D refolded conformations can be chosen [Nano Lett. **12** (5), 2452 (2012)]

Electron Microscopy: e-interaction

Electron Microscopy: e-interaction



Secondary Electron detector (SE) captures the energy from the secondary electrons generated in the material. It provides information on the most superficial **texture/topography**

X-ray detector (EDX, EDS or EDAX) captures the energy from the X-rays generated on the surface. They are characteristic of each element and will provide information on **elemental composition**

Backscattered Electron detector (BSE) captures the energy coming from the backscattered electrons. It is sensitive to **variations in the atomic number** of surface elements, therefore in **composition**

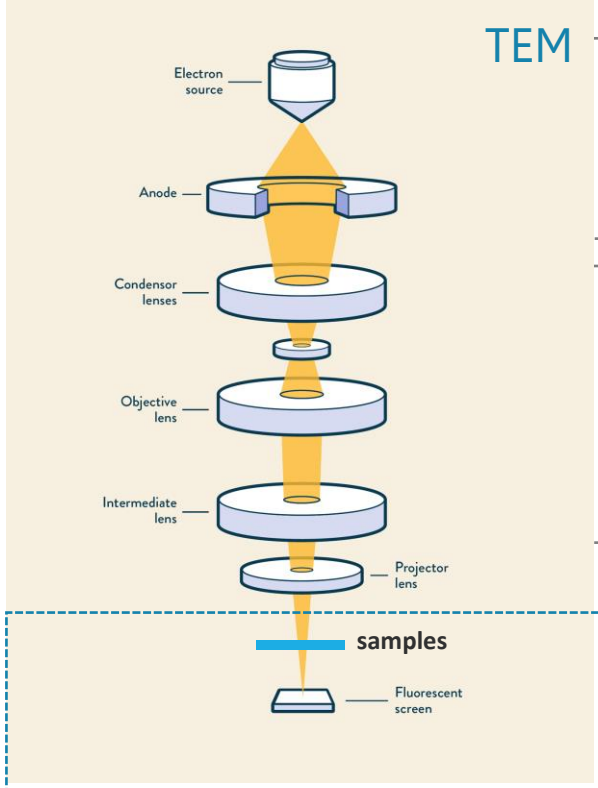
X-ray Detector (WDS) similar to EDX, but it only measures the **signal generated by a single element**.

Diffracted Backscattered Electron detector (BSED) captures the energy of electrons diffracted by the surface (Bragg's law), providing information on the **crystal structure**

interaction volume increases with ***increasing atomic number*** and ***accelerating voltage***

Electron Microscope(s)

Depending on *signal detection*, electron microscopy can be categorized in:
transmission electron microscopy **(TEM)** & ***scanning electron microscopy*** **(SEM)**

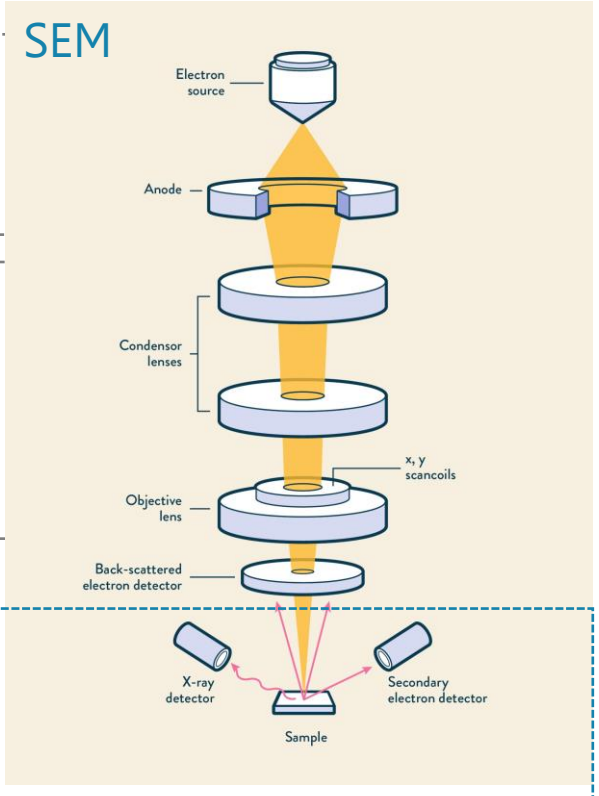


transmitted e^-

electron gun

- source-filament producing an electrons cloud
- Wehnelt cylinder to form the beam
- anode to accelerate the beam

