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Central European Institute of Technology

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BioAFM imaging

Nové směry v bioanalytické chemii

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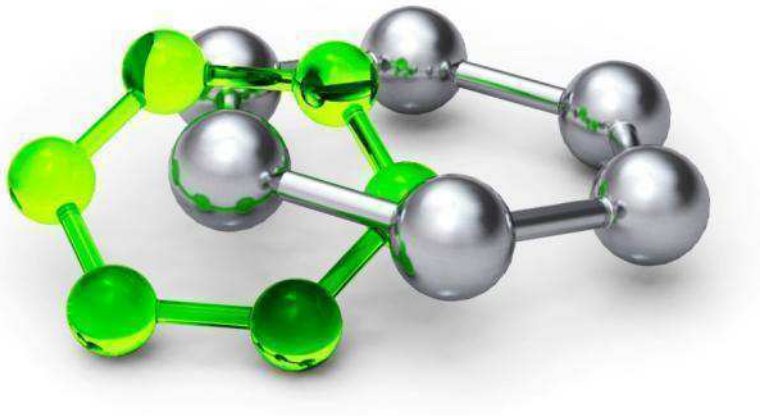

EUROPEAN UNION
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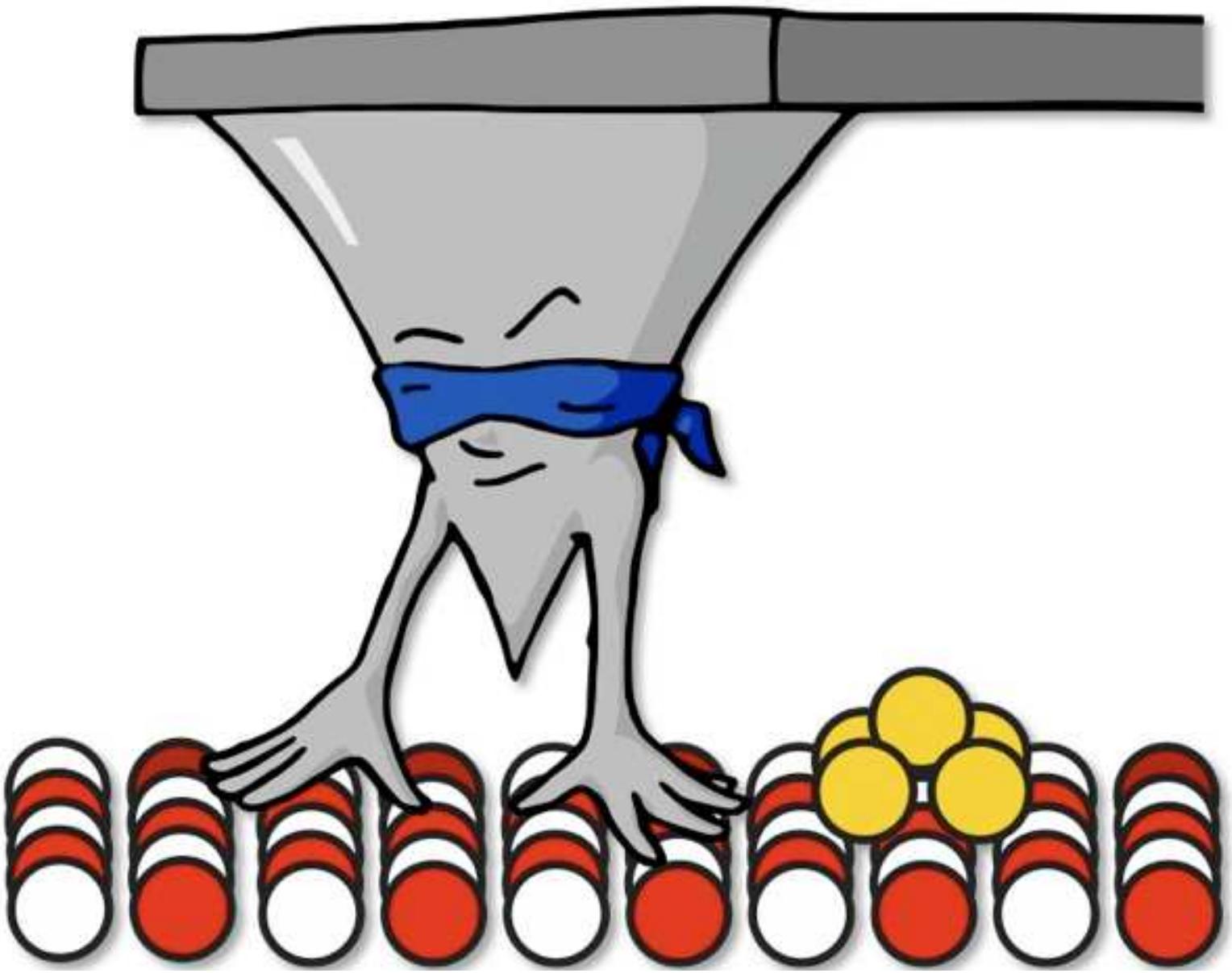
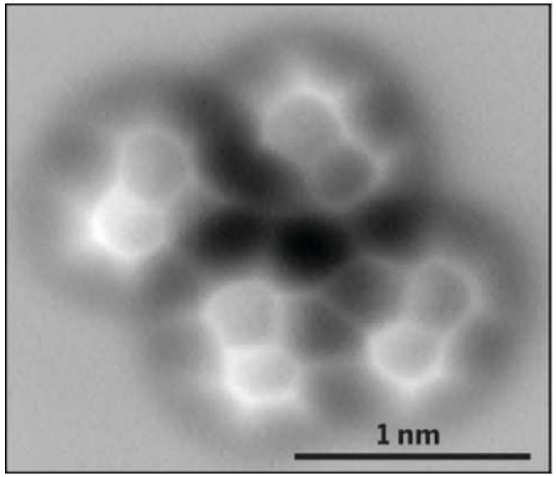
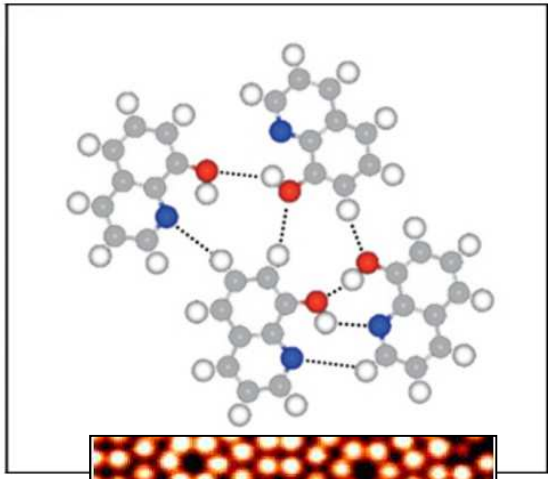
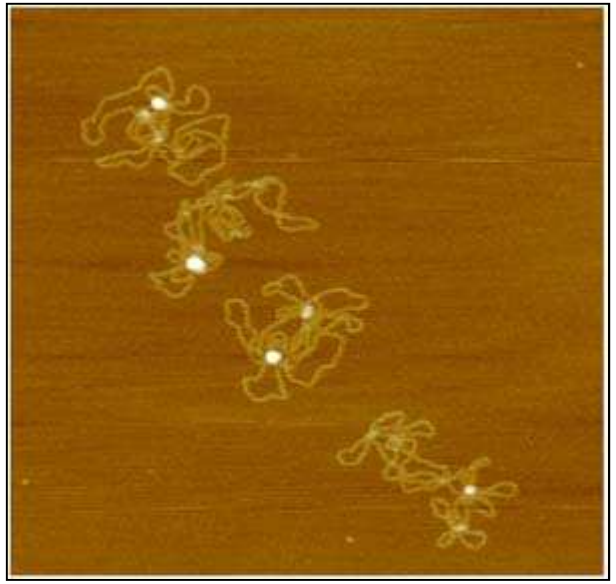
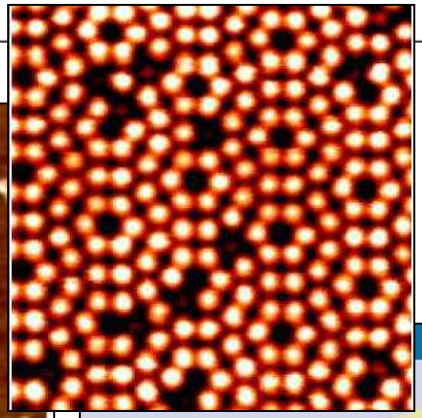
2007–13
OP Research and Development for Innovation

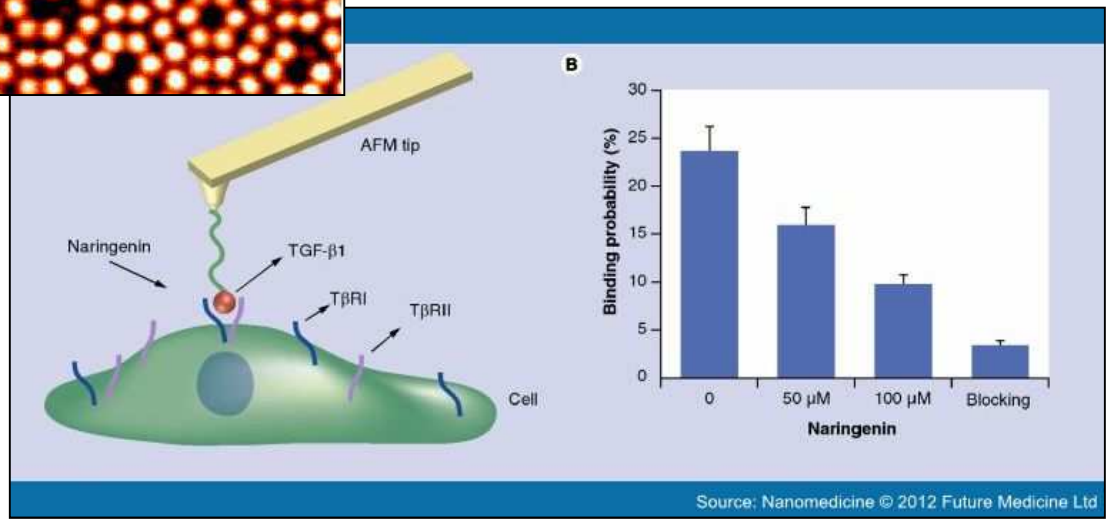



1 nm








AFM tip
Naringenin
TGF-β1
TβRI
TβRII
Cell
B
Binding probability (%)
30
25
20
15
10
5
0
0
50 μM
100 μM
Blocking
Naringenin
Source: Nanomedicine © 2012 Future Medicine Ltd

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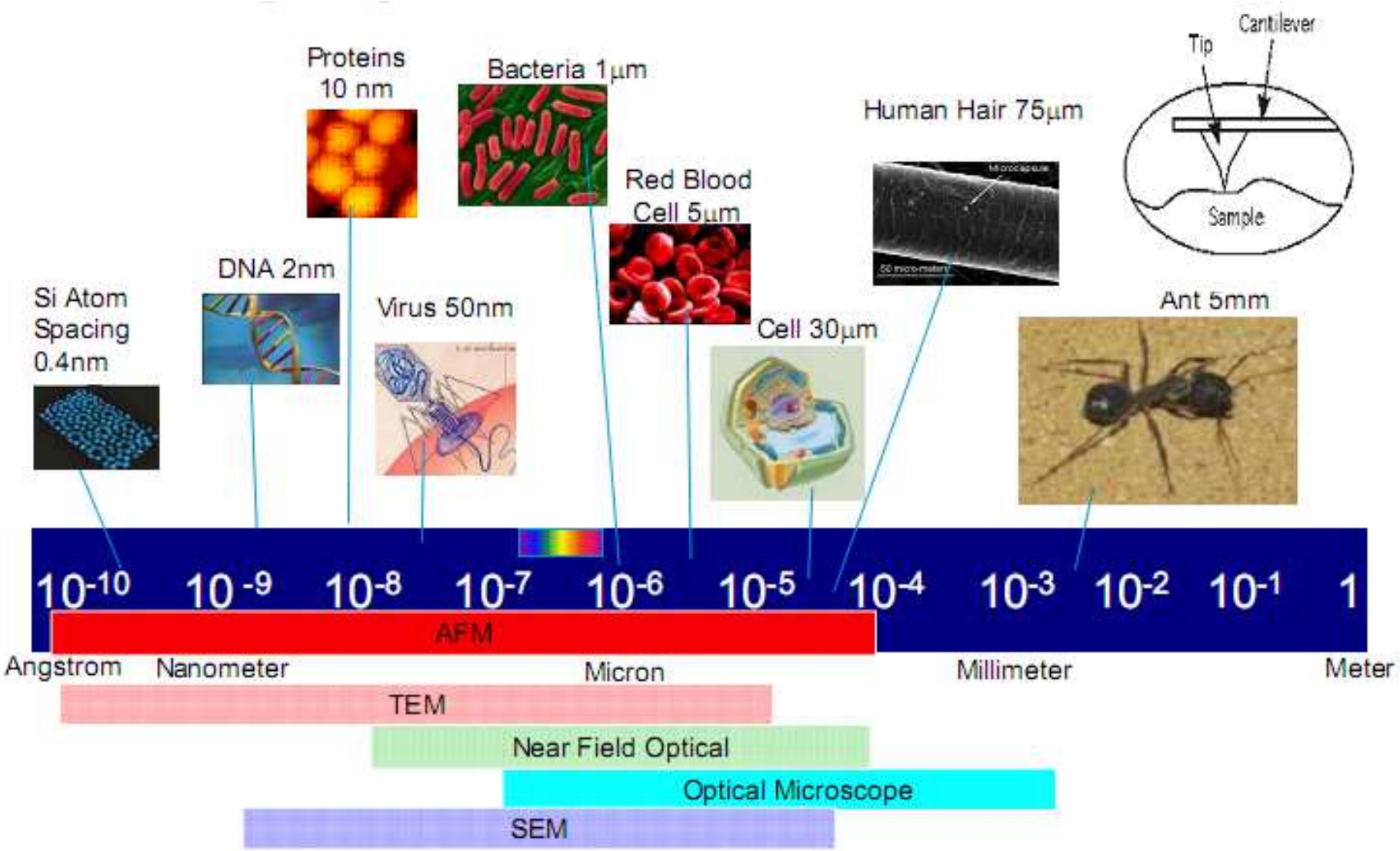
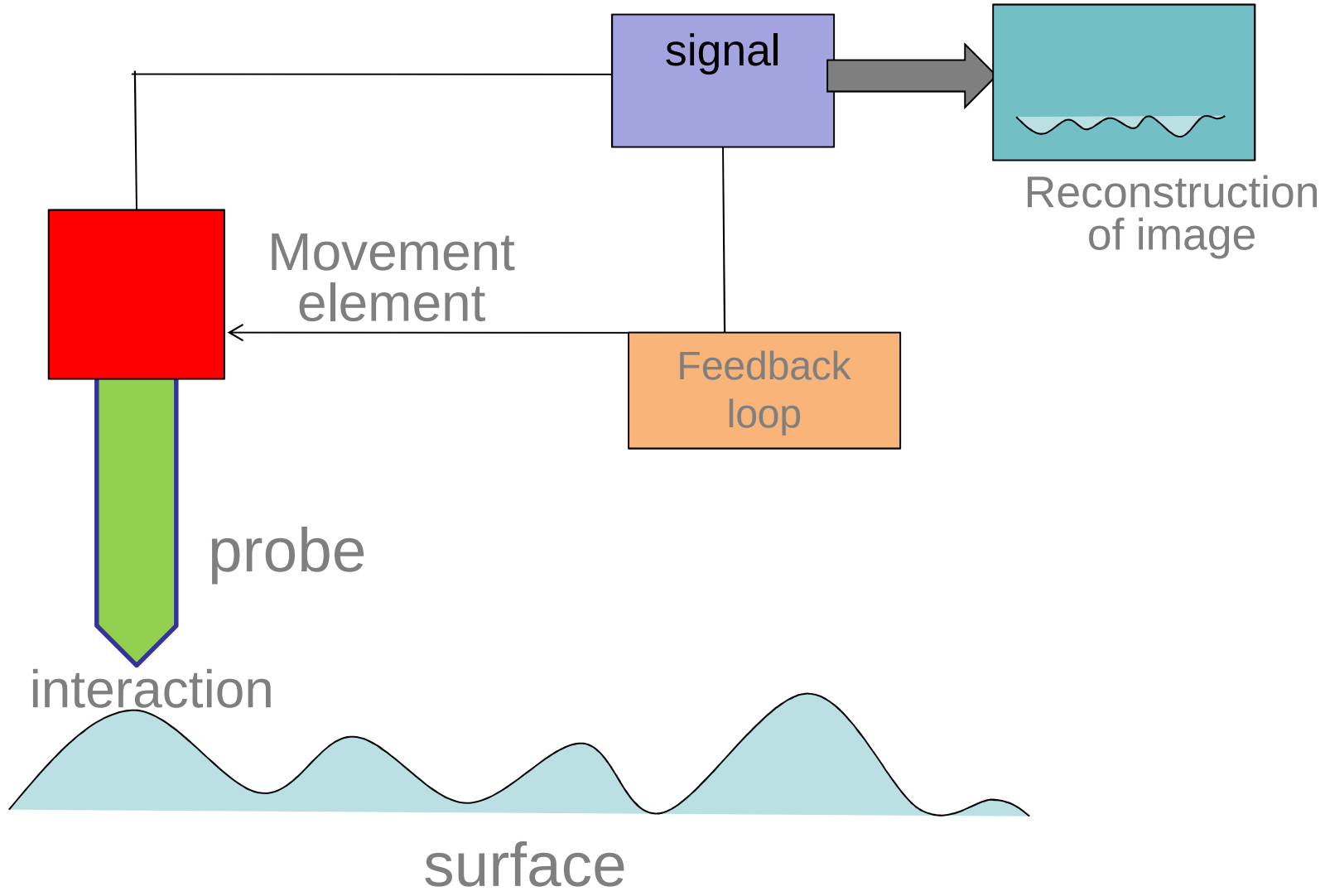
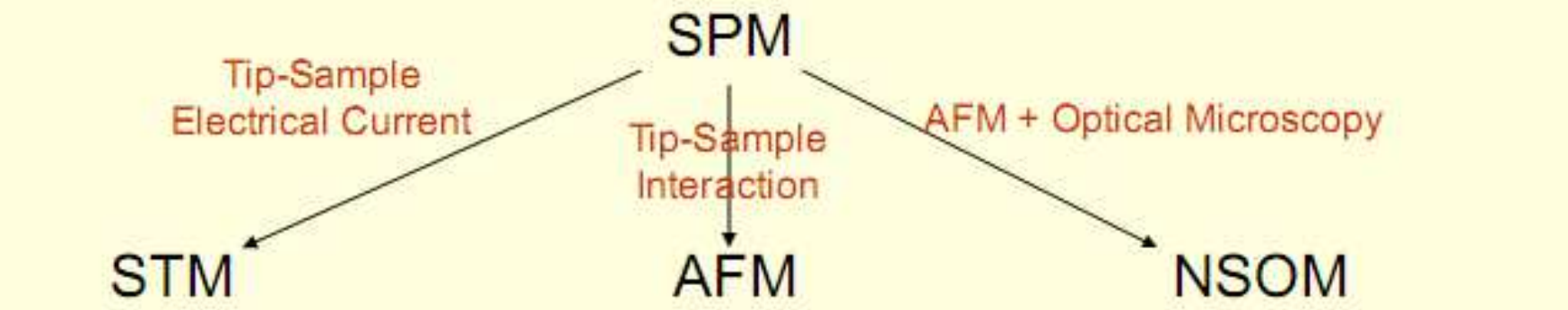
Microscopy techniques - resolution



Scanning Probe Microscope
basic scheme



SPM Family



Extremely high Resolution at UHV

Mechanic Force:

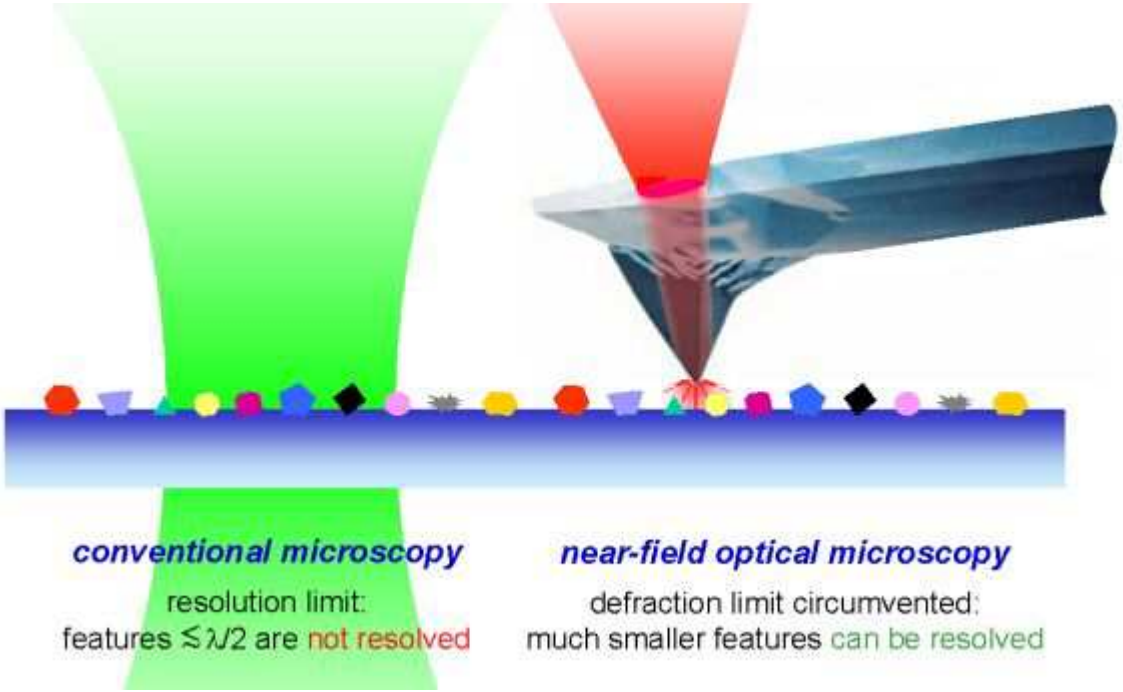
- **Contact mode**
- **Non-contact mode**
- **Tapping (intermittent) mode**

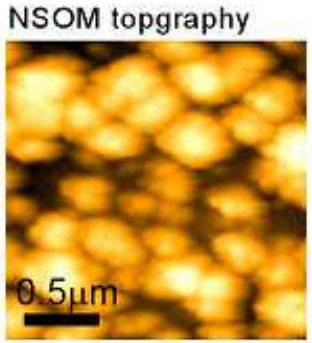
Other Interactions:

- **Electrostatic mode (scanning electrostatic potential microscope)**
- **Magnetic mode**
- **Chemical Force mode**

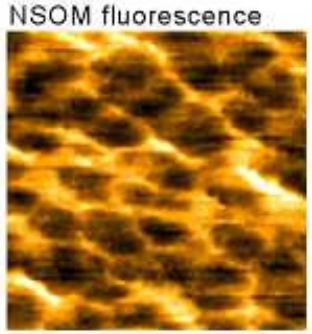
SNOM (=NSOM)
Scanning NearField Optical Microscopy