electromagnetic lenses
to focus the electron beam to obtain a collimated beam



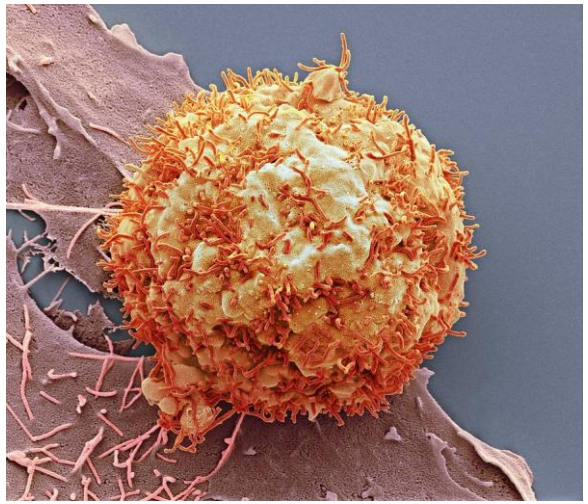
scattered e^-

e-sample interaction
beam can be transmitted (TEM) or scattered (SEM) upon interaction with the sample

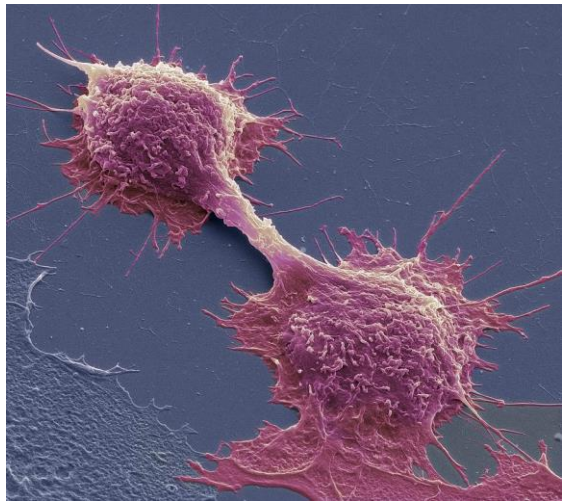
EM Challenges: Biomolecules & Biological Samples

vacuum	EM operation in vacuum causes issues for biological tissue, such as evaporating water destroying structures being imaged. Living tissue cannot be images with EM
lack of contrast	biological tissue do not diffract many electrons
transparency	electron beam must be able to pass through the sample (TEM); some samples are small enough that they can be imaged as a whole, but most cells and tissues are larger (>1μm)
charging	biological samples are non-conductive, which create issues when bombarded by a negatively charged electron beam, causing instability, drift, blurring and image distortion

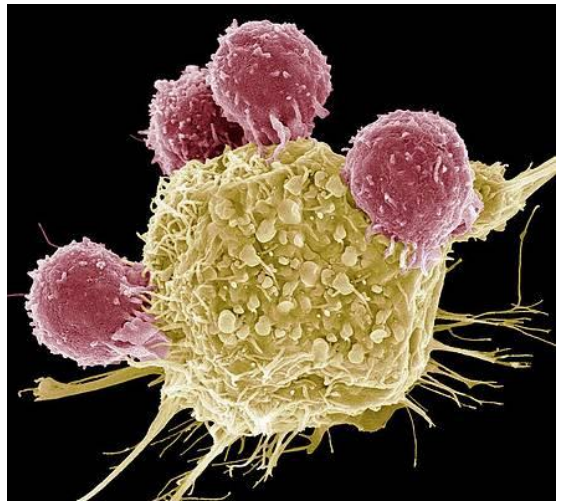
SEM: Cancer Cells



Liver cancer cell: colored image of a hepatocellular carcinoma (HCC) cell, showing the numerous filopodia (*hair*-like) covering its surface. Magnification x4000 when printed at 10cm wide.



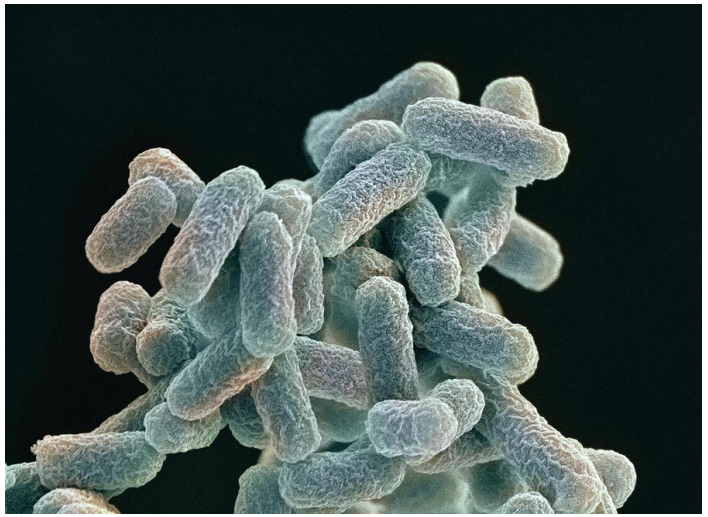
Dividing cancer cell: colored image of a colorectal cancer cell undergoing mitosis (nuclear division). Magnification x4000 when printed at 10cm wide.



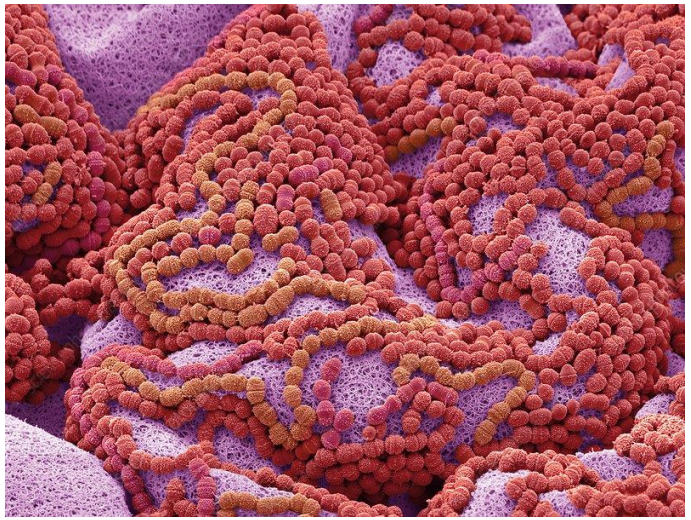
T-lymphocytes & cancer cell: colored image of T lymphocyte cells (pink) attached to a cancer cell. T lymphocytes are a type of white blood cell that recognize a specific site (antigen) on the surface of cancer cells to bind to it. Magnification x2300 when printed at 10cm wide.

Images from FineArtAmerica (images are on grey scale in original format)

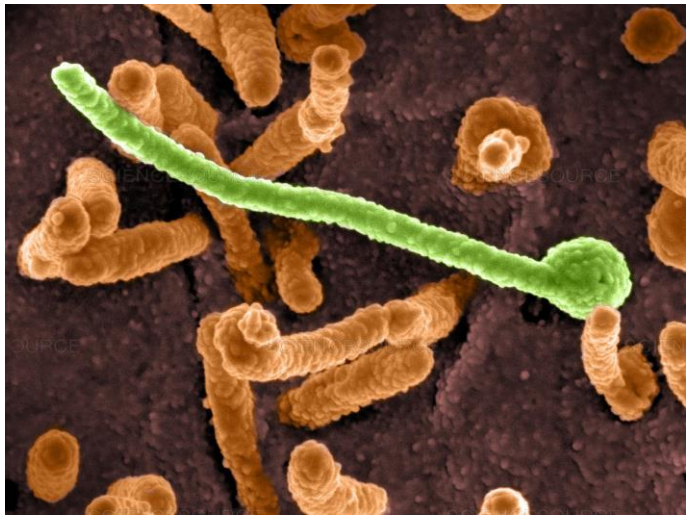
SEM: Viruses & Bacteria



E. coli bacteria: colored SEM image of Escherichia coli bacteria, rod-shaped bacteria part of the normal flora of the human gut. Magnification x14,000 when printed at 10cm wide.



Tooth bacteria: colored SEM image of bacteria from the surface of a human tooth. Mixture of bacteria, saliva and carbohydrates is known as plaque. Magnification X3,500 when printed at 10cm wide.