SNOM – basic principles





TiO_2 particles wrapped in PPV film



Fluorescence quenching by TiO_2 particles

- **Light diffraction limit** - conventional optical microscopy: λ/2 ~ 250 nm (→ Abbe diffraction limit)
- Real cases - **optical resolution** ~ λ, 500 nm
- **SNOM** offers higher resolution around 50 nm (or even < 30 nm), depending on tip aperture size.
- **Near-field** = distance << wavelegth

- **SNOM**- simultaneous measurements of the:
  - topography
  - + optical properties (fluorescence)
  - → direct correlation between surface nanofeatures and optical/electronic properties.
- Useful for the **studying**:

  inhomogeneous material surfaces (nanoparticles, polymer blends, porous silicon, biological systems)

History of NSOM

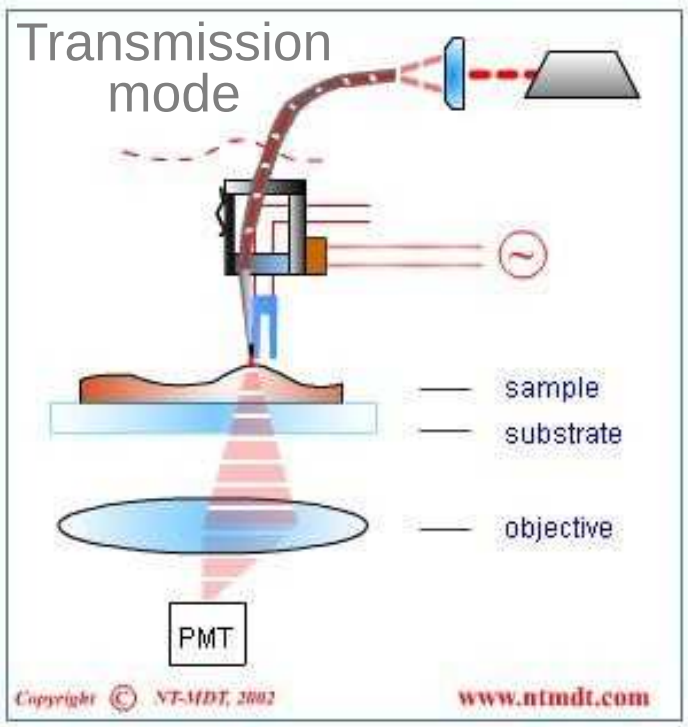
1928 roots trace back – letters between Edward Hutchinson Synge and Albert Einstein

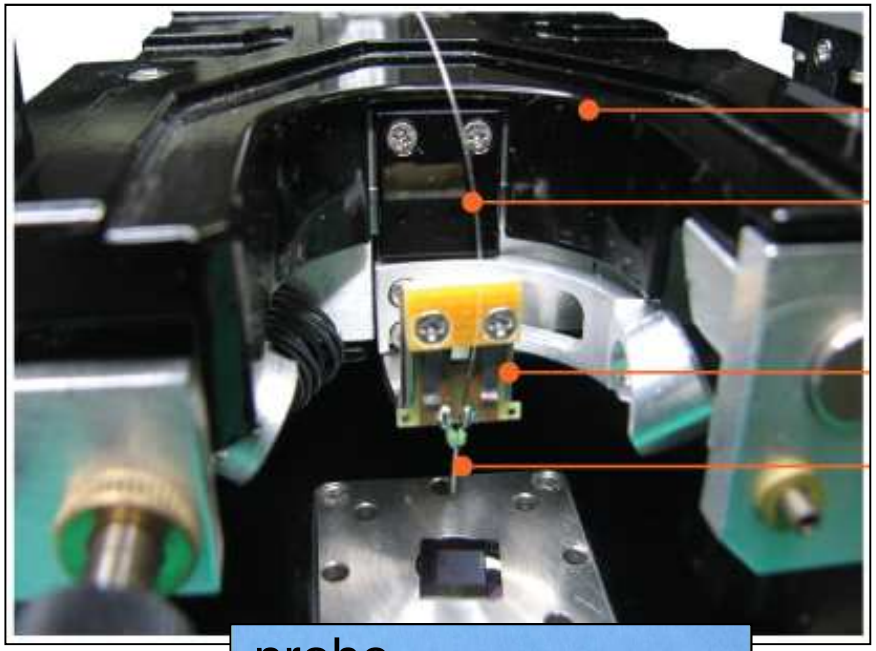
Technology developed in 1990's:

- Eric Betzig, et al. Science, 262, 1422-1425 (1993).

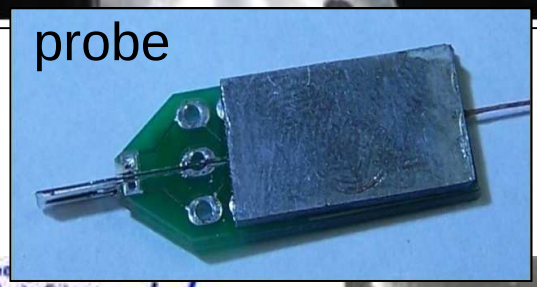
Prototype commercial available since 2000's

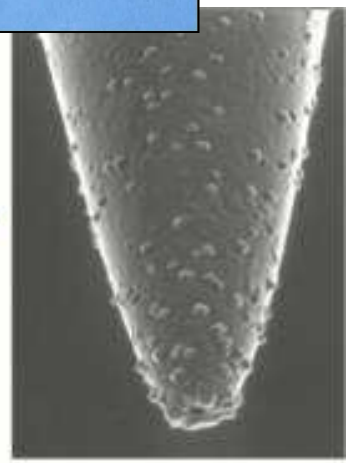
Scheme of SNOM apparatus

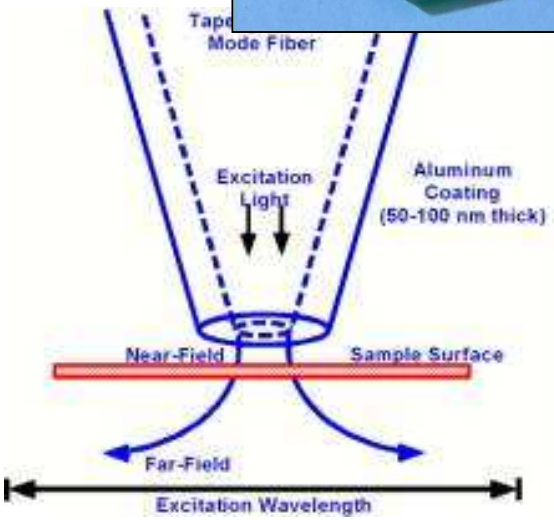




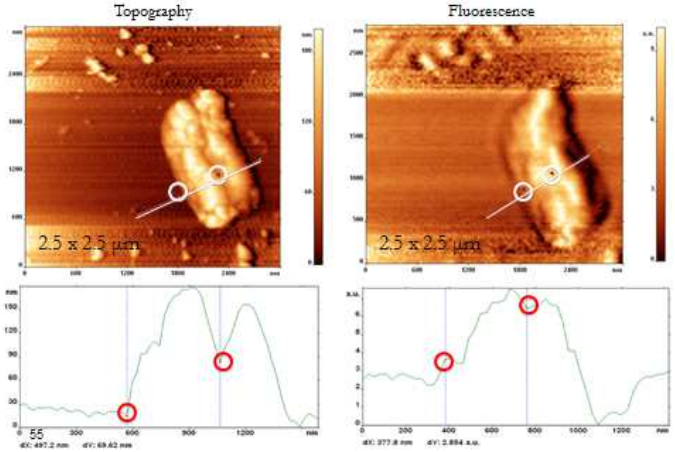
probe



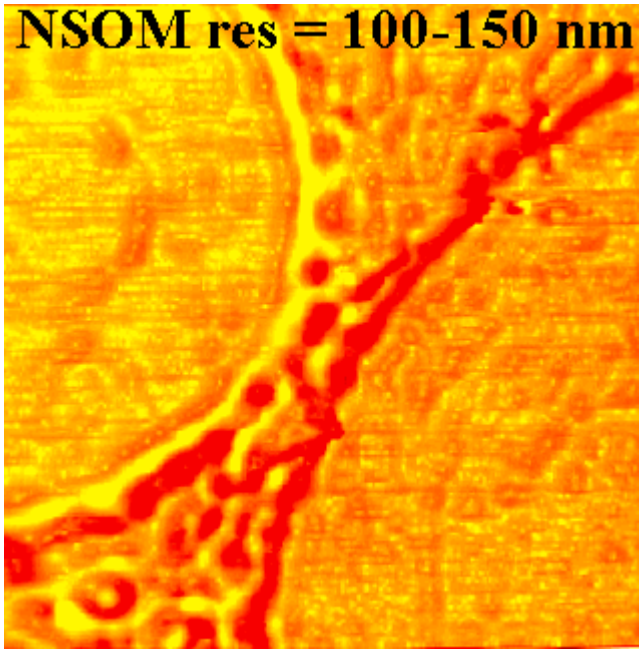


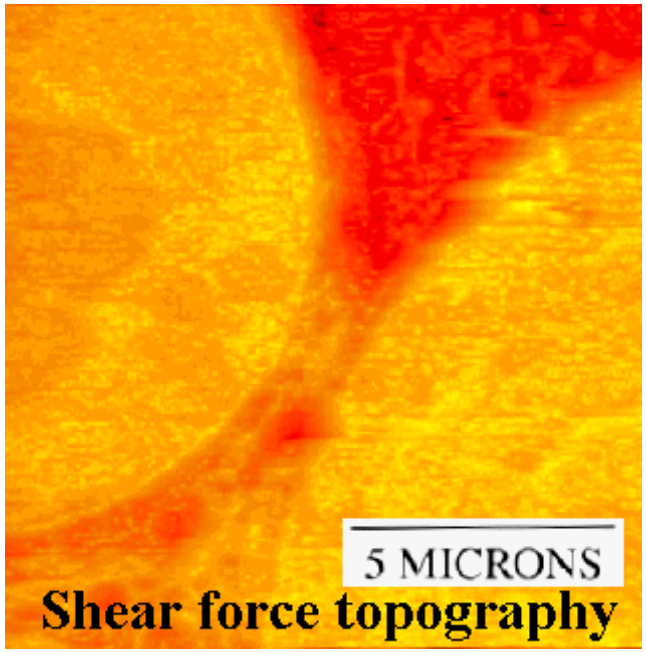


Picocyanobacteria (PCC 7942)

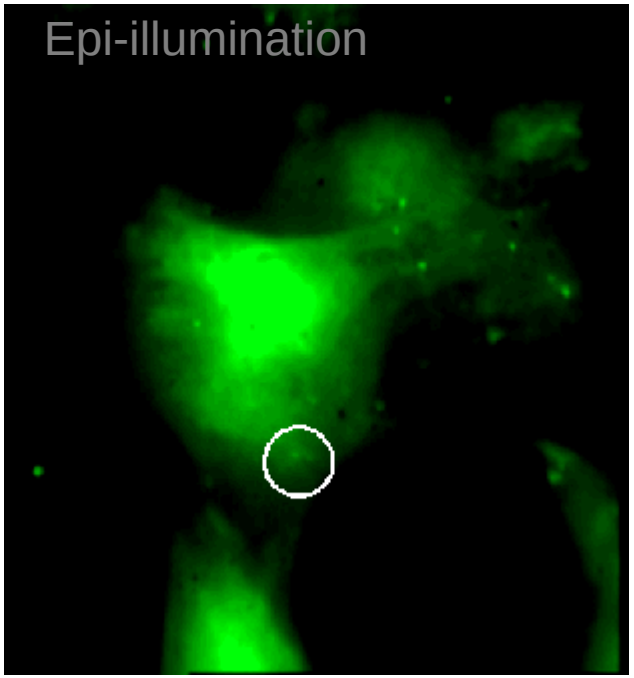


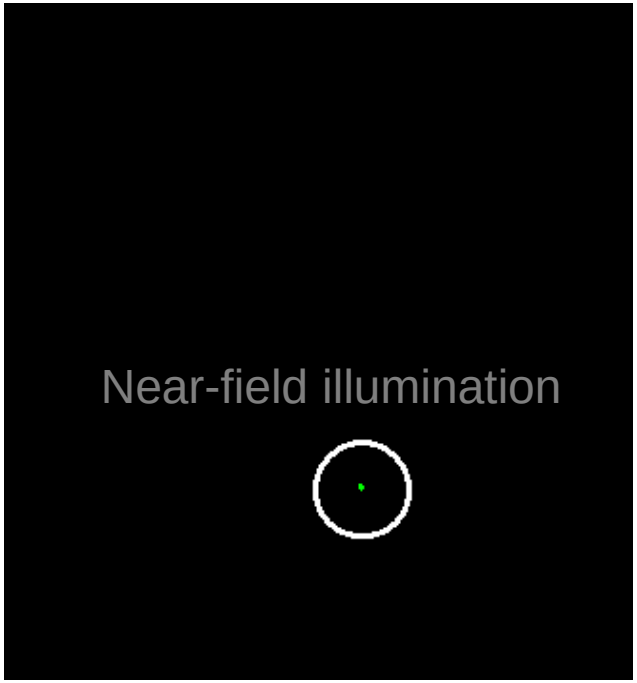
NSOM images





Tissues images





Living cells

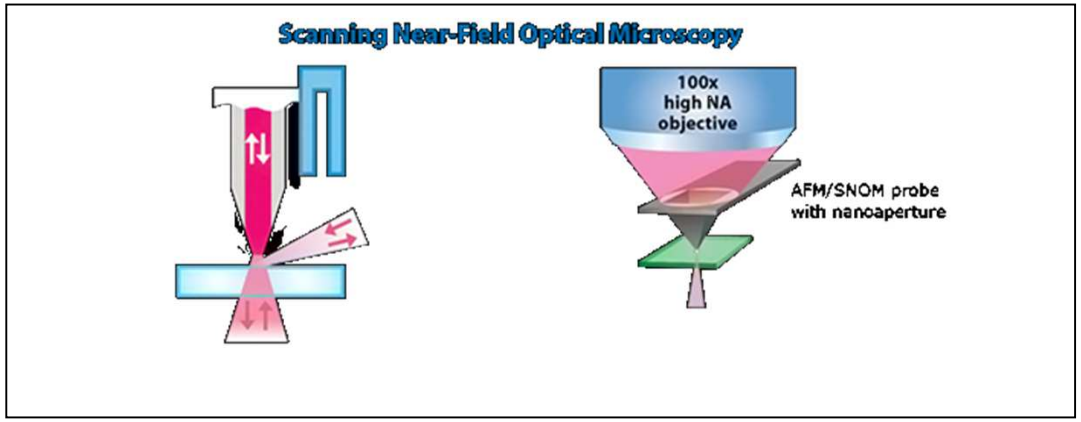
Real instrument example

Ntgra Vita AURA, Ntgra Vita SPECTRA (NTMDT, Zelenograd, Russia)

Bringing light close to the surface

Optical fiber
+ tuning fork

AFM transparent probe
+ light via objective



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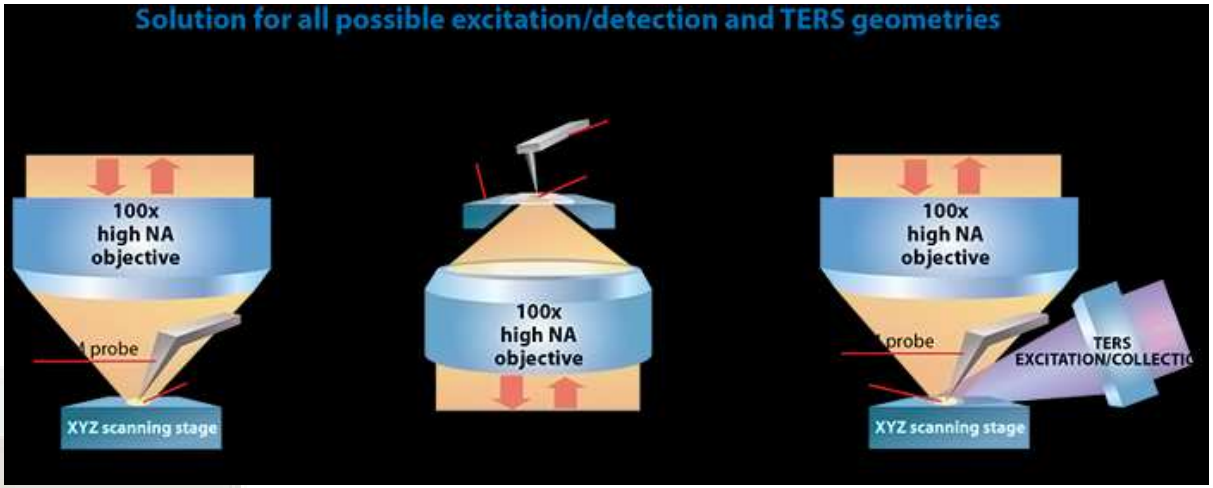
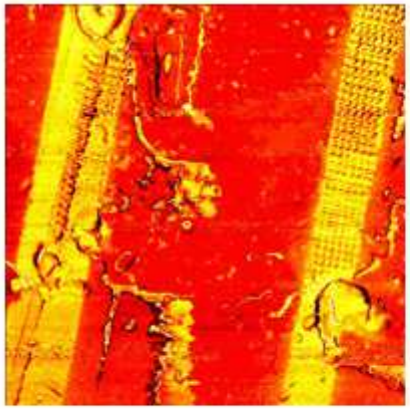
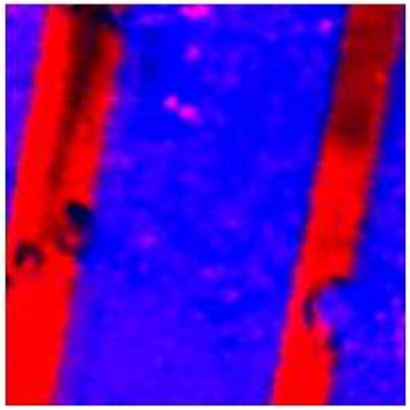


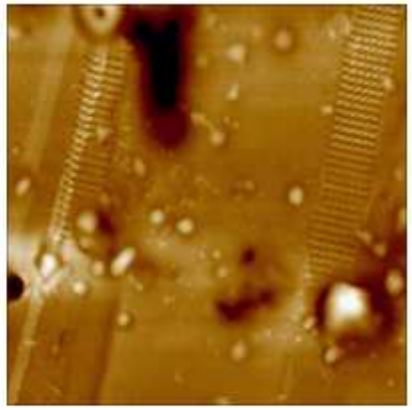
Tip Enhanced Raman Spectroscopy



Stiffness of HDPE/LDPE polymer sandwich cut by microtome



Overlap of Raman maps: HDPE (red), LDPE (blue)



AFM topography

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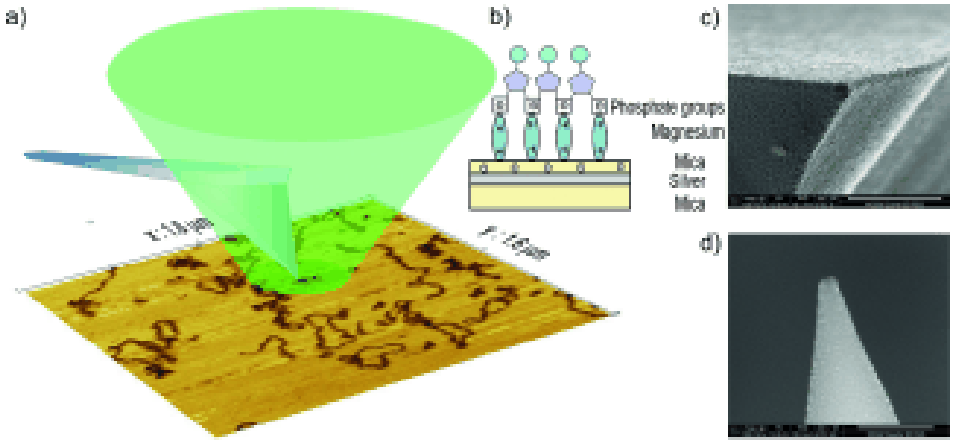
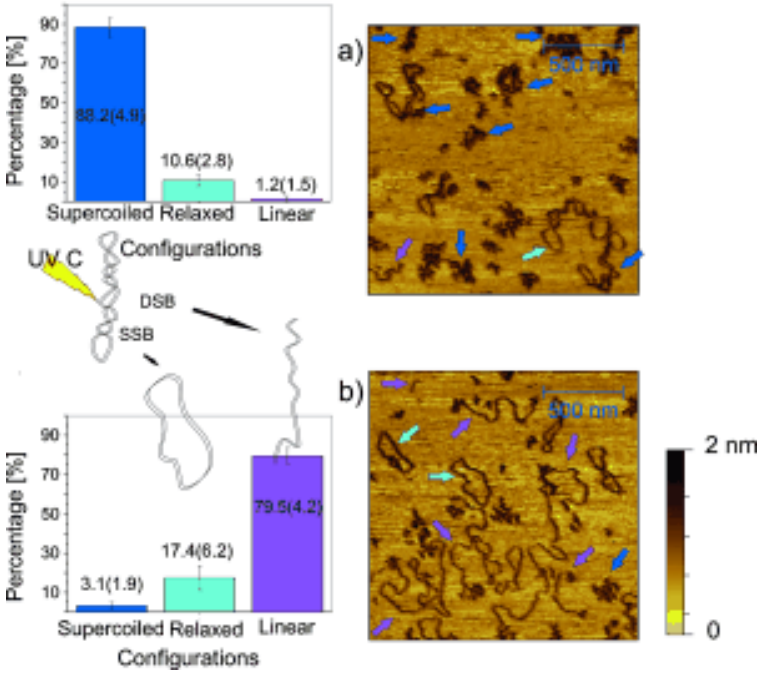
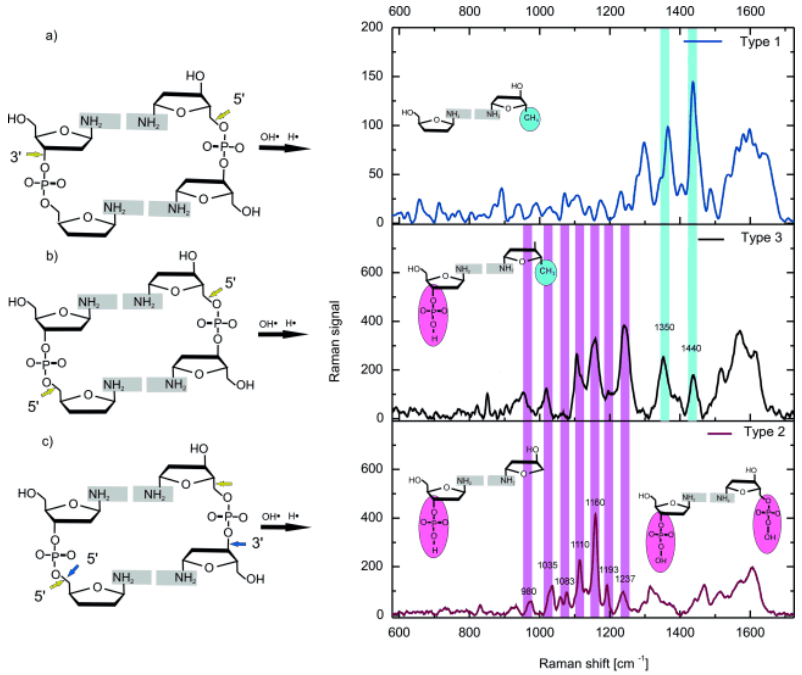
Examples

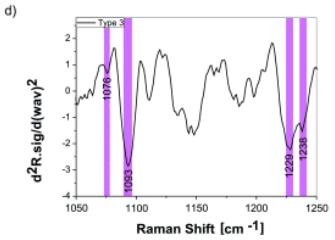
Use TERS technology for DNA structure study