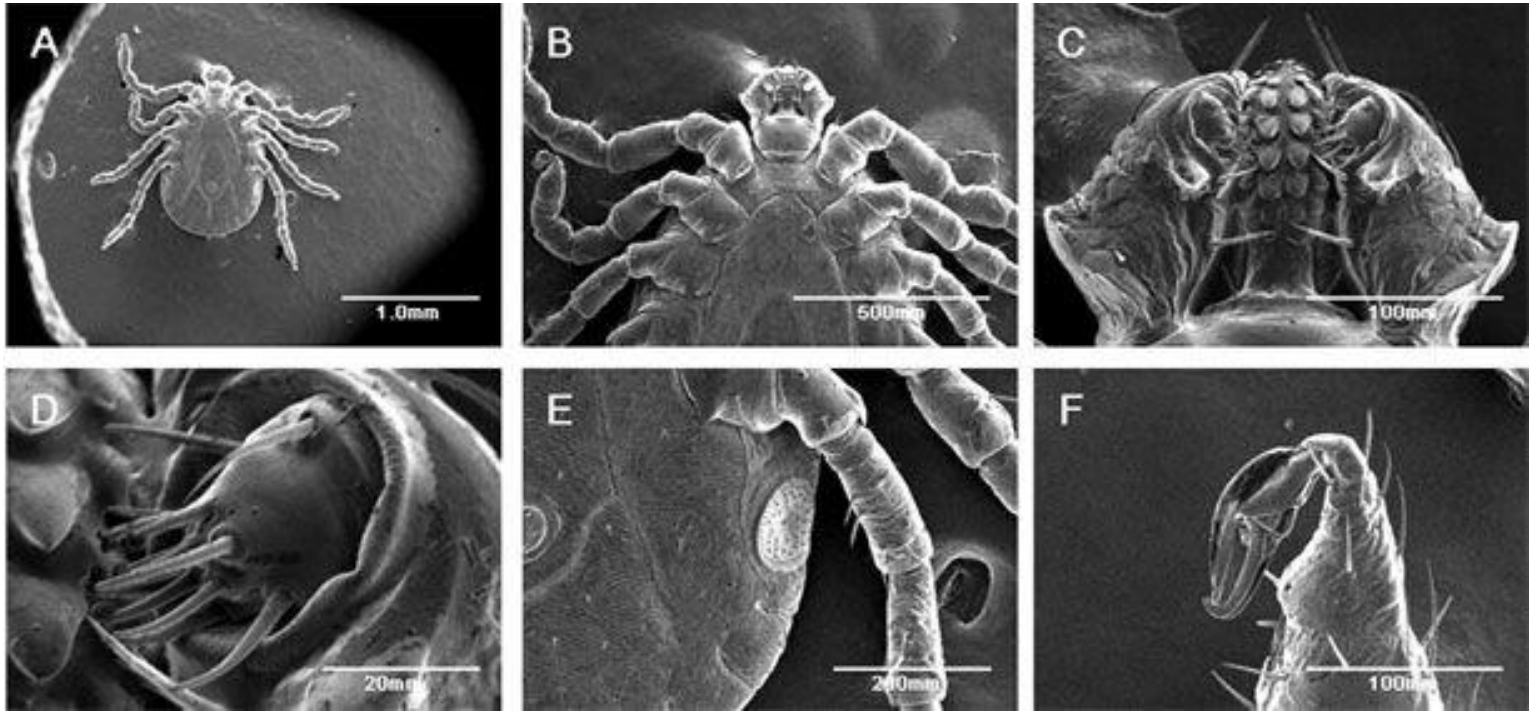


Ebola virus Makona: colored SEM image of Ebola virus Makona from the West African epidemic, on the surface of Vero cells. Magnification x10,000 when printed on 10cm wide

Images from FineArtAmerica (images are on grey scale in original format)

Still Alive in an SEM: a Tick Survives

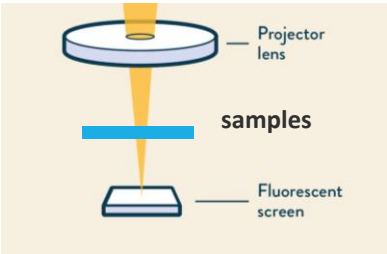
Bombarded with electrons and sealed in a vacuum,
the noble tick survived the ordeal!!!!