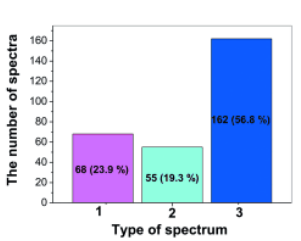
Molecular Characterization of DNA Double Strand Breaks with Tip-Enhanced Raman Scattering











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http://onlinelibrary.wiley.com/doi/10.1002/anie.201307271/full#fig1

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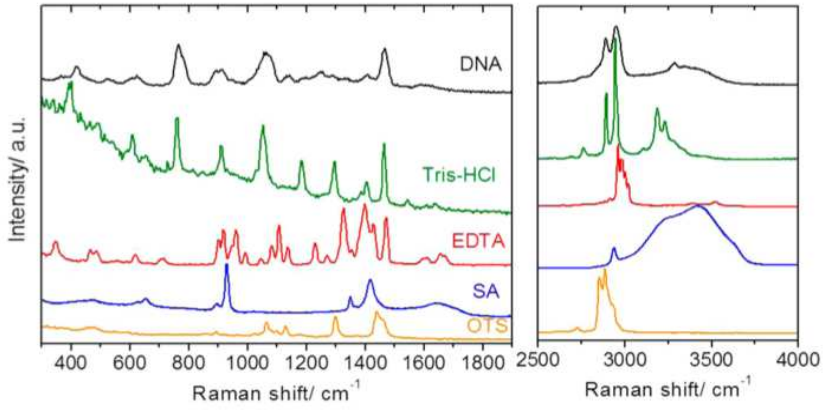
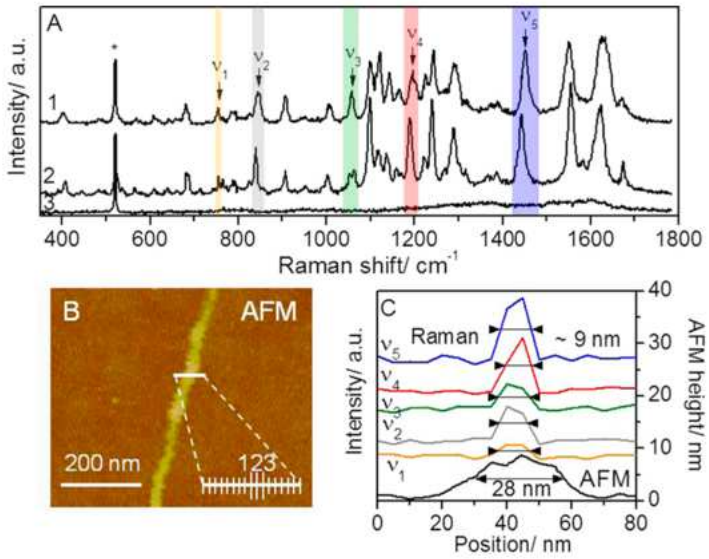
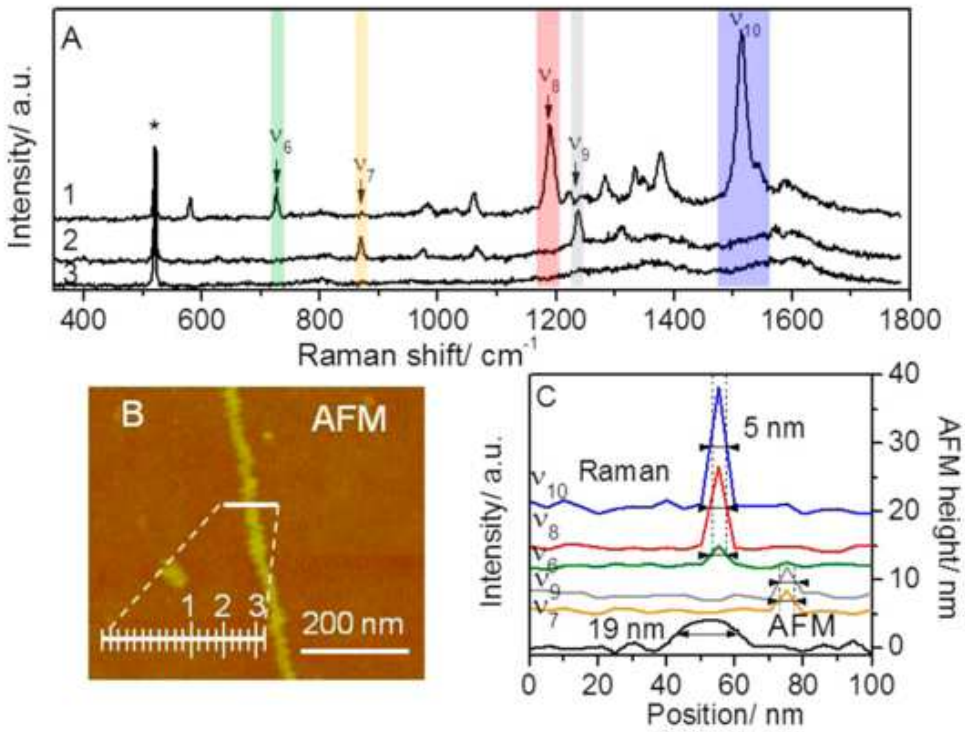
Tip-Enhanced Raman Spectroscopy of Combed Double-Stranded DNA Bundles







J. Phys. Chem. C, **2014**, *118* (2), pp 1174–1181

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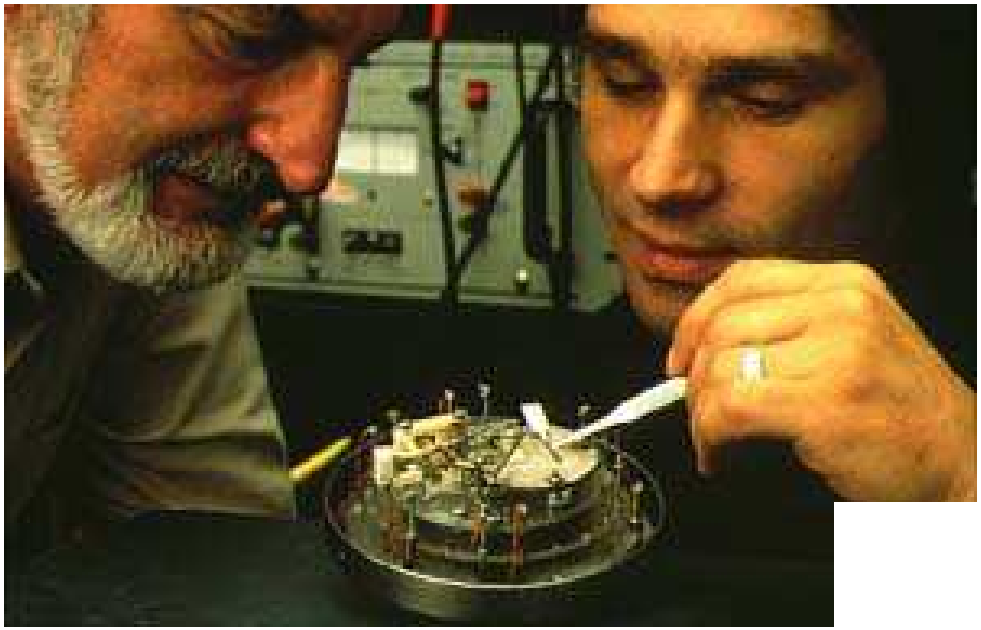
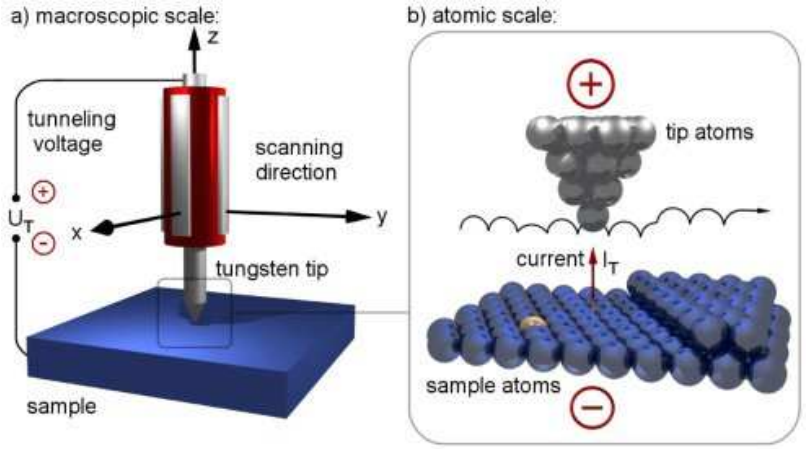
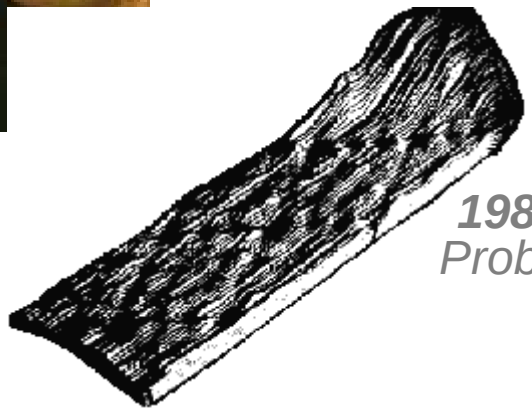
Scanning Tunnelling Microscopy
STM

- **STM** - the **first** member of **SPM** family
- Developed in **1982** by Gerd Binnig and Heinrich Rohrer members of IBM in Zurich (Phys. Rev. Lett., 1982, vol 49, p57)
- **1986 -** Nobel prize in physics for their brilliant invention







***1982** - Triumph of Scanning Probe Microscopy - image of silicon surface 7x7 reconstruction.*

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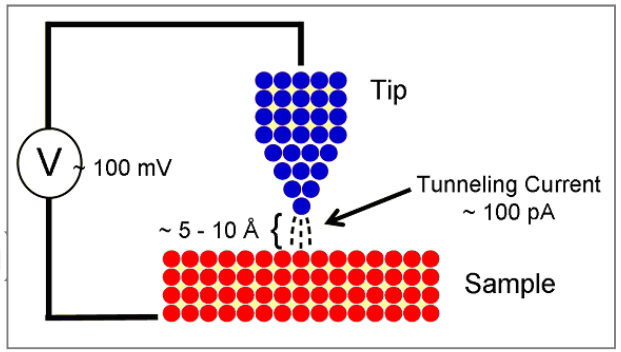
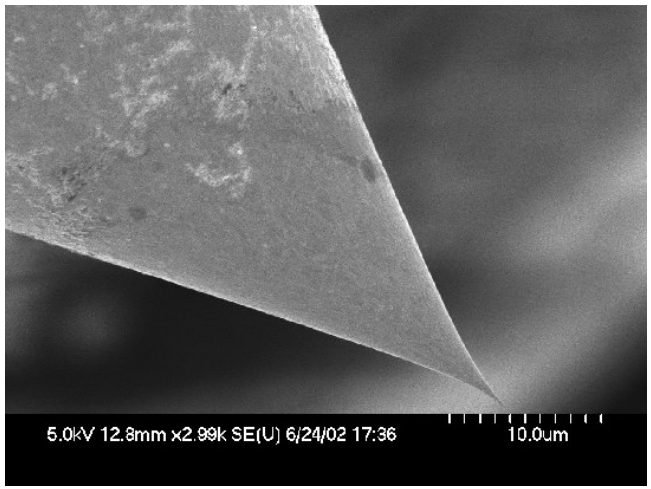
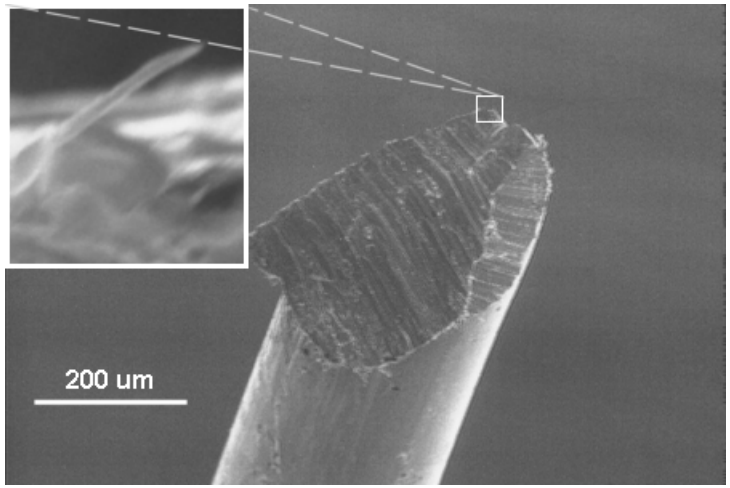
STM tip

- **STM tip** - conductive (metals - Pt, W, Pt/Ir)
- **STM** microscopy uses the very top (outermost) atom at the tip and the nearest atom on sample

  Tip is not necessarily very sharp in shape (different from AFM)
- **Tip preparation**:
  - Cutting with scissors
  - Electrochemical etching
  - Other techniques such as FIB (and combination)







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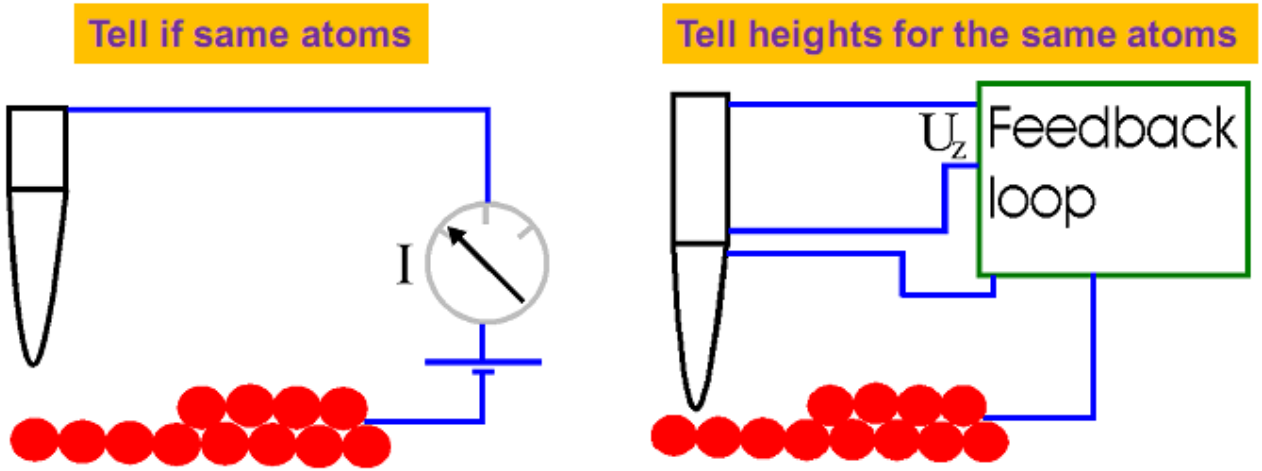
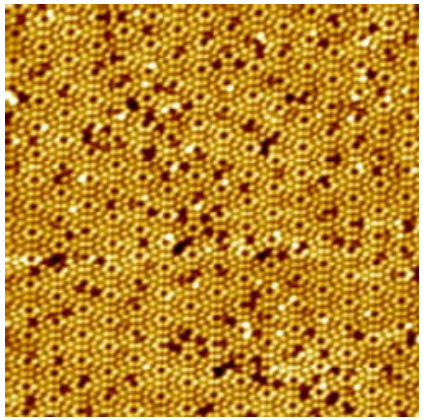
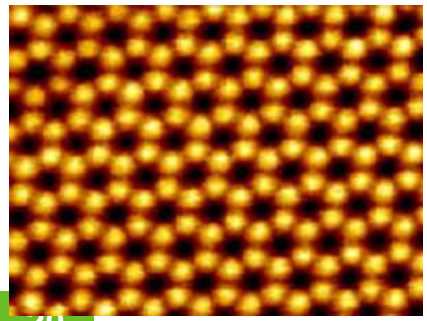
STM modes



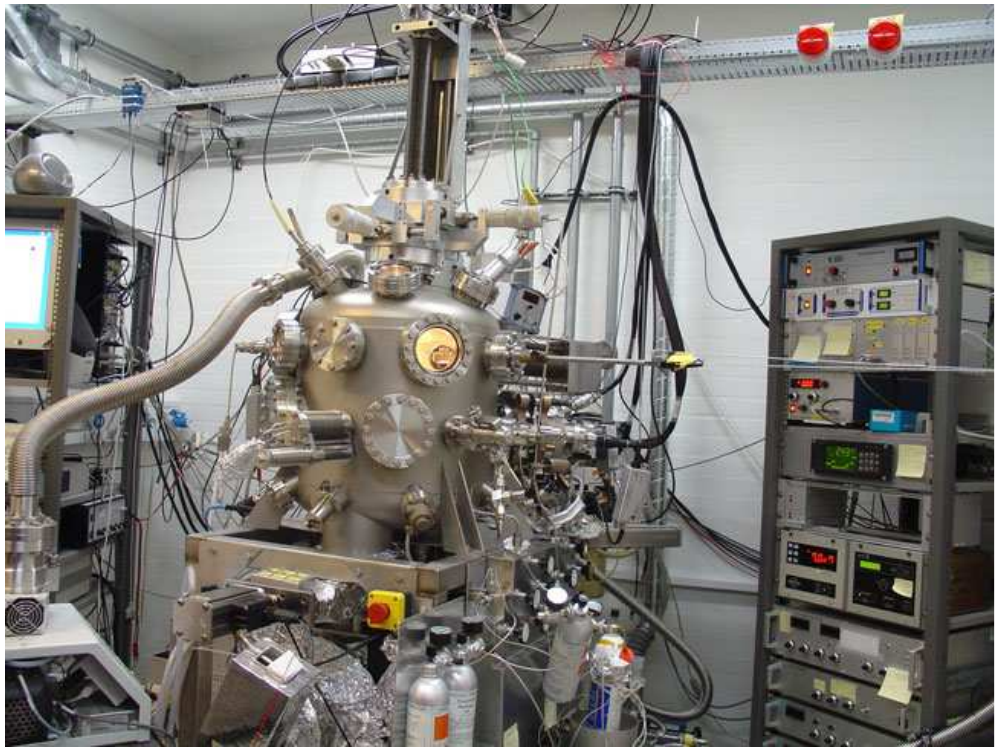
Constant height / constant current



Si (111) 7x7, 40nm
empty states image, room temperature, dark spots represent missing atoms or adsorbates