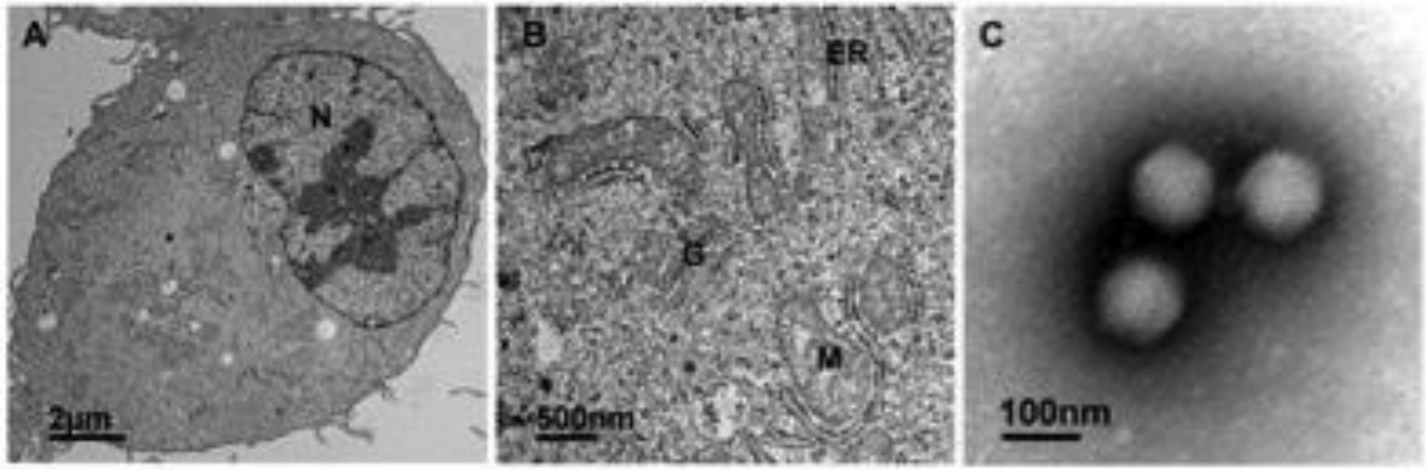
Click here if you want to
see the tick alive in an SEM

[PLoS ONE **7**(3): e32676]

Transmission Electron Microscopy



- TEM is appropriate for imaging either very small samples (*i.e.* molecules) or the inside of cells and tissues
- larger samples have to be sectioned (50-200nm) to become *e*-transparent
- TEM image is a 2D projection through the sample (everything in the image is in focus because of the large depth of field)



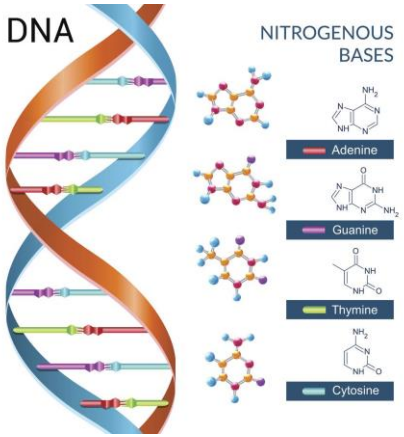
TEM images of a (A) fibroblast cell, (B) organelles within a fibroblast cell and (C) adenoviruses. The nucleus (N), endoplasmic reticulum (ER), Golgi apparatus (G) and mitochondria (M) can be seen in the cell.

TEM Sample Preparation

fixation	stabilize the cell to avoid changes/damages to the cell (snapshot in time of the living cell). This can be performed through ***chemical fixation*** or ***cryofixation***
rinsing	remove residues from fixation process
secondary fixation	fixation (*i.e.* OsO_4) to transform proteins in gel and enhance contrast
dehydration	replace water content with an organic solvent
infiltration	infiltrate and polymerize epoxy resin to harden the sample
polishing	reduce scratches and asperity on surface
slicing	cut sample in thin and *e*-transparent slices (<100nm)
staining	heavy metals (*i.e.* U, Pb, or W) to increase the contrast between different structures in the specimen