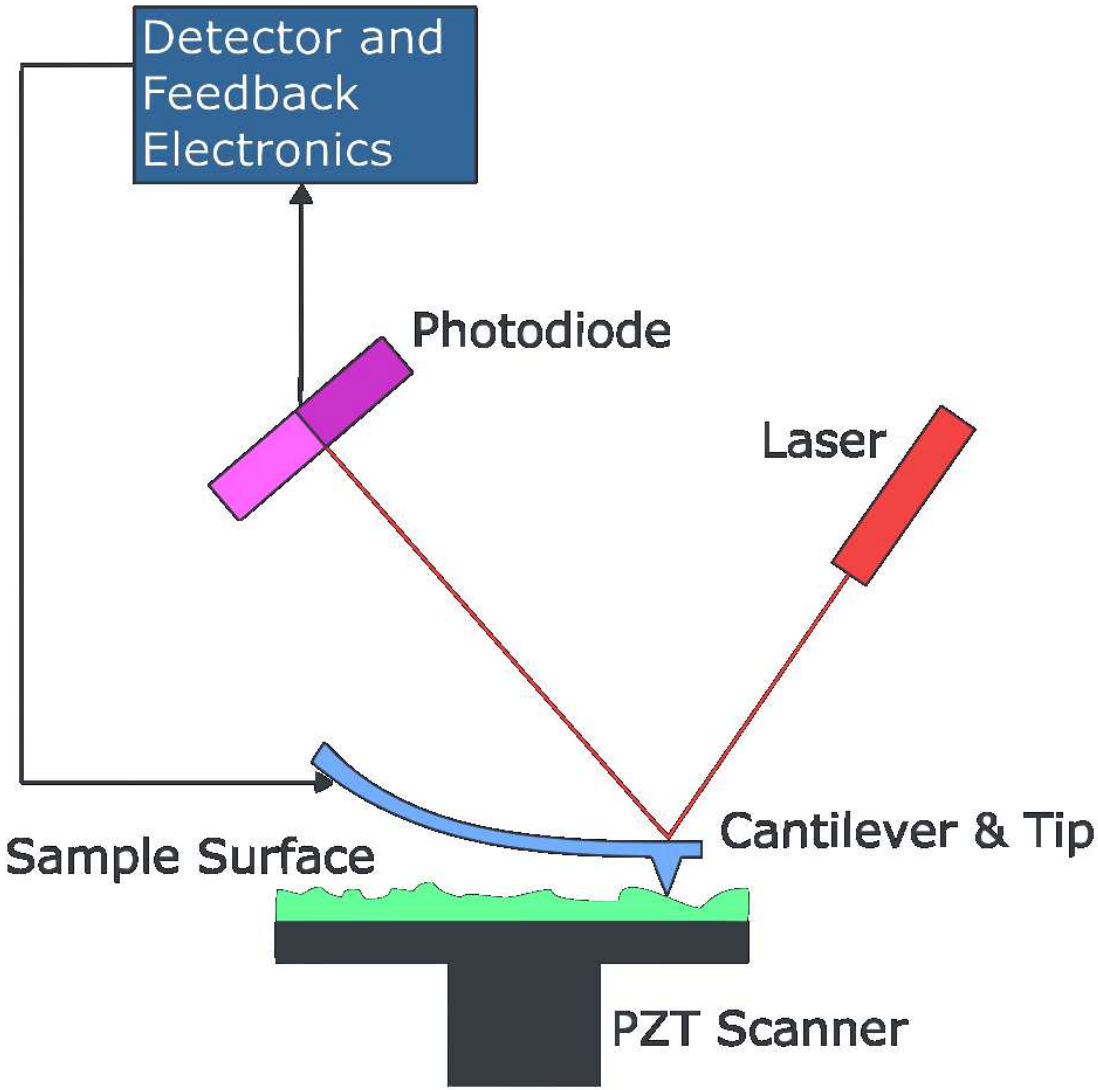


Ag-Si (111) 10nm



Atomic Force Microscopy
AFM

AFM microscope basic scheme



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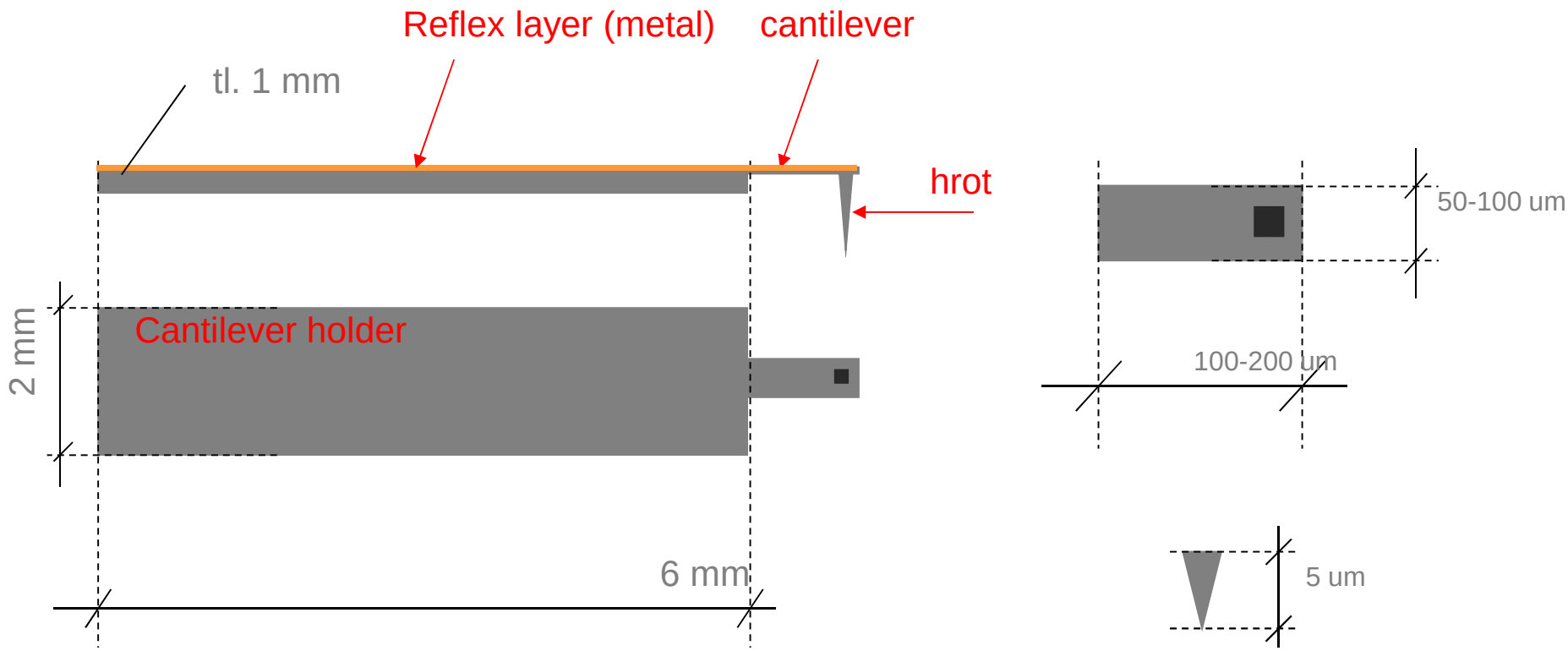
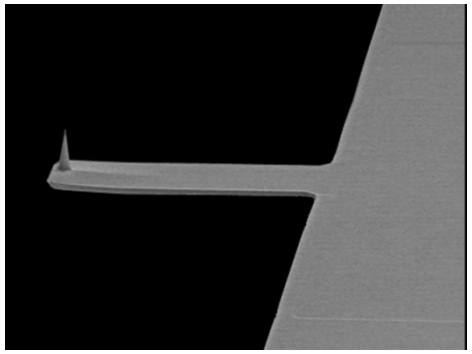
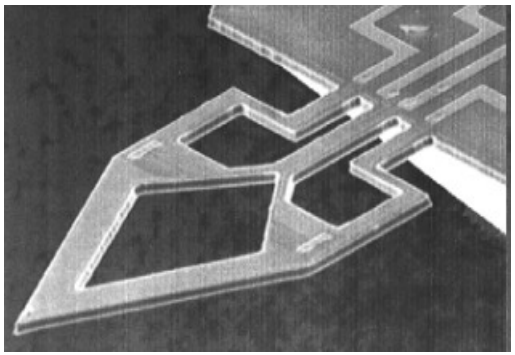
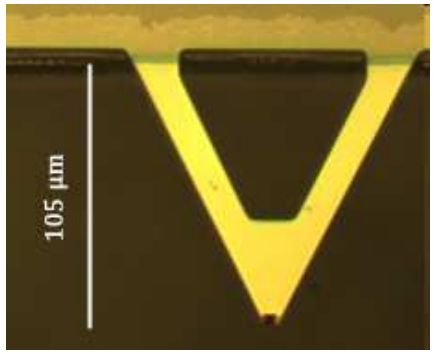
Tip and cantilever

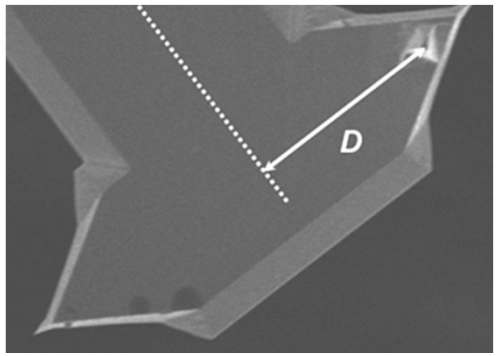
Cantilever and tip



- Cantilever holder is quite universal
- Cantilever and tip – a variety of various types

Cantilevers



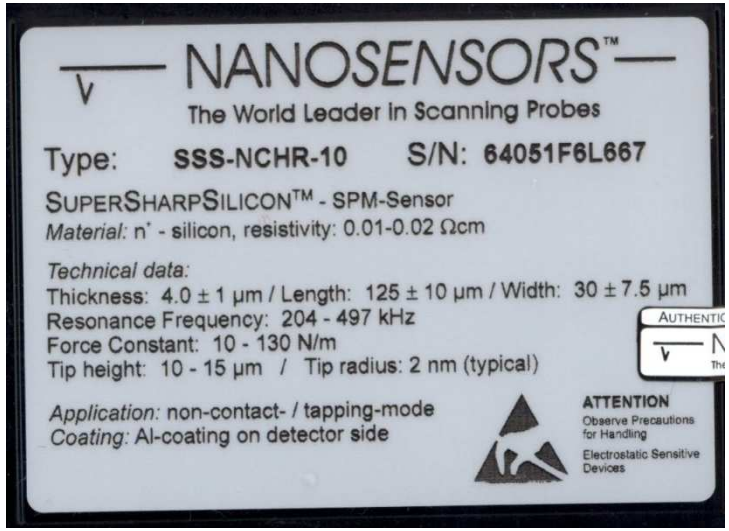
Material properties – Stiffness **Force Constant [N/m]**

Force const.[N/m]	10-130	1-10	0.1-1.0	0.005-0.1
Material	cryst. silicon	pol. silicon	glass	Si_3N_4
Res. f. [kHz]	200-500	100-200	15-100	1-20

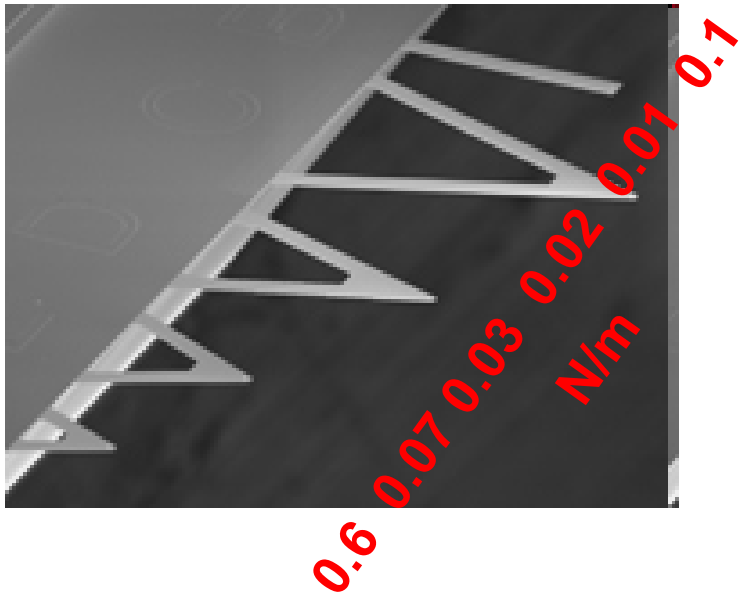
Special applications – conductive, colloid, magnetic, tip less, …

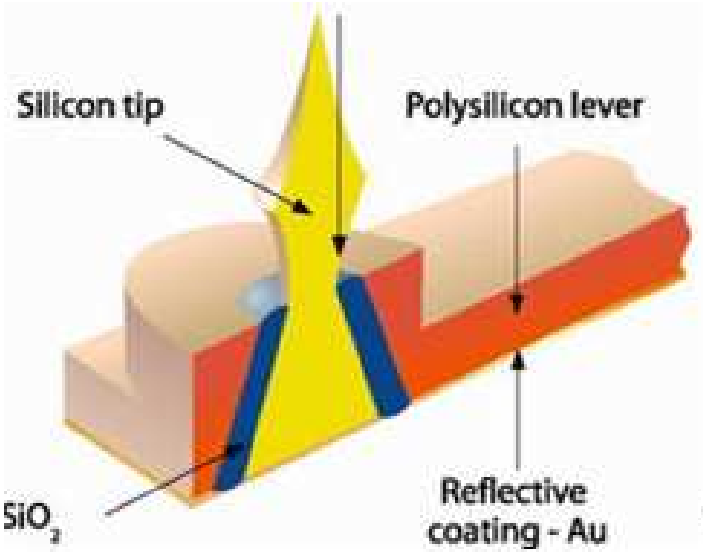
25

Cantilever
characterization you may find on box



Cantilever field
choose the one you like/need





AFM probes (**micro)fabrication** is quite complex

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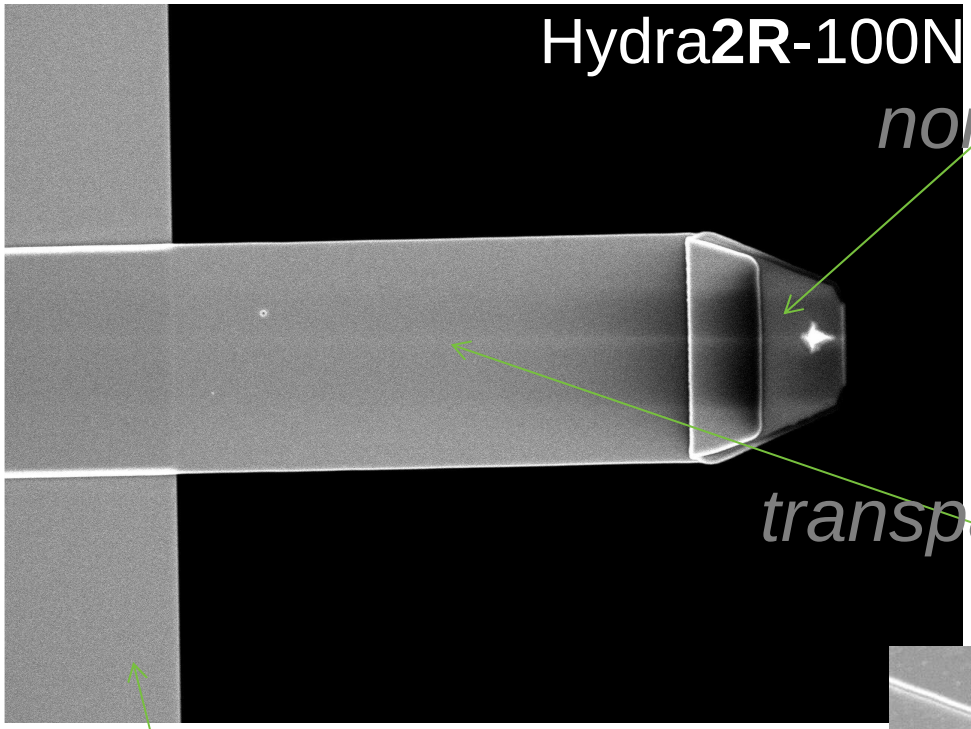
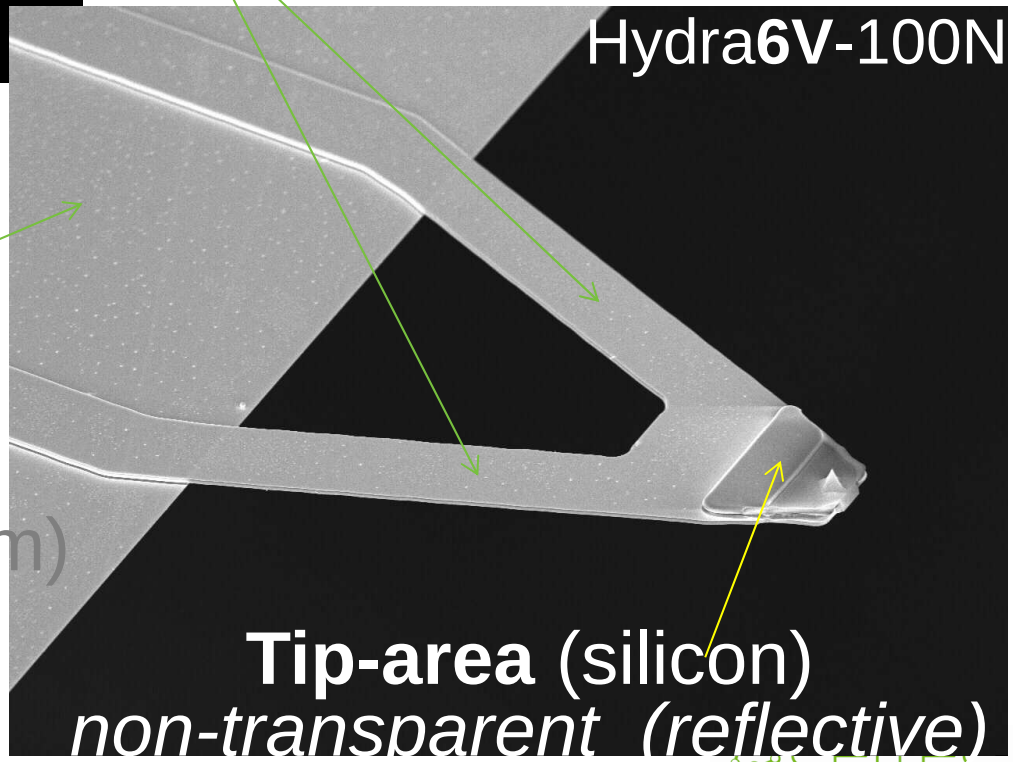


Tip-area (silicon)
non-transparent (reflective)

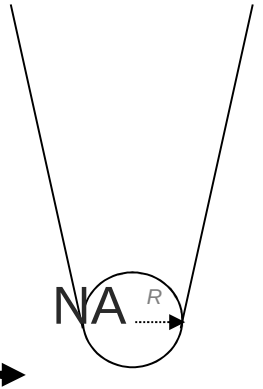
Cantilever (silicon nitride)
transparent (length 100um, thick. 2



Cantilever holder (4x2 mm)

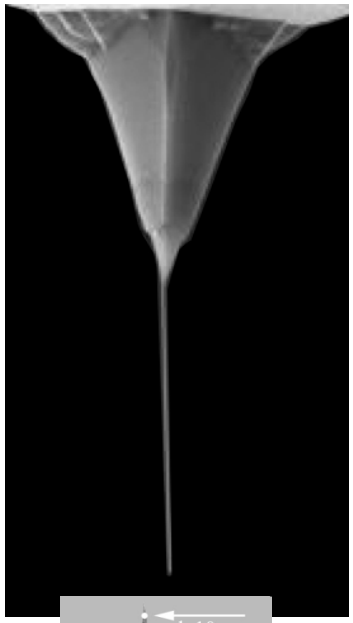
Tip properties

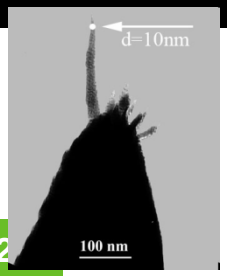
Shape – **Curvature Radius *R [nm]***

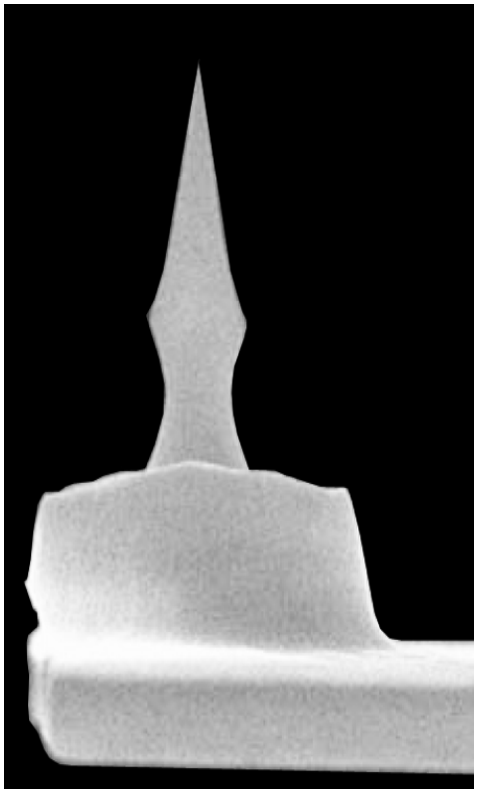


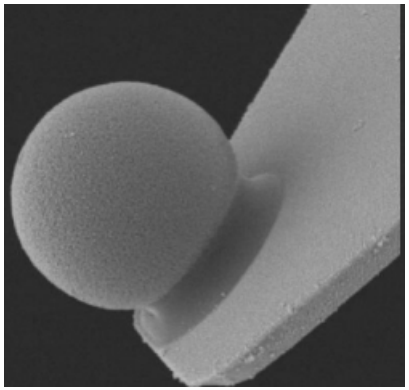
R 1 nm 10 nm 100 nm NA

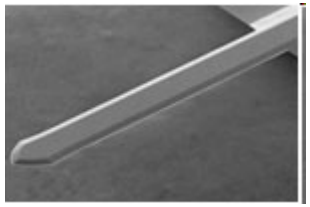
Supersharp Standard Special app. Tip less











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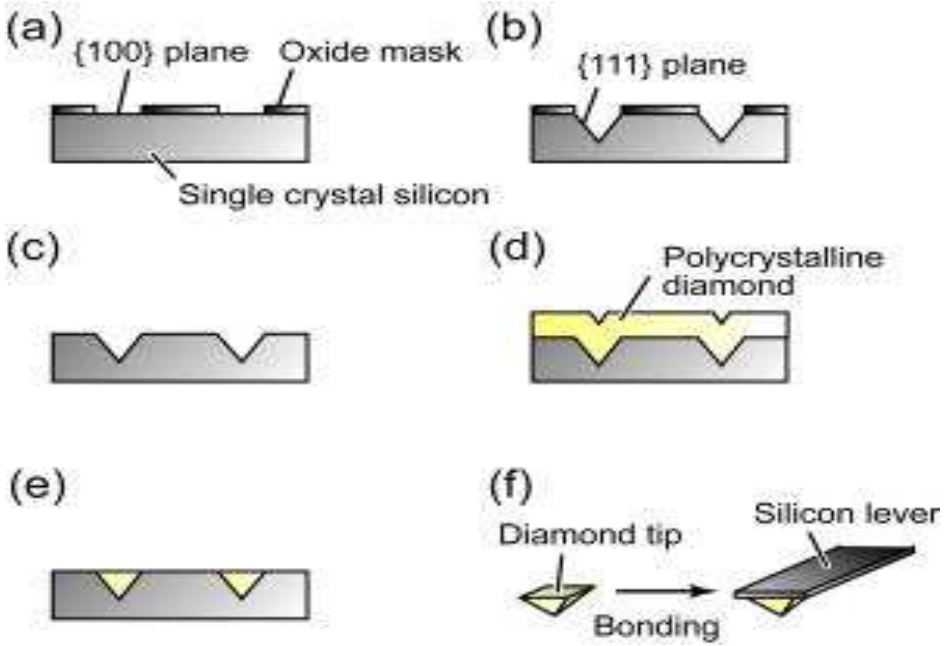
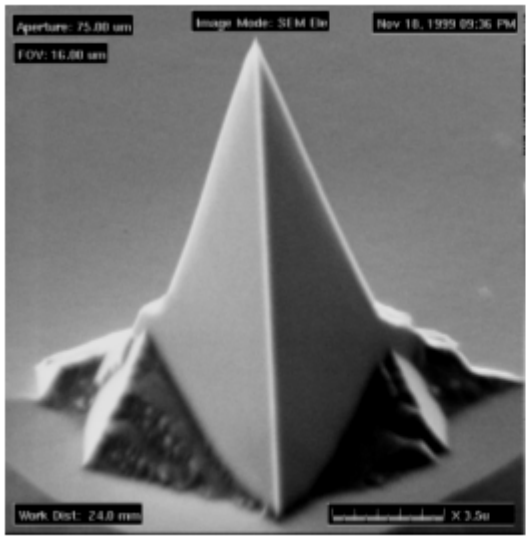
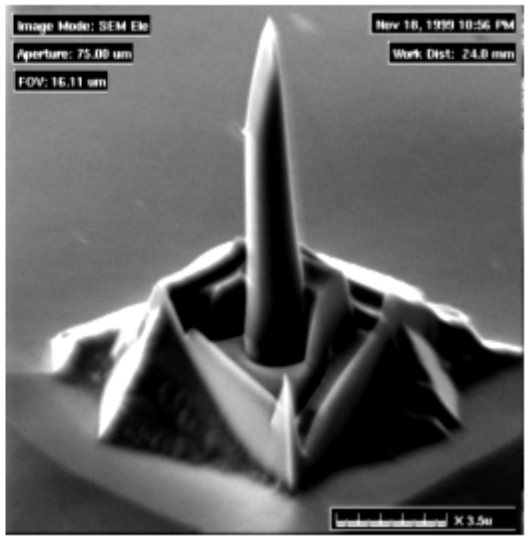
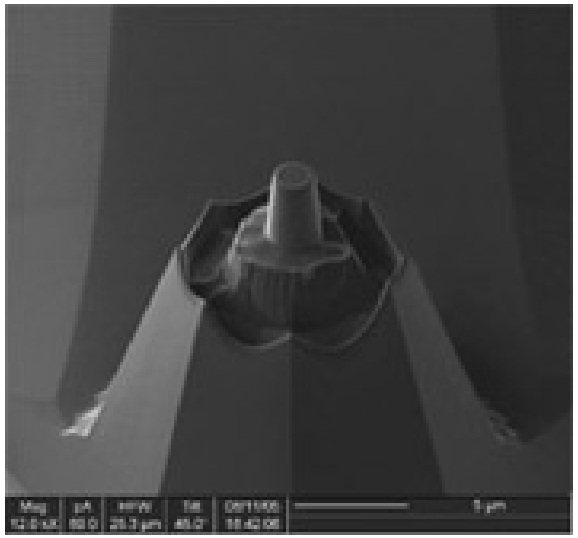
Cantilever **fabrication**



FIB (Focus Ion Beam) post-fabrication of AFM probes (tip)







Plateau Tip

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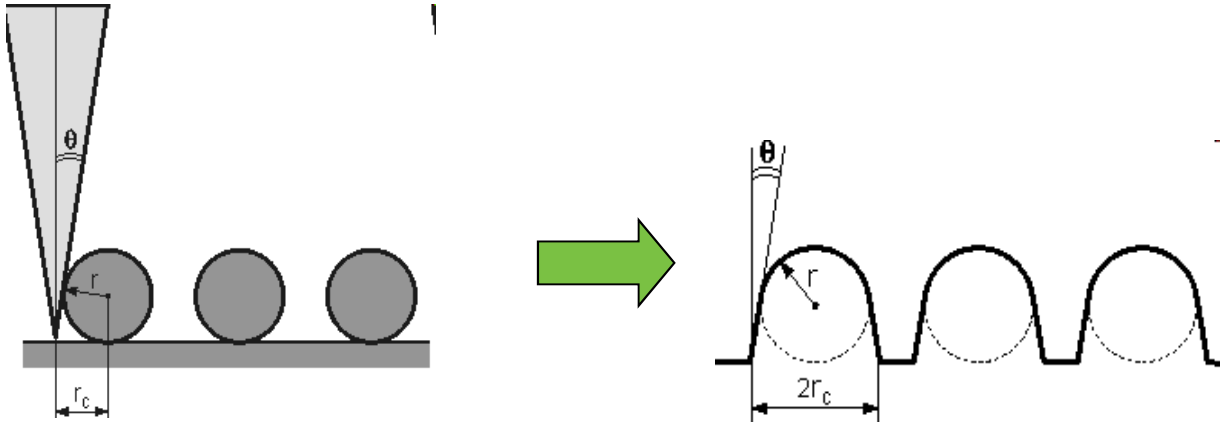
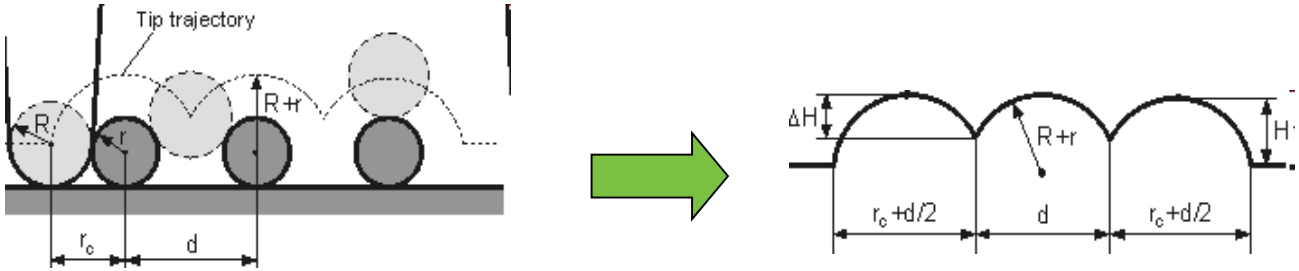
Curvature radius (R) effect

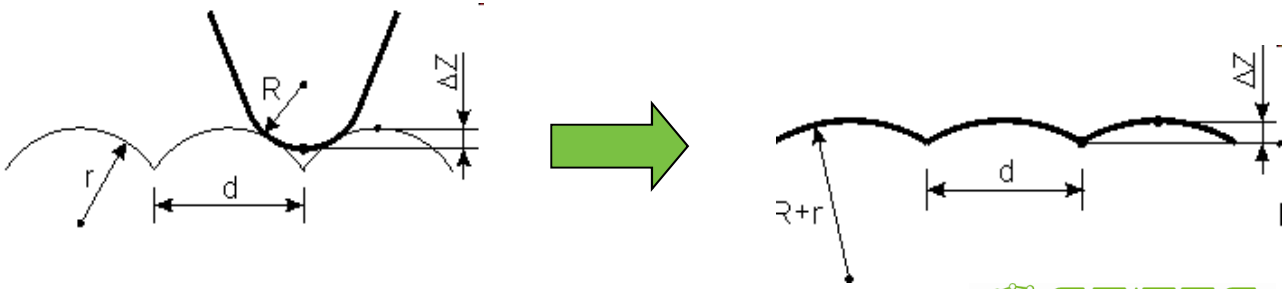
SuperSharp tip
= real image



Standard tip
= R ~ 5-10nm



Blunt tip
= affecting real shape and size



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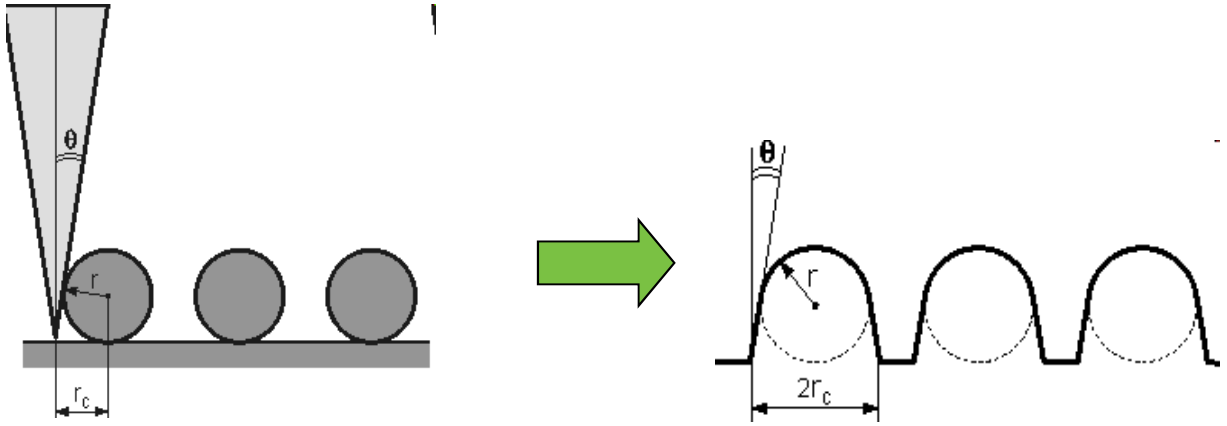
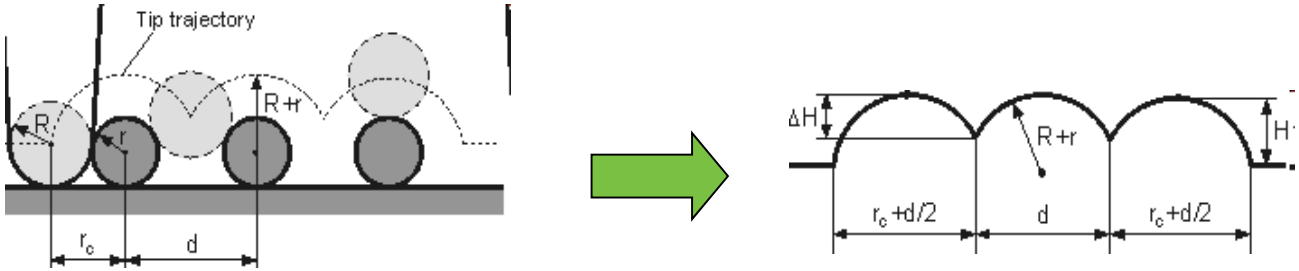
Curvature radius (R) effect

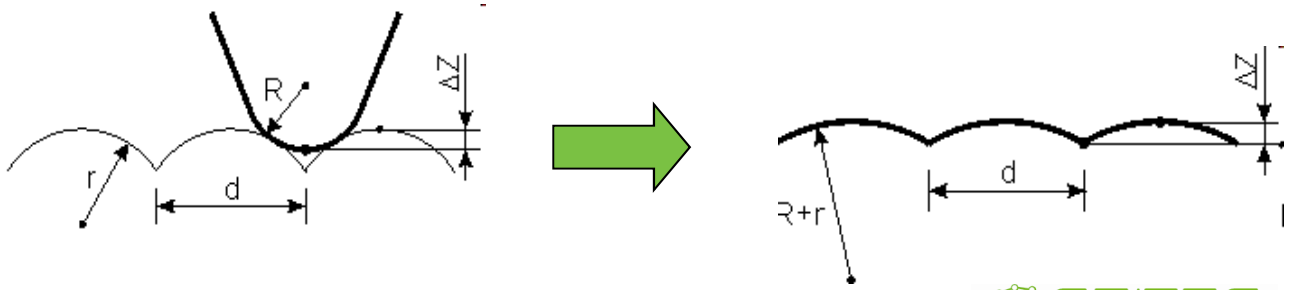
SuperSharp tip
= real image



Standard tip
= R ~ 5-10nm



Blunt tip
= affecting real shape and size



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Laser, photodiode a cantilever

Laser + photodiode

→

Detection of cantilever bending

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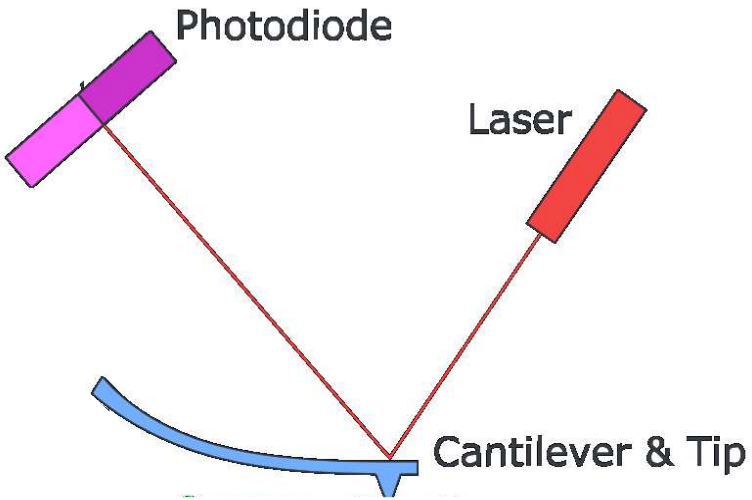
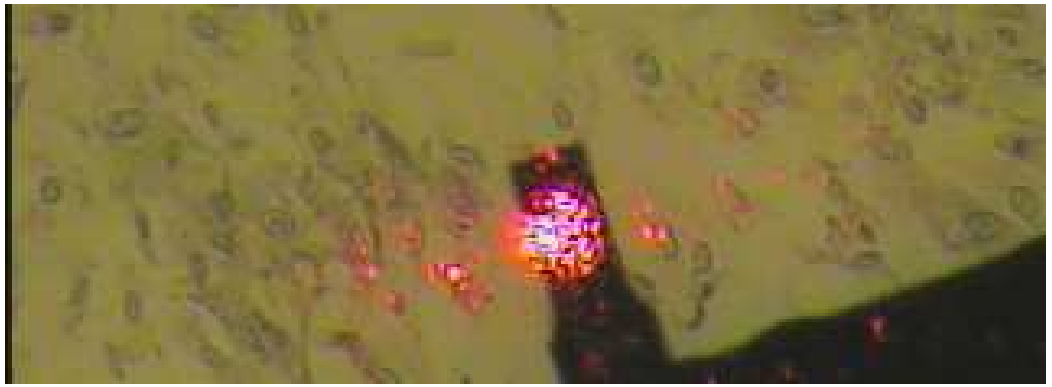
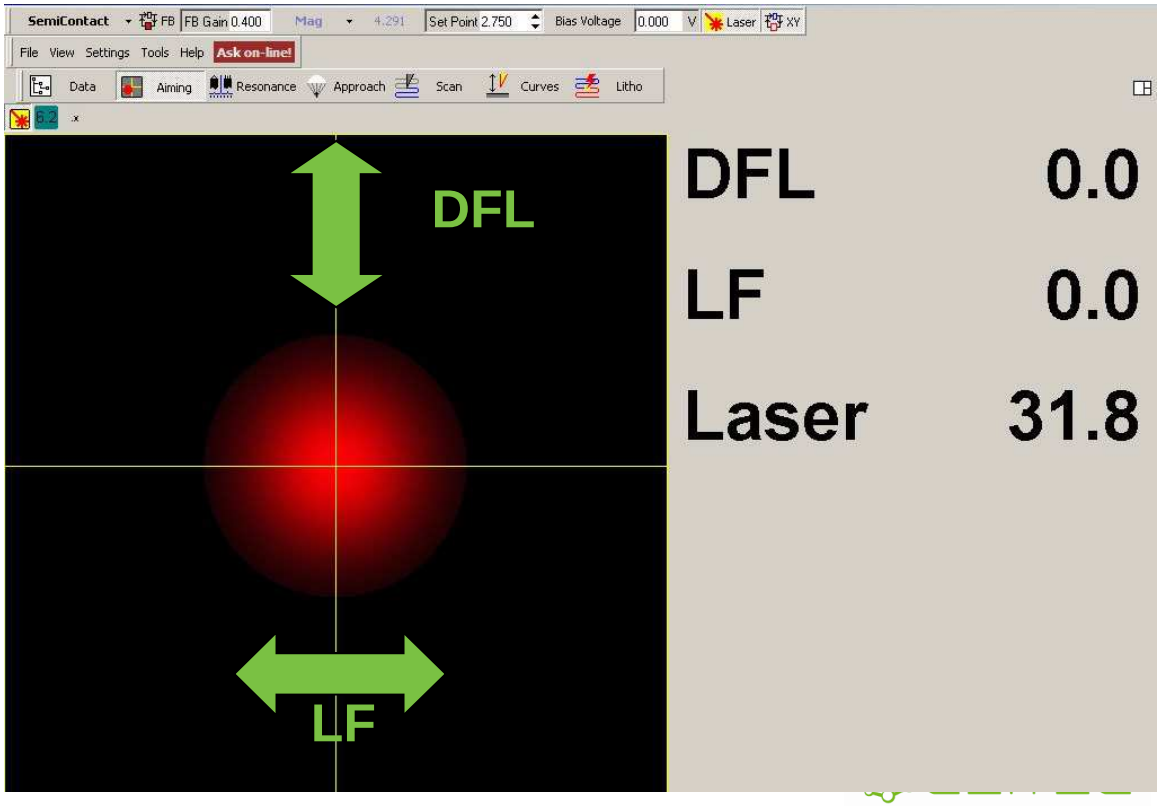
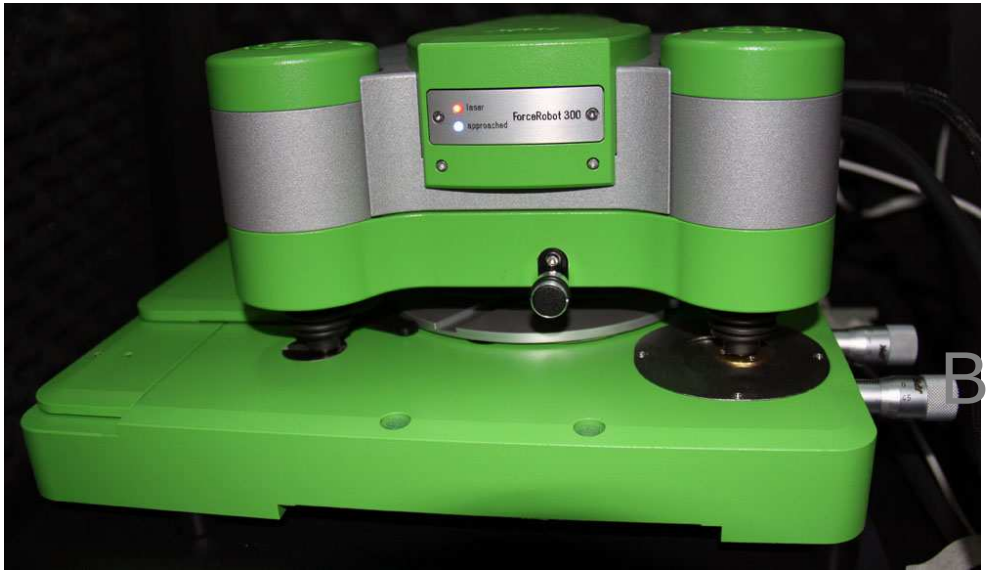

Photodiode
Laser
Cantilever & Tip




SemiContact
FB
FB Gain 0.400
Mag
4.291
Set Point 2.750
Bias Voltage
0.000
V
Laser
XY
File
View
Settings
Tools
Help
Ask on-line!
Data
Aiming
Resonance
Approach
Scan
Curves
Litho
DFL
LF
DFL 0.0
LF 0.0
Laser 31.8

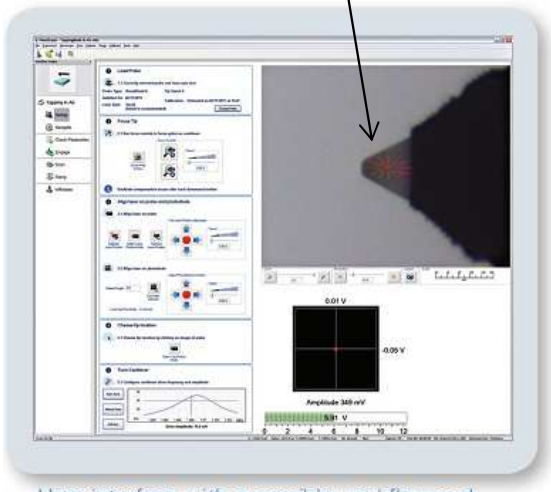
ıutomatic adjustment available



JPK Force Robot head

Bruker Icon/FastScan

NTMDT Solver Next



User interface with a sensible workflow and automatic setup.

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AFM modes of operation

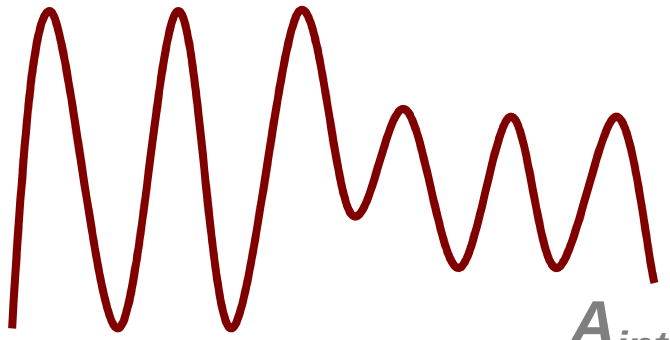
Contact mode

- Measured parameter - cantilever **bending** (= **deflection**, **DFL**)
- Deflection ~ tip sample **force interaction**
- **Hook`s law:** $F = - k * \Delta h$

F – force, ***k*** force constant (stiffness), ***Δh*** – height (=deflection)

Semicontact mode *(tapping mode, AC mode, oscillation mode, …)*

- Measured parameter **amplitude of oscillation** (= **magnitude**, **MAG, …**)



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NT-MDT

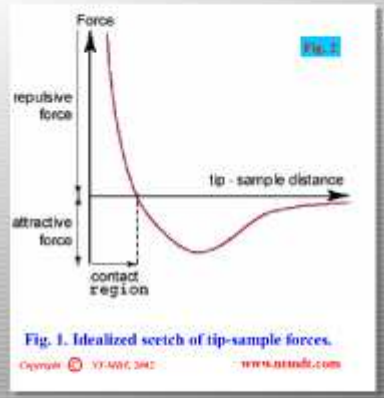
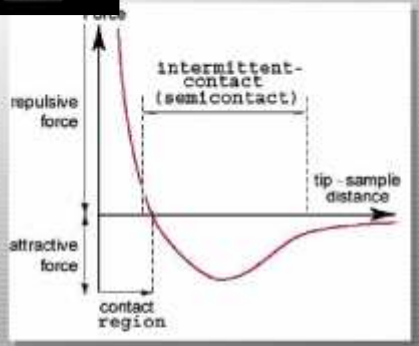


Fig. 1. Idealized sketch of tip-sample forces.

dc Contact techniques:

- Constant Height mode
- Constant Force mode
- Constant Error mode
- Lateral Force imaging
- Spreading Resistance imaging

In **Contact mode** of operation the cantilever deflection under scanning reflects repulsive force and is used as **such**, in **feedback circuitry** or in **their combination** to imagine the sample surface profile. Simultaneously with topography acquisition under scanning one can imagine some other characteristics of the investigated sample.



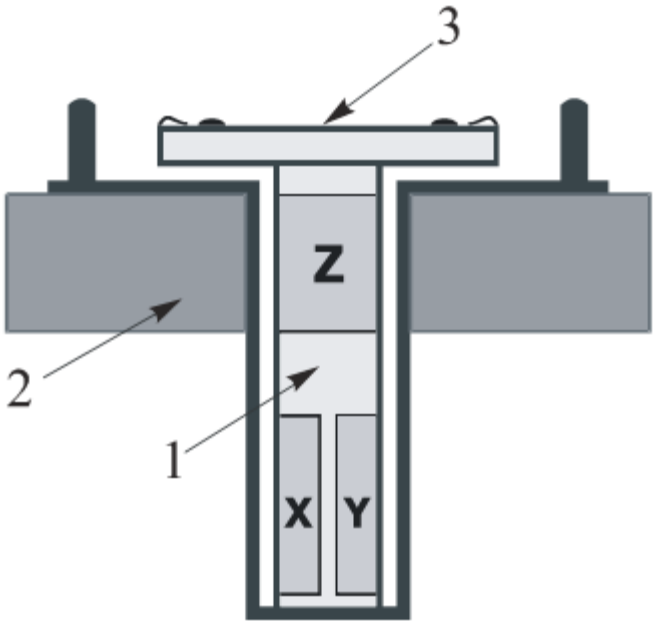
Semicontact techniques:

- Semicontact mode
- Phase Imaging mode
- Semicontact Error mode

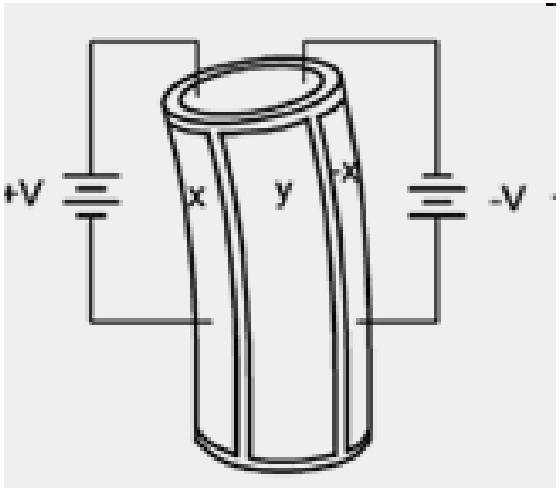
Usage of **SFM** with oscillating cantilever was firstly anticipated by **Binnig**. Relatively small shift of cantilever oscillating frequency with sensing repulsive forces means that contact of cantilever tip with sample surface under oscillation is not constant. Only during small part of oscillating period the tip "feels" contact repulsive force. Scanning sample surface with cantilever oscillated in this manner is not non-contact, but intermittent contact (semicontact).