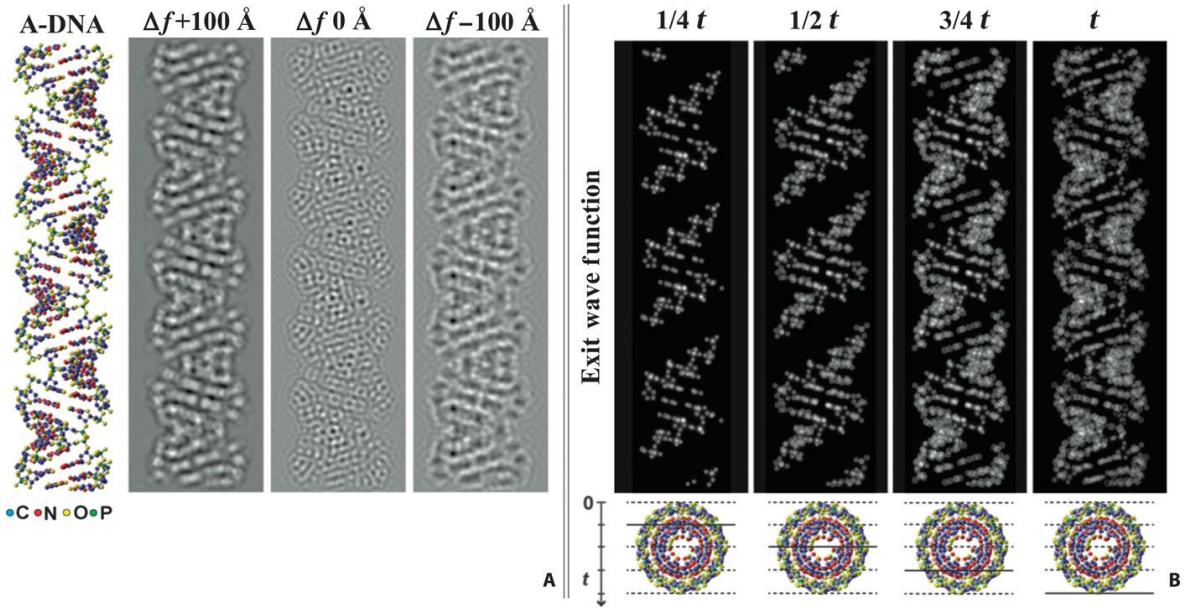
TEM: A Closer Look at DNA

DNA structure is studied by x-ray diffraction (indirect measurement). A direct image of DNA allows for a quantitative evaluation of all characteristic lengths and properties (nucleotides ~1nm).



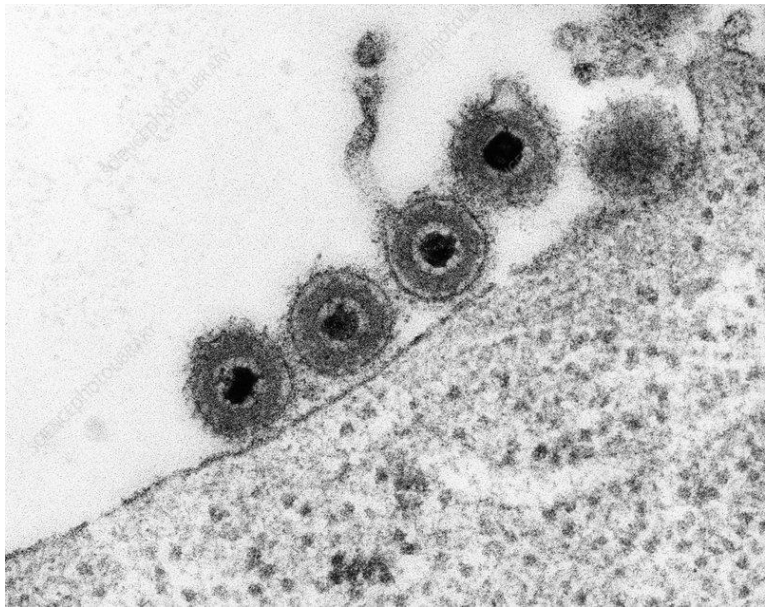
challenges:

- ***intrinsic very low contrast*** of the elements
- ***sample preparation*** while preserving pristine shape/size