PZT

Piezoelectric tubes



Piezoelectric tubes PZT
Piezoelectrodes

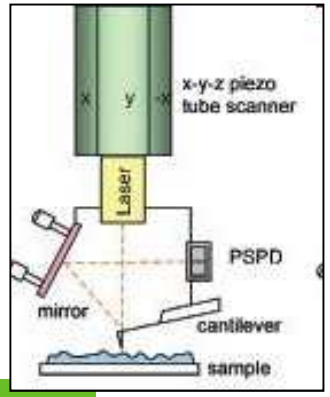


- **Hollow ceramic** tubes
- **Metal covered** in selected parts
- Voltage application → change of size

Notes + cautions

- *Fragile*
- *High voltage applied*

PZT – construction approaches of AFM

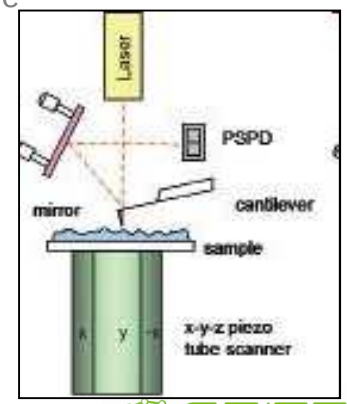


Scanning by probe
construction

- *x,y,z* axes movement by head
- Oscillator in head
- Range x,y 100-150 um
- Range *z* 10-15 um

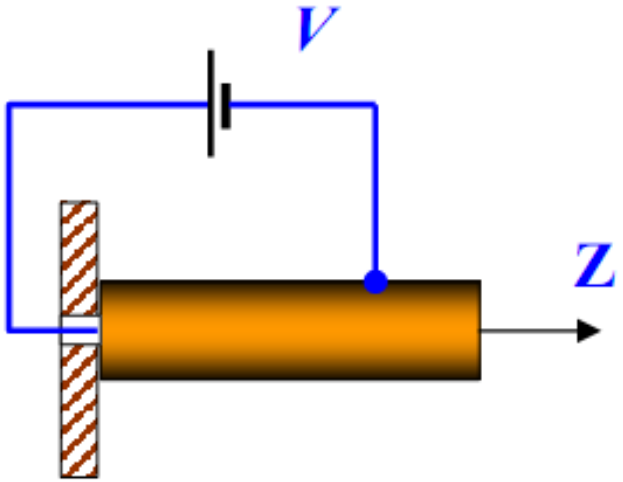
Scanning by sample construction

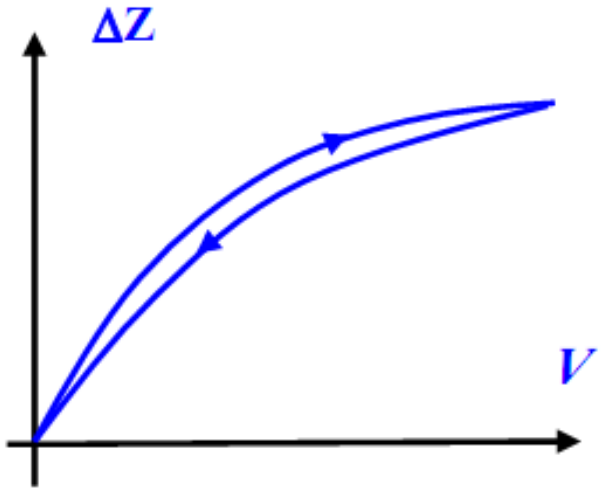
- *x,y,z* axes movement by sample
- Oscillator in head
- Range x,y 1-10 um
- Range *z* 1-3 um
- Low noise



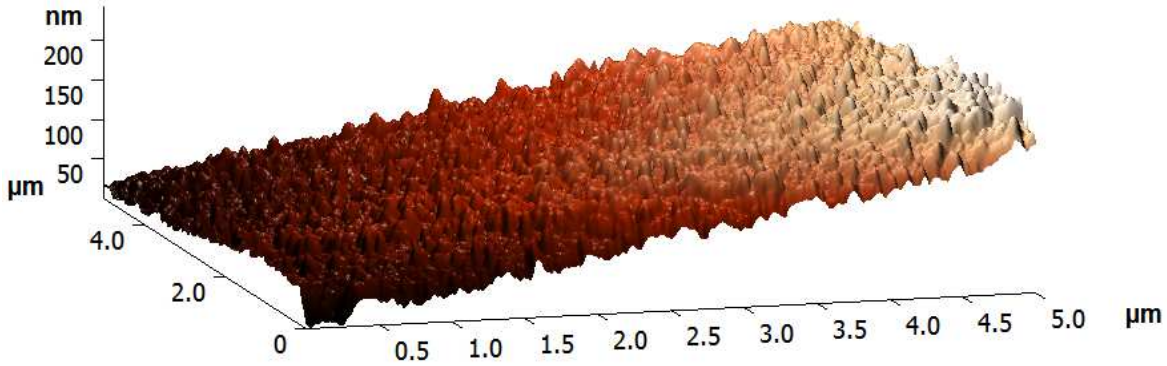
PZT: voltage-extension dependency

non-linear





Native (raw) AFM **data** are **shifted**. Removed e.g. by polynomal regression of data.



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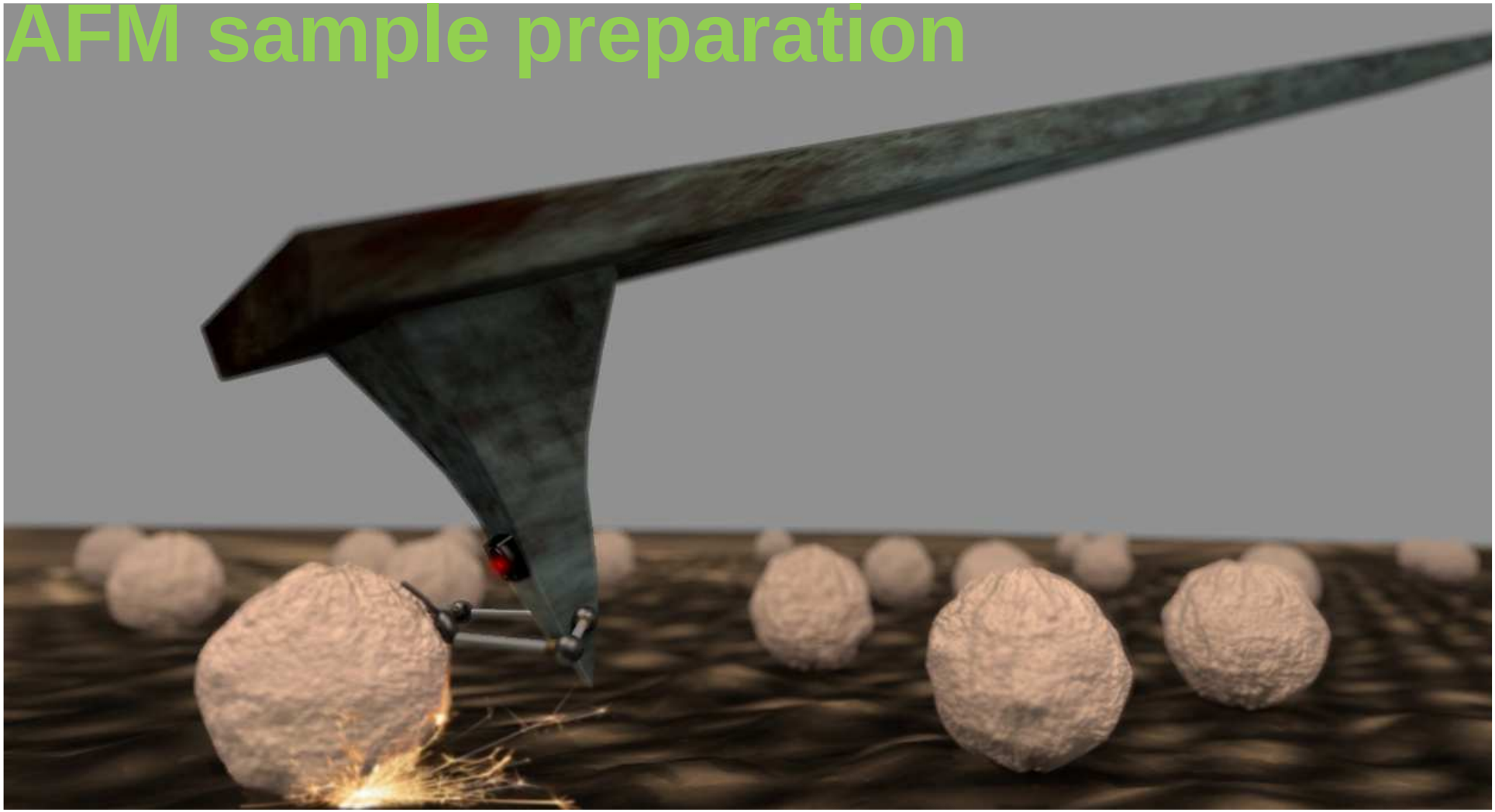
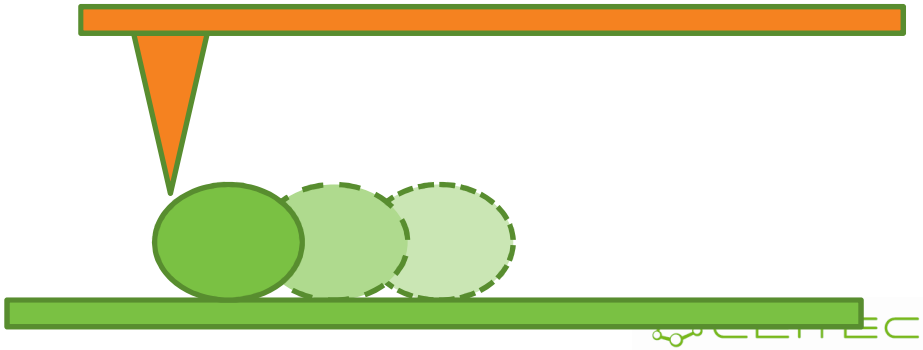
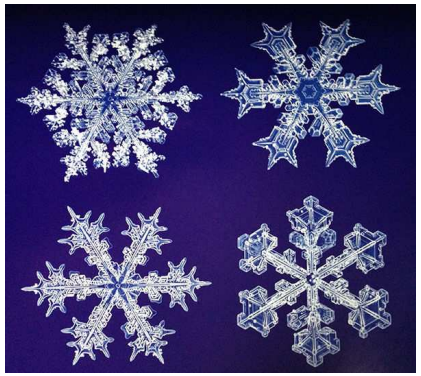
Sample preparation for AFM

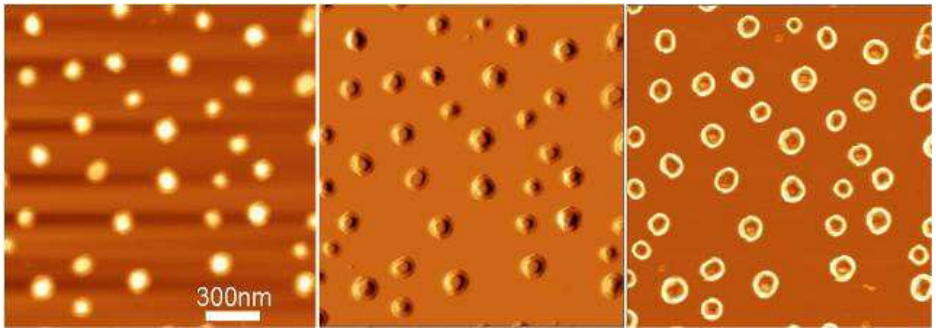
AFM sample preparation

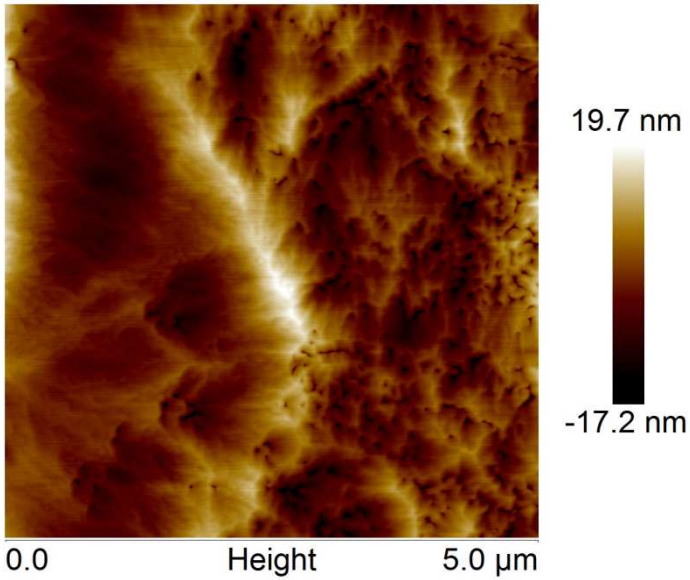


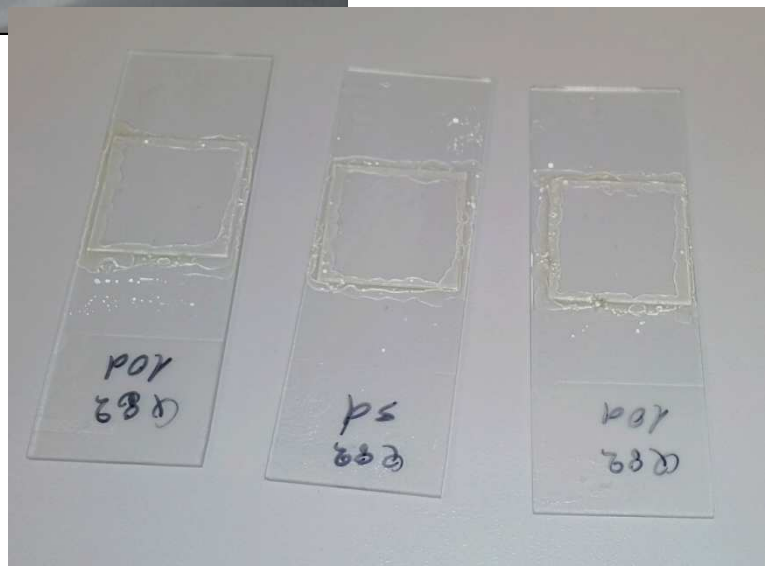


Concentration – surface density









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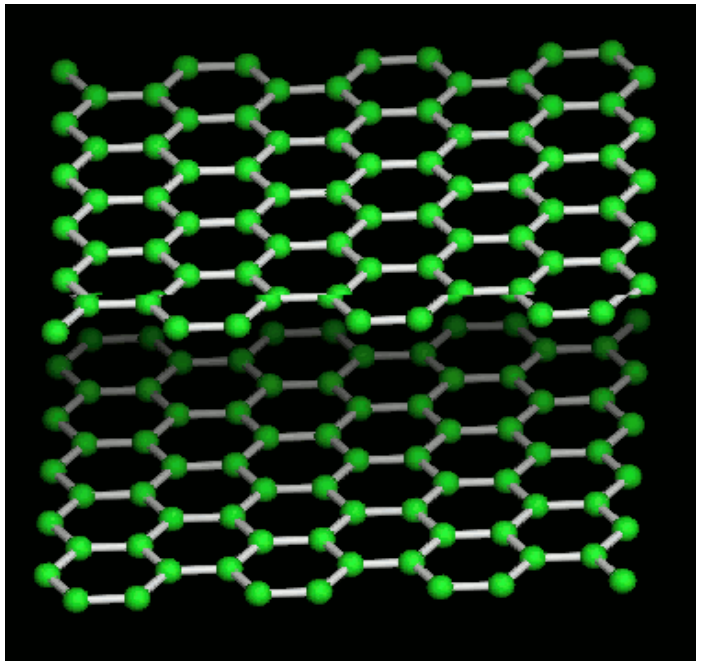
Substrates for preparation of AFM samples

Atomically flat surfaces

1. **HOPG** Highly Ordered Pyrolytic Graphite

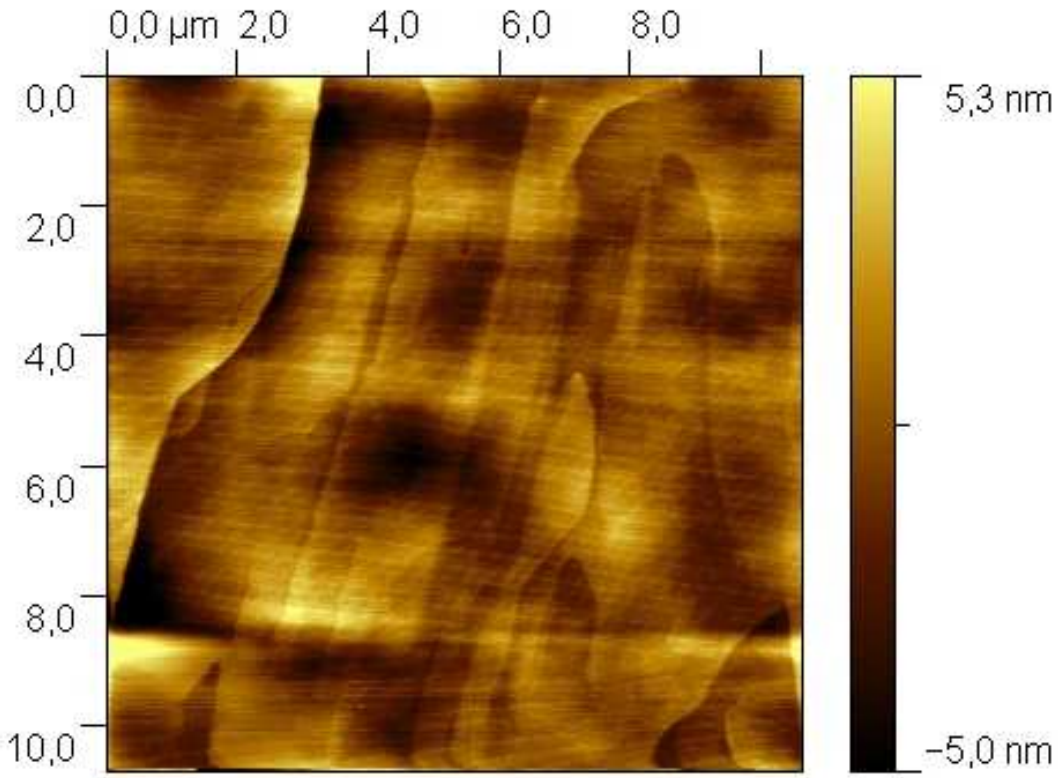


- Kish's graphite, waste in steel production
- Hexagonal planar structure
- C-C bond142 pm, layer-layer distance335 pm
- Conductive, highly hydrophobic
- Planar structure
- Synthetic form of graphite, high chemical purity
- Traditionally – substrate for SEM, STM i AFM (→ **conductivity**)
- **Immobilization** – spontaneous adsorption (→ **hydrophobicity**)

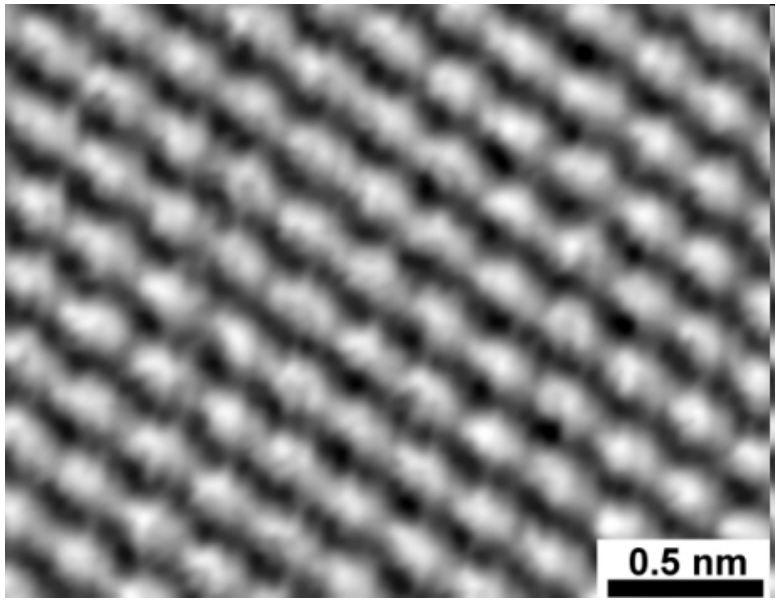
Atomically flat surfaces

1. **HOPG** Highly Ordered Pyrolytic Graphite

Large areas
visible layers



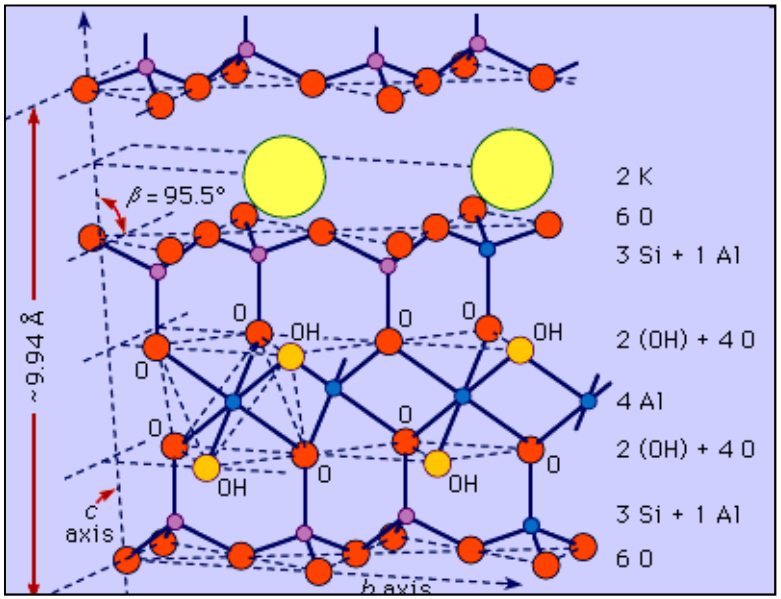
Small areas
atomically flat



Atomically flat surfaces

2. Mica (muscovite)

- „Cat's silver", muscovite acc. to city of Moscow
- Chem. structure: $K_2O \cdot Al_2O_3 \cdot SiO_2$
- Hydrophilic surface
- Easy to be modified by chemical synthesis
- Immobilization by **chemical bonding** as well as **ionic interaction**
- pKa ~ 3, physiological pH → negative surface charge
- Mica = silicate, hydrated SiO_2 (~ Si-OH) from the chemical point of view



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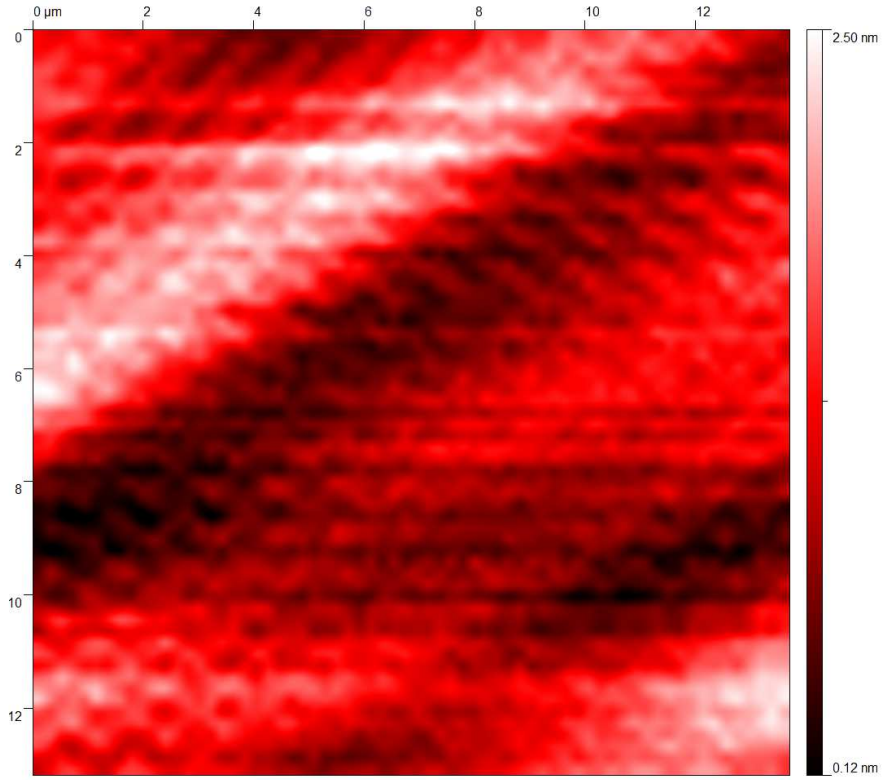
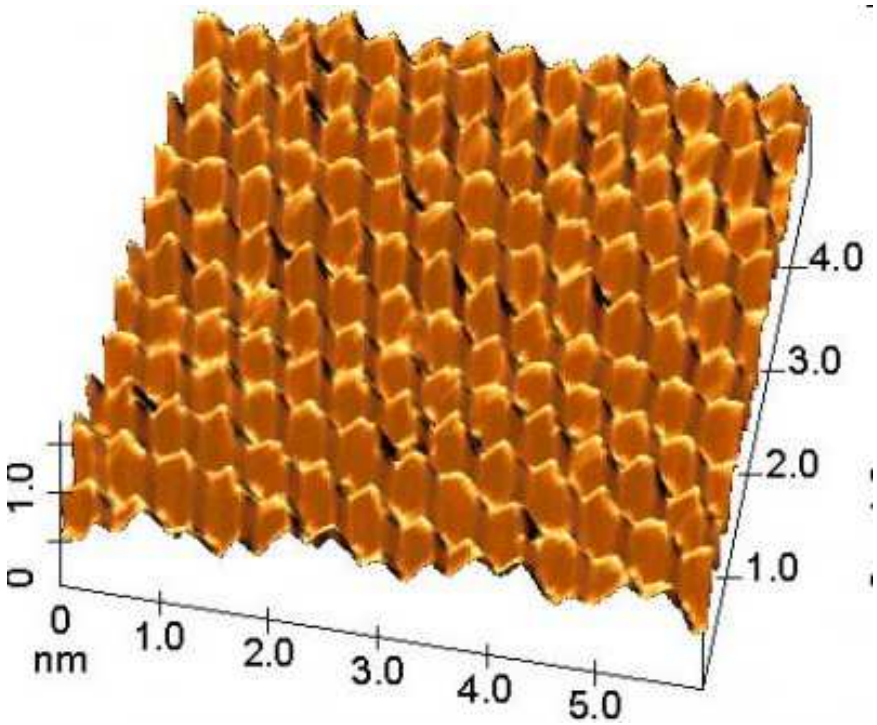
Atomically flat surfaces

2. Mica (muscovite)





Extremely flat on small and larger areas

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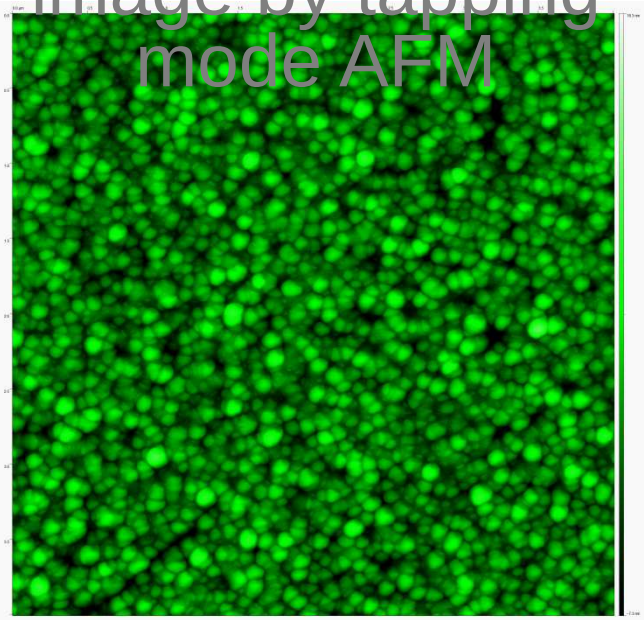
Other surfaces

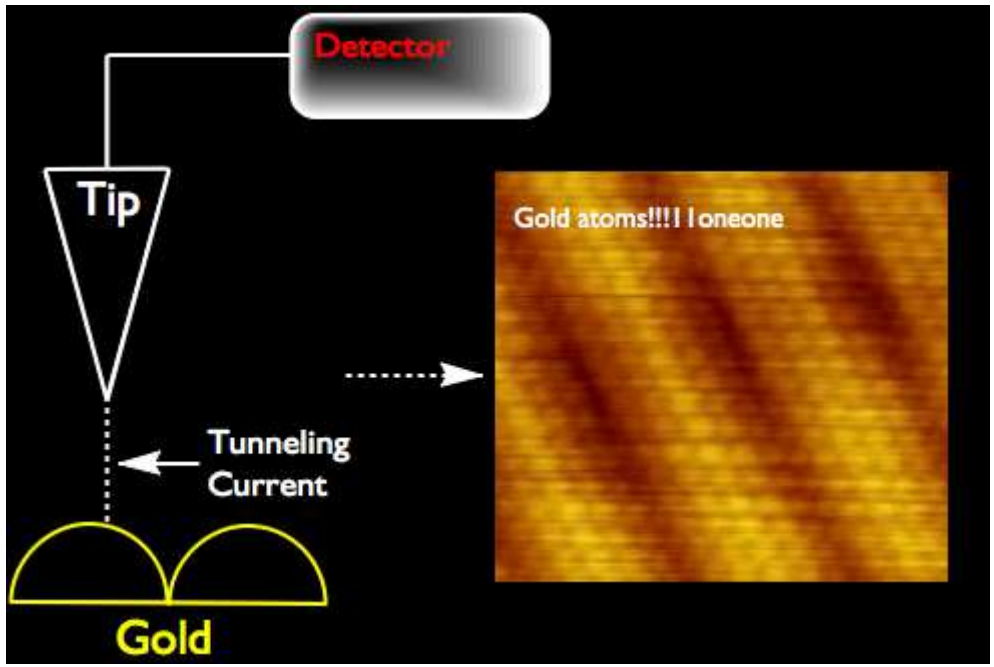
3. Gold

- Inert metal
- Traditionally in (bio)electrochemistry (i.e. biosensors) - electrodes
- Conductive - STM + AFM
  - Hydrophobic: spontaneous non-selective adsorption of molecules (proteins, DNA, …)
- Specific chemical binding of thiols (-SH) – organic molecules + cysteine
- Prepared usually by evaporation
- Adhesion layer for operation in liquids (Al/Cr/Ti)

Sputtered gold laye

image by tapping mode AFM

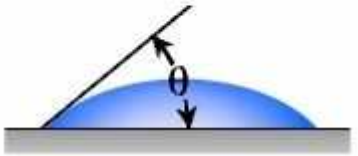




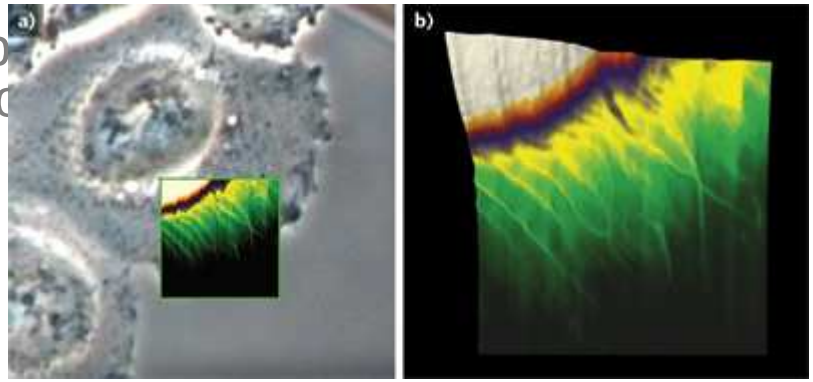
Other surfaces

4. Glass

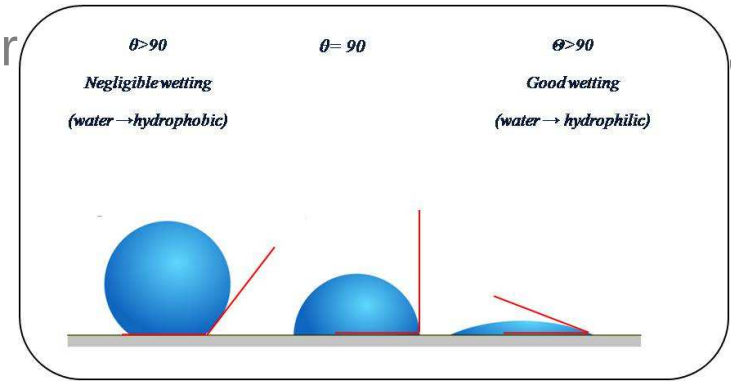
Contact Angle



- Amorphous noncrystalline structure
- Lab glass composition: 75% SiO_2 plus Na_2O, CaO, borate and minor additives
- Si-OH → from chemical point of view
- Less hydrophilic comparing to mica
- Roughness much higher comparing to mica (production by pressing)
- **Not** suitable for **individual molecules** imaging with AFM
- Typ… micr… who…



AFM – optical image overlap



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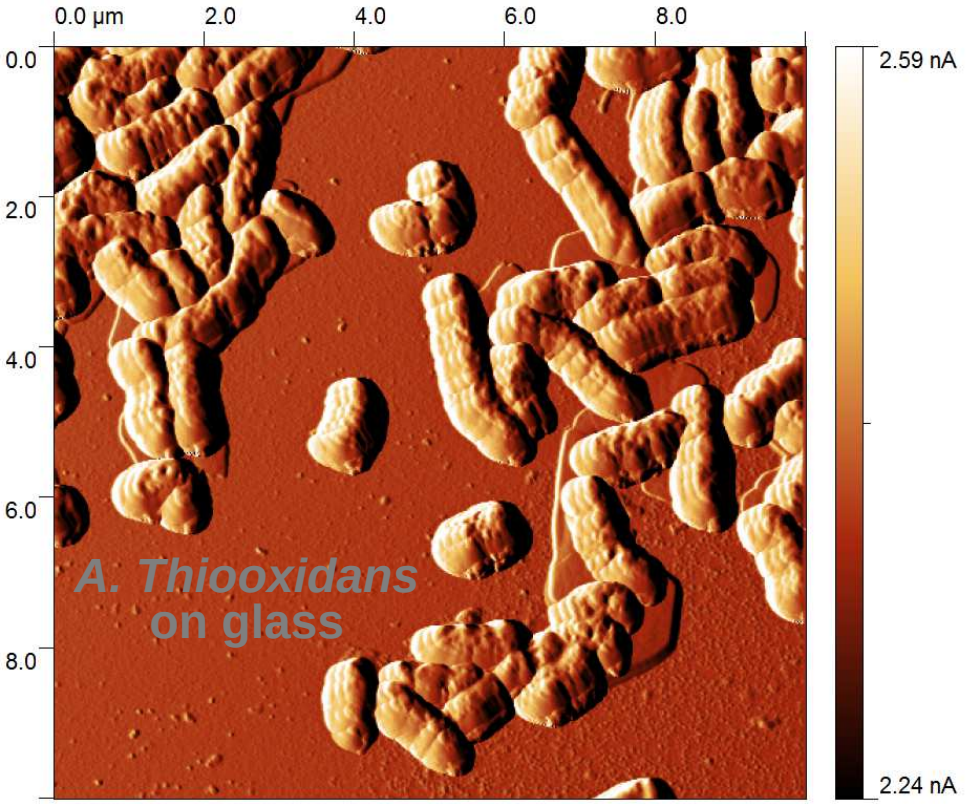
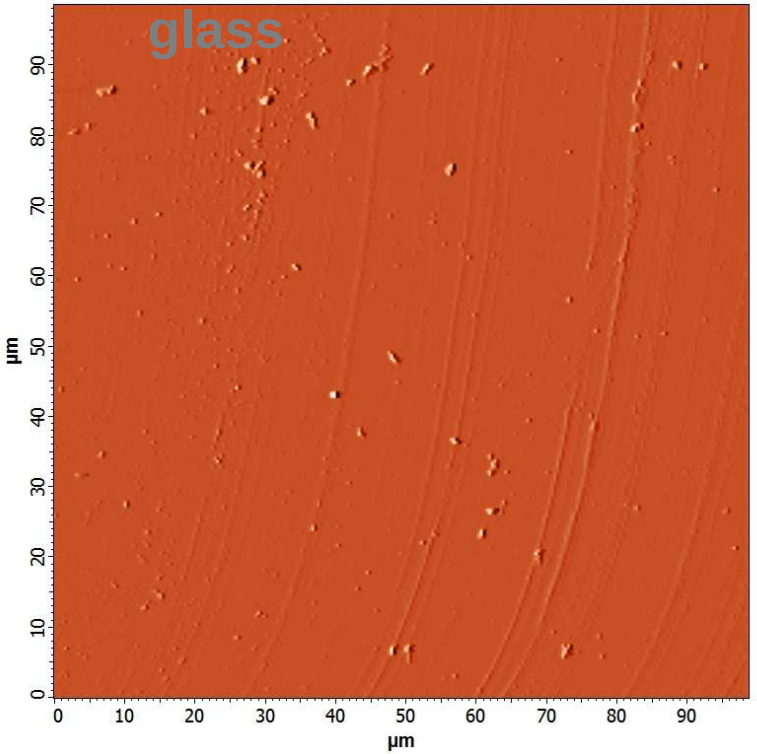
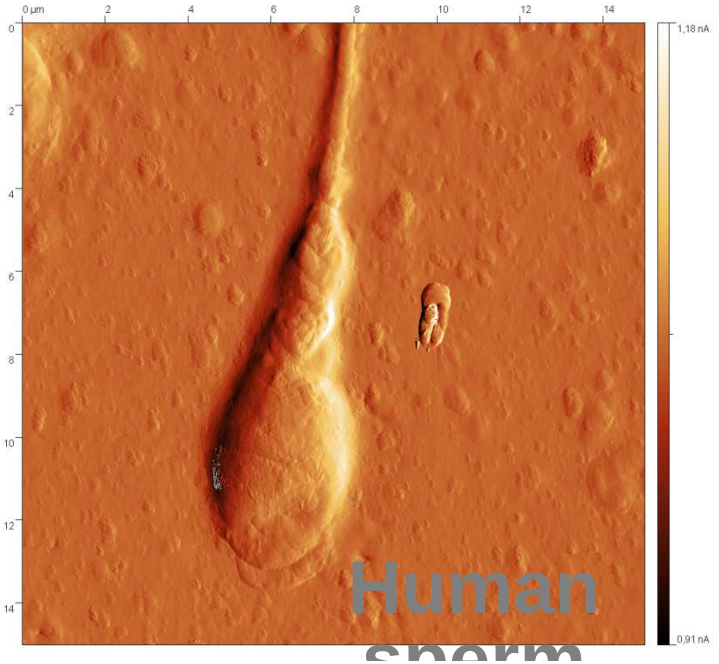
Whole cells on glass
under AFM

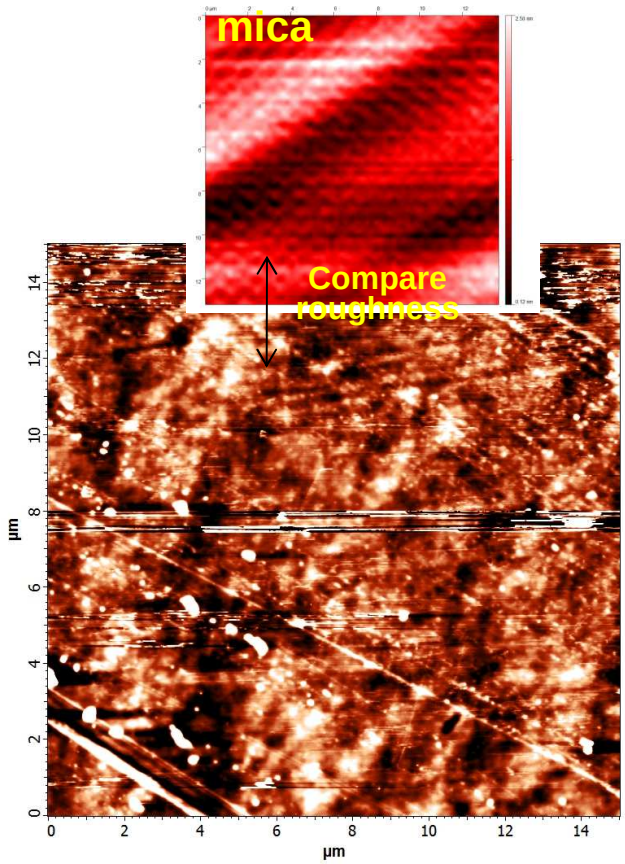


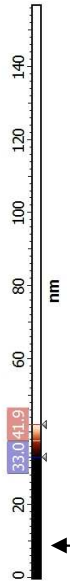


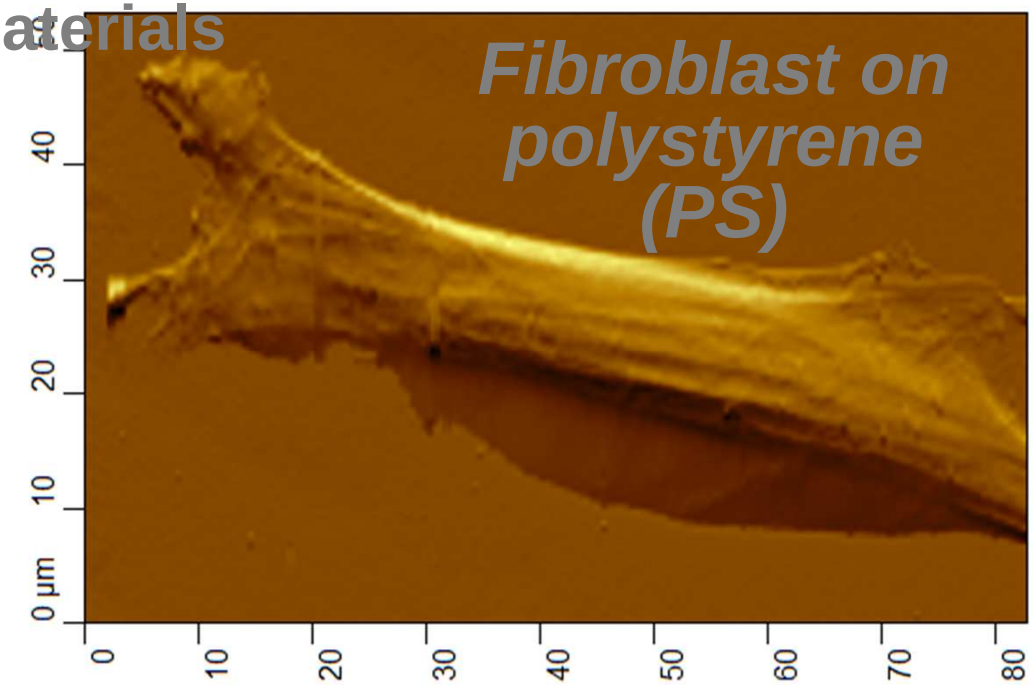


Other surfaces

5. Plastic materials







Non-modified polystyrene (PS)

- Most of lab supplies made of plastic (**PP, PE, PS**)
- **No functional groups** to be used in covalent binding
- **PS** – **hydrophobic** → spontaneous non-specific adsorption of proteins → usually as underlying support (i.e. for cell attachment)

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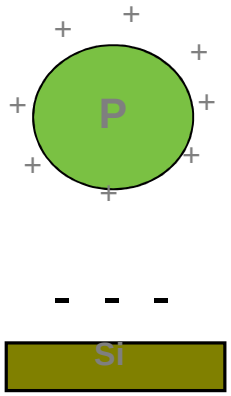
Immobilization procedures

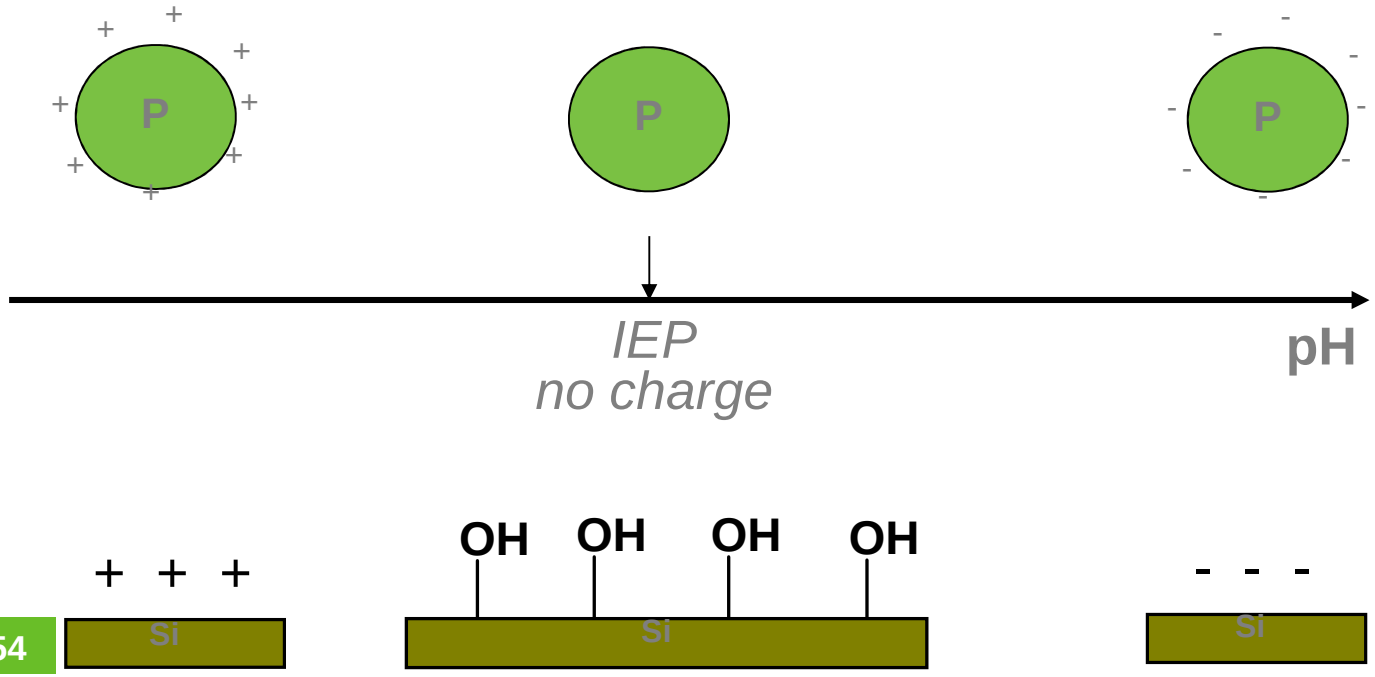
1. Proteins

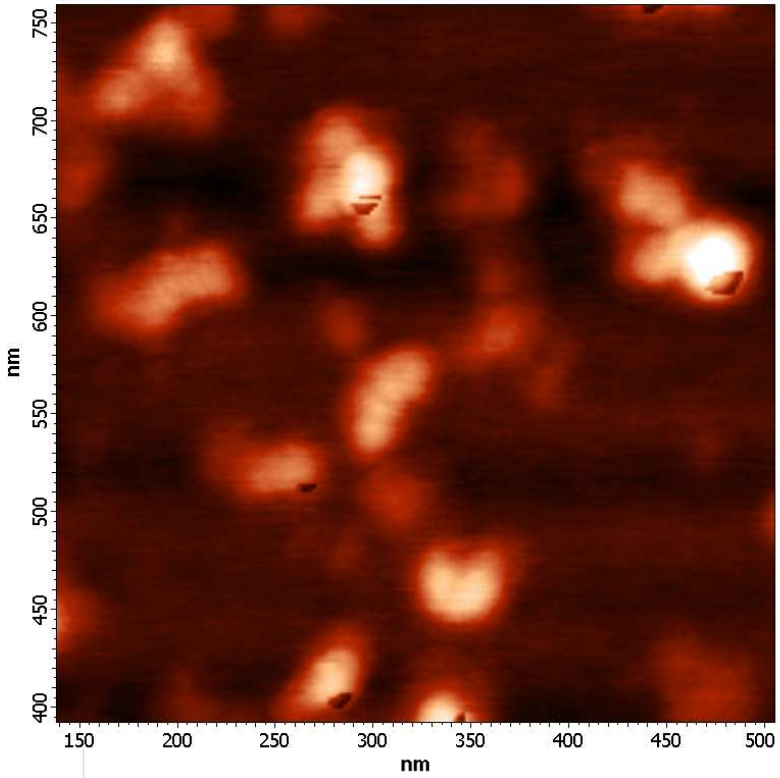
Surface: **mica or HOPG** (extremely flat)