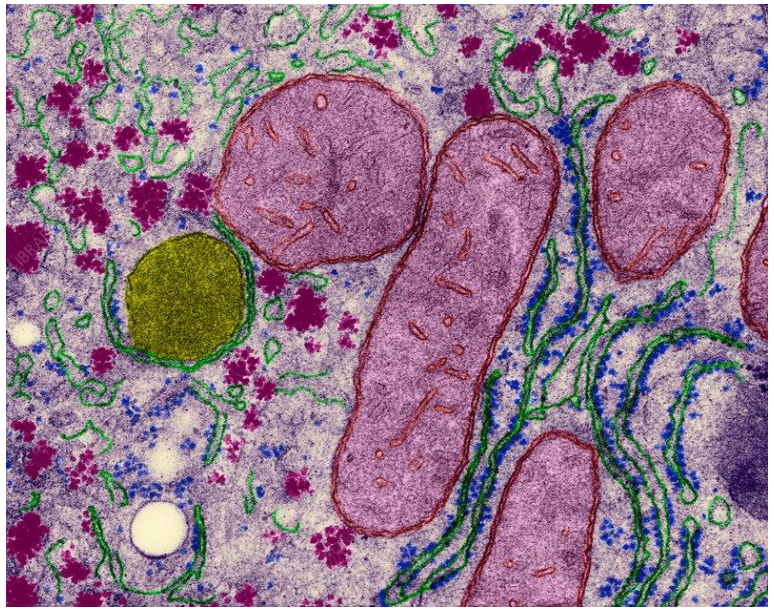


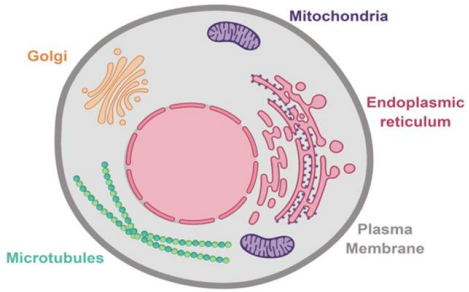
High-resolution TEM image (80 keV, resolution of 1.5Å) allows for the measurement of all relevant lengths of DNA. [Sci. Adv. **1**, e1500734 (2015)]

Transmission Electron Microscopy



Herpes simplex virus (HSV-2, DNA virus) on a peripheral blood lymphocyte. Herpes simplex is a viral infection, wihth HSV-2 causing mosst of the genital herpes cases. Magnification: x23,280 when shortest axis printed at 25mm.





Cellular organelles from a hepatocyte (liver cell): TEM micrograph showing mitochondria (light purple), endoplasmic reticulum (green), ribosomes (blue), glycogen granules (dark purple), peroxisome (yellow) and lipid bodies (white). Magnification: x11,440 when shortest axis printed at 25mm.

Cryo-Microscopy: A Nobel Story (2017)

*" We may soon have detailed images of life's complex machineries in atomic resolution. The Nobel Prize in Chemistry 2017 is awarded to **Jacques Dubochet, Joachim Frank** and **Richard Henderson** for the development of cryo-electron microscopy, which both simplifies and improves the imaging of biomolecules. This method has moved biochemistry into a new era"* (Royal Swedish Academy of Sciences)

https://www.nobelprize.org/prizes/chemistry/2017/press-release/

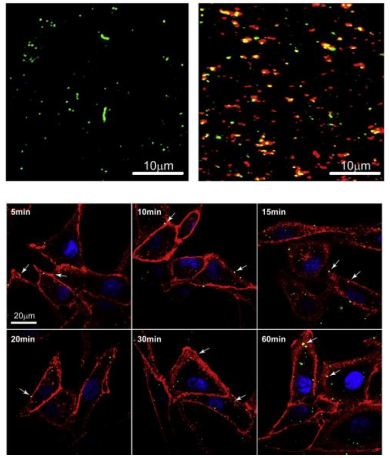
Jacques Dubochet | Joachim Frank | Richard Henderson

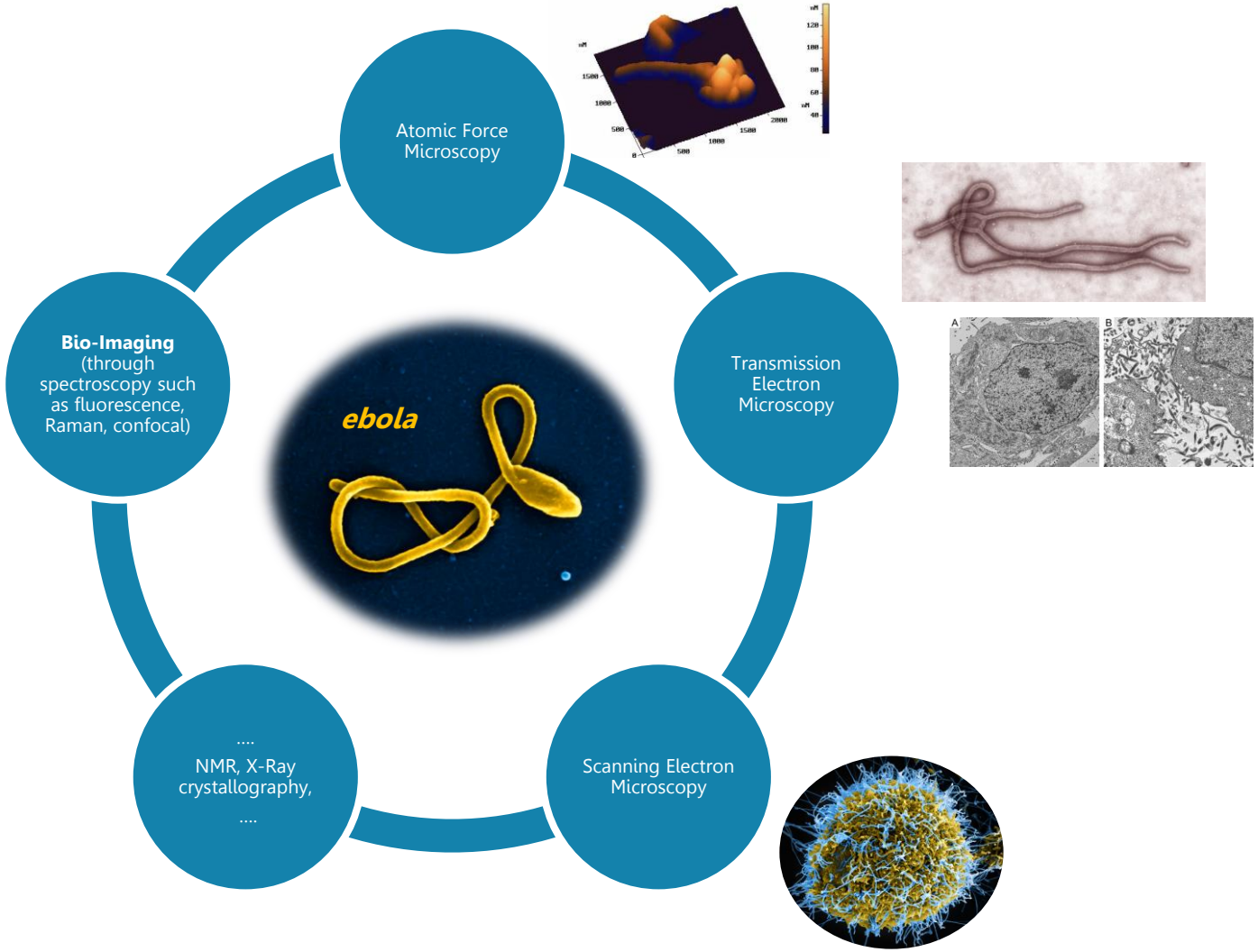
Richard Henderson succeeded in generating *a **3D image of a protein at atomic resolution*** with an electron microscope (1990). This proved the technology's potential

Joachim Frank made the ***technology generally applicable***, while developing between 1975 and 1986, an ***image processing method*** in which the electron microscope's fuzzy 2D mages are analyzed and merged to reveal a sharp 3D structure

Jacques Dubochet added ***water to electron microscopy***. Liquid water evaporates in vacuum, making biomolecules collapsing. In the early 1980s, Dubochet succeeded in vitrifying water – he cooled water so rapidly that it solidified in its liquid form around a biological sample, allowing the biomolecules to retain their natural shape even in a vacuum

Complementary Imaging Techniques





Summary of Lecture

- **Microscopy:** *going beyond visible light resolution*
  - study of surfaces
    - using Coulomb interaction (Atomic Force Microscopy)
    - using tunneling current (Scanning Tunneling Microscopy)
  - study of materials/surfaces with electrons
    - scattered electrons from the sample (SEM)
    - transmitted through the sample (TEM)