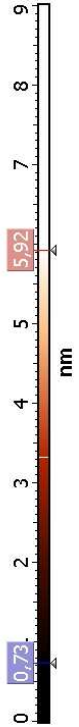


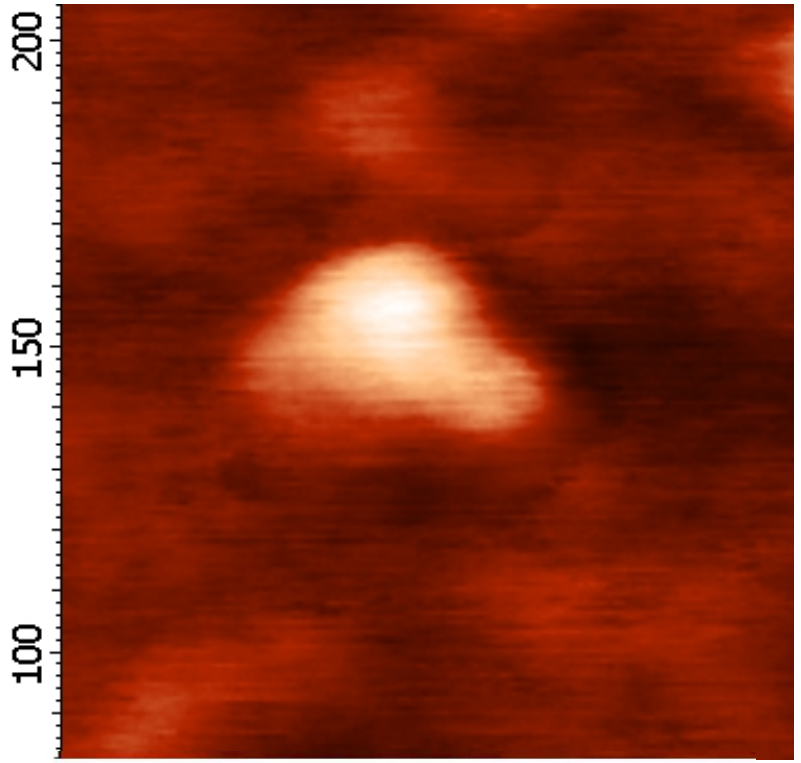
Protein: **charge** is given by IEP + pH

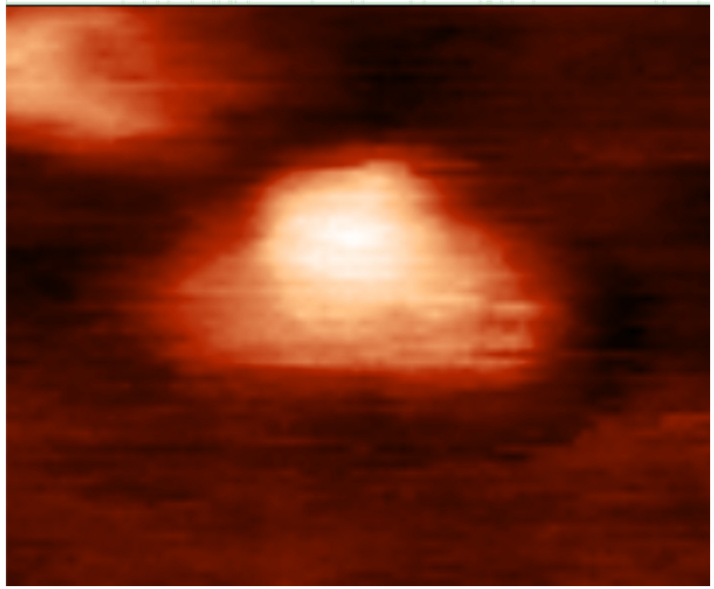
Immobilization on mica: **pKa (mica) < pH < IEP**

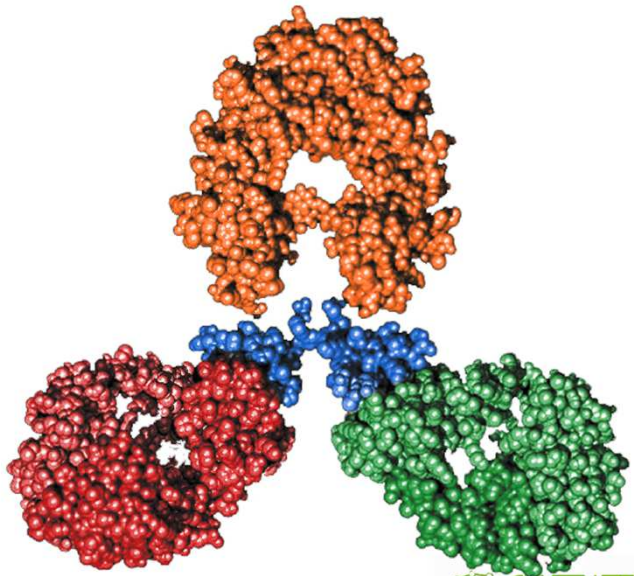



nm
750
700
650
600
550
500
450
400
150
200
250
300
350
400
450
500
nm


9
8
7
5,92
5
4
3
2
0,73
0
nm

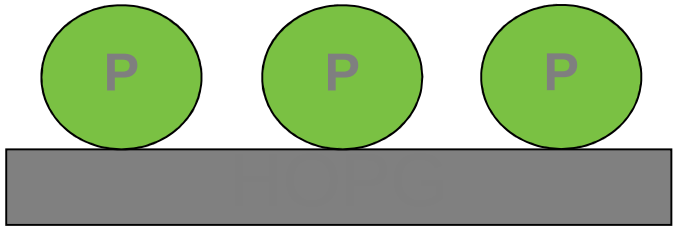

200
150
100

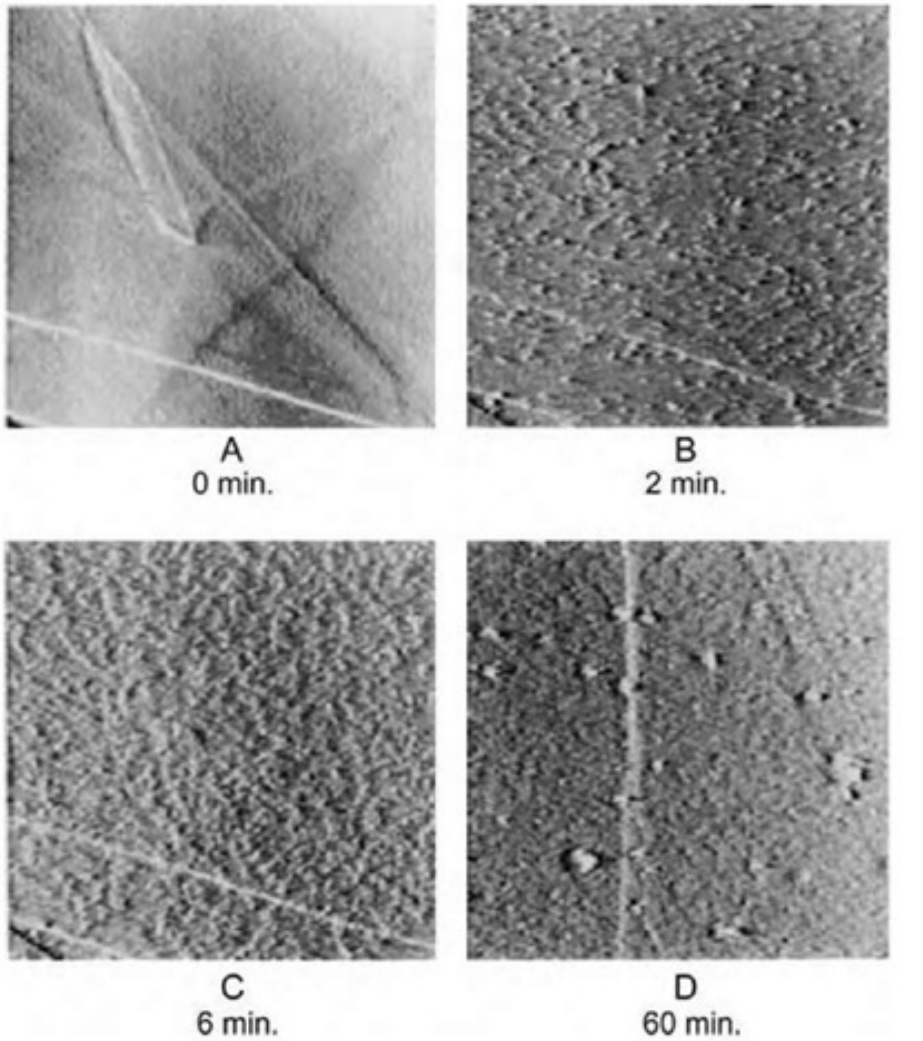




Protein immobilization on HOPG

A. **Spontaneous** (non-specific) **adsorption** of protein → hydrophobic surface (best results at zero charge *pH = IEP)*





Lysozyme molecules on HOPG

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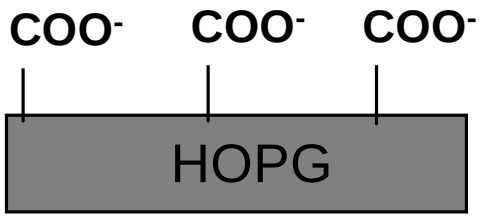
B. **Ionic** (specific) **binding** of molecules → creation of charge/chem. groups on HOPG surface

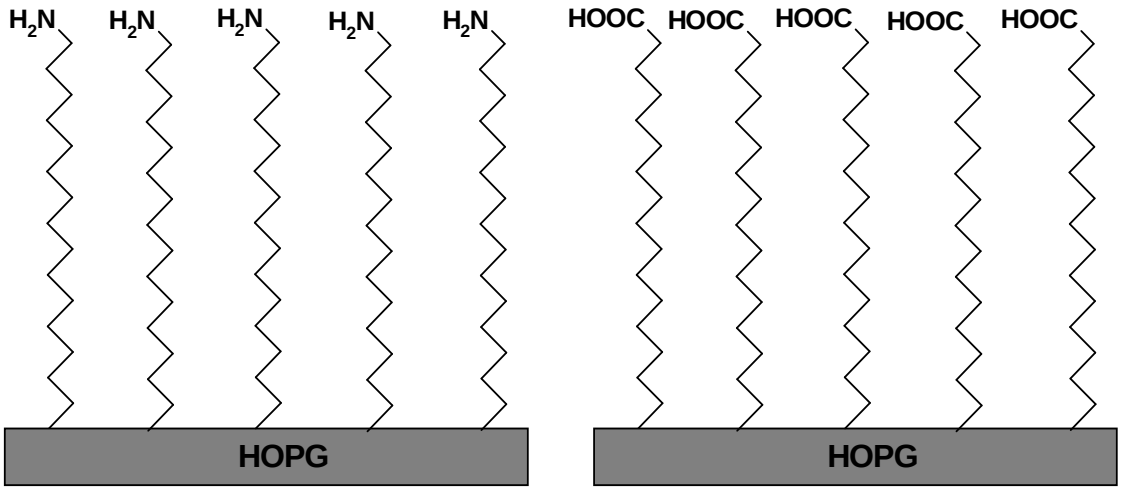
Electrochemical oxidation
(E ~ 2 V vs. Ag/AgCl)

Adsorption of long chain double-sided ions
(C_{16}/C_{18})



- Ionic interation

- Covalent binding (i.e. via NHS-esters)



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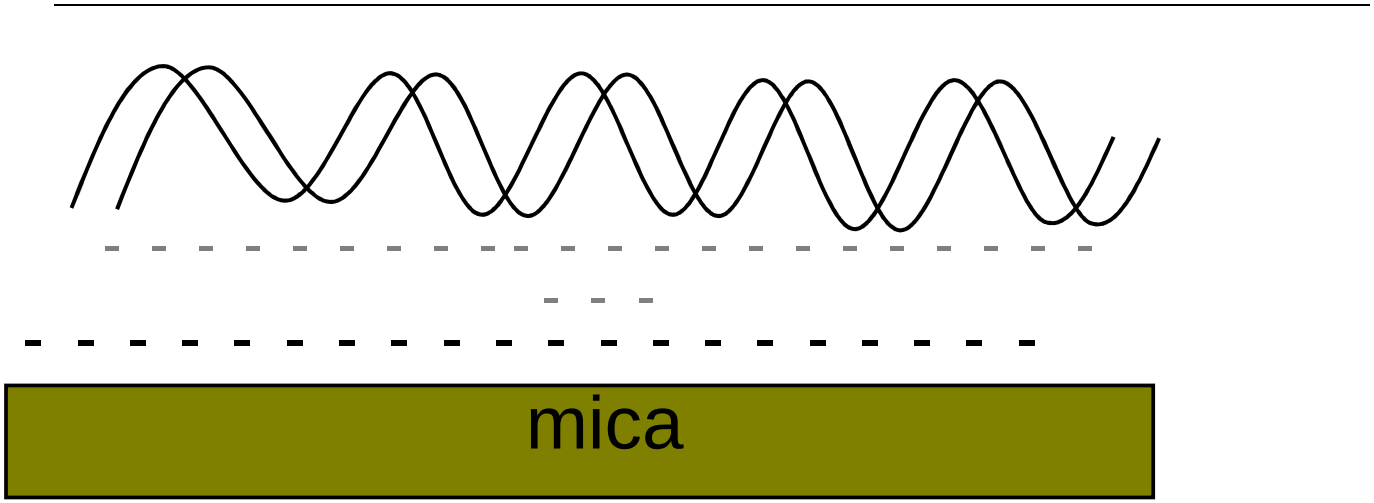
2. DNA

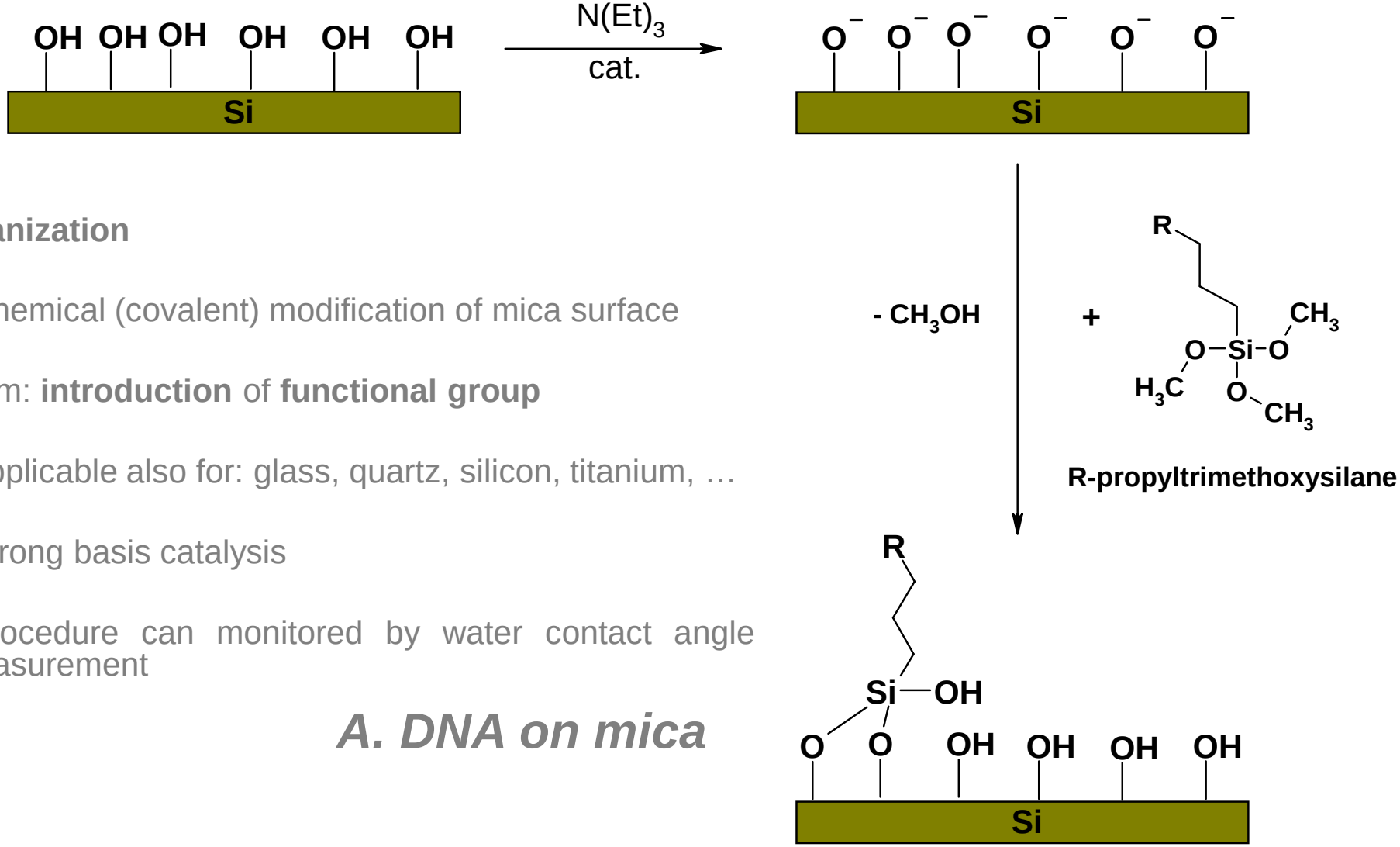
Surface: **mica or HOPG** (extremely flat)



Immobilization problem:

DNA (sugar-phosphate bone) as well as **mica – negative charge** under physiological pH

→ surface introduction of **positive charge**



Silanization

= chemical (covalent) modification of mica surface

- Aim: **introduction** of **functional group**

- Applicable also for: glass, quartz, silicon, titanium, …

- Strong basis catalysis

- Procedure can monitored by water contact angle measurement

A. DNA on mica

silanization

hydrophobization

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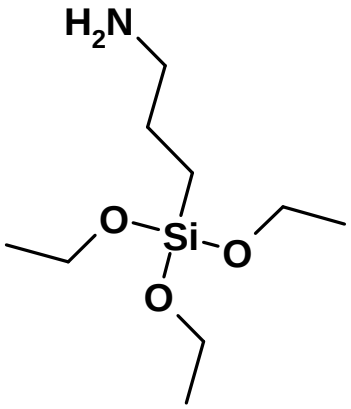
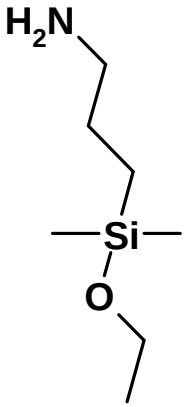
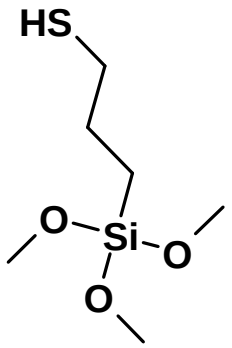
Examples of **alkoxysiloxanes**



(3-Aminopropyl)trimethoxysilane
APTES

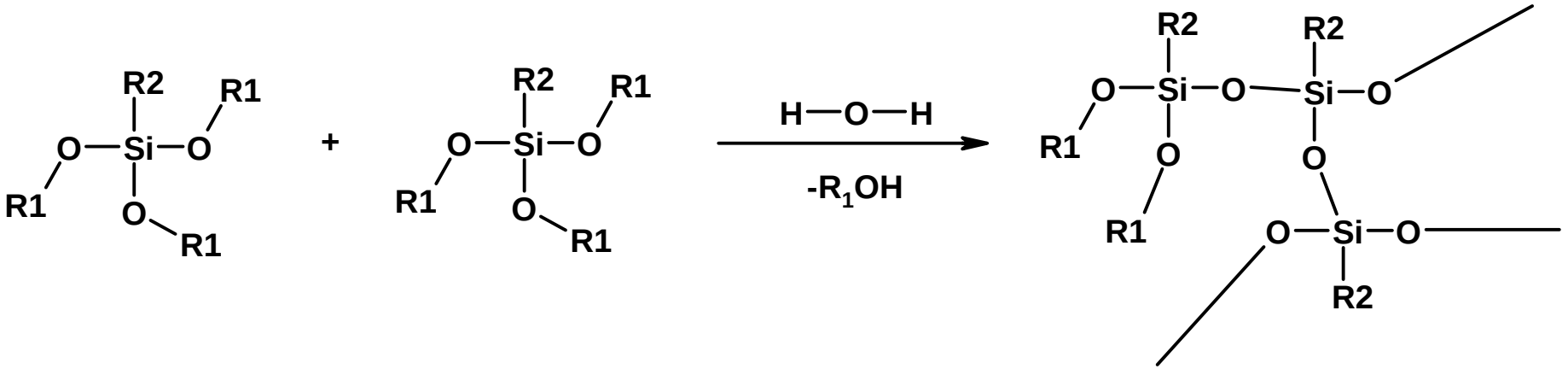


3-(Ethoxydimethylsilyl)propylamine
APDMES



(3-Mercaptopropyl)trimethoxysilane
MPTS

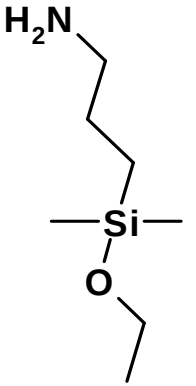
Self-polymerization
practical complication



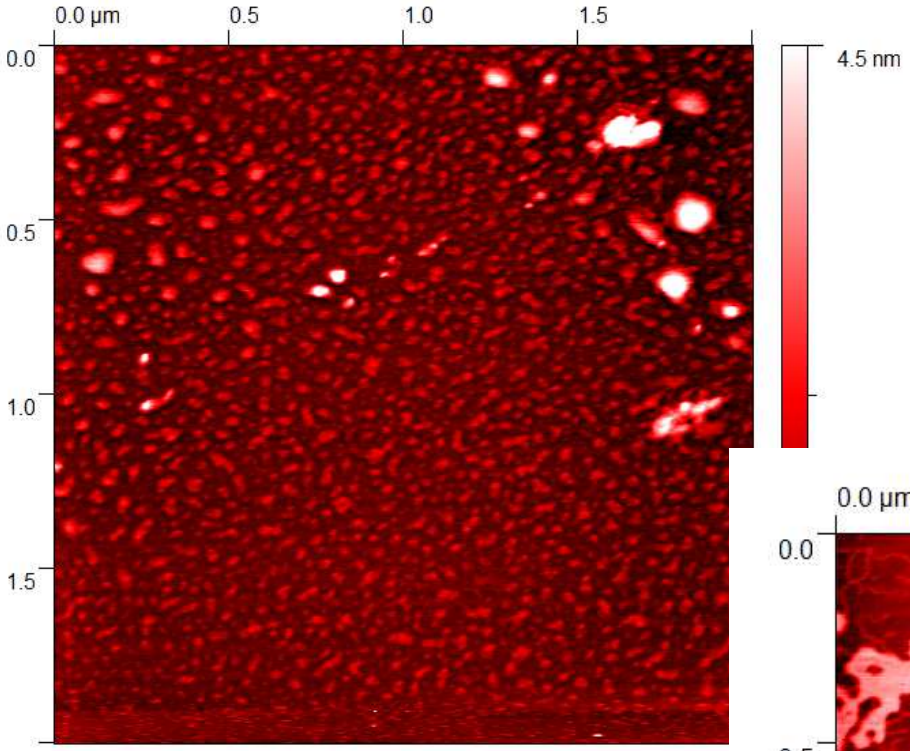
- Especially with **APTES** during liquid silanization
- Even vapors of water can cause this effect
- Fixation for **optical** microscopy – **expected** factor
- In contrary – in fixation for **AFM** – very **disturbing**
- Solution:

- silanization in **vapours** under **vacuum** (i.e. in desiccators)

- **mono**alkoxysilanes – can not poly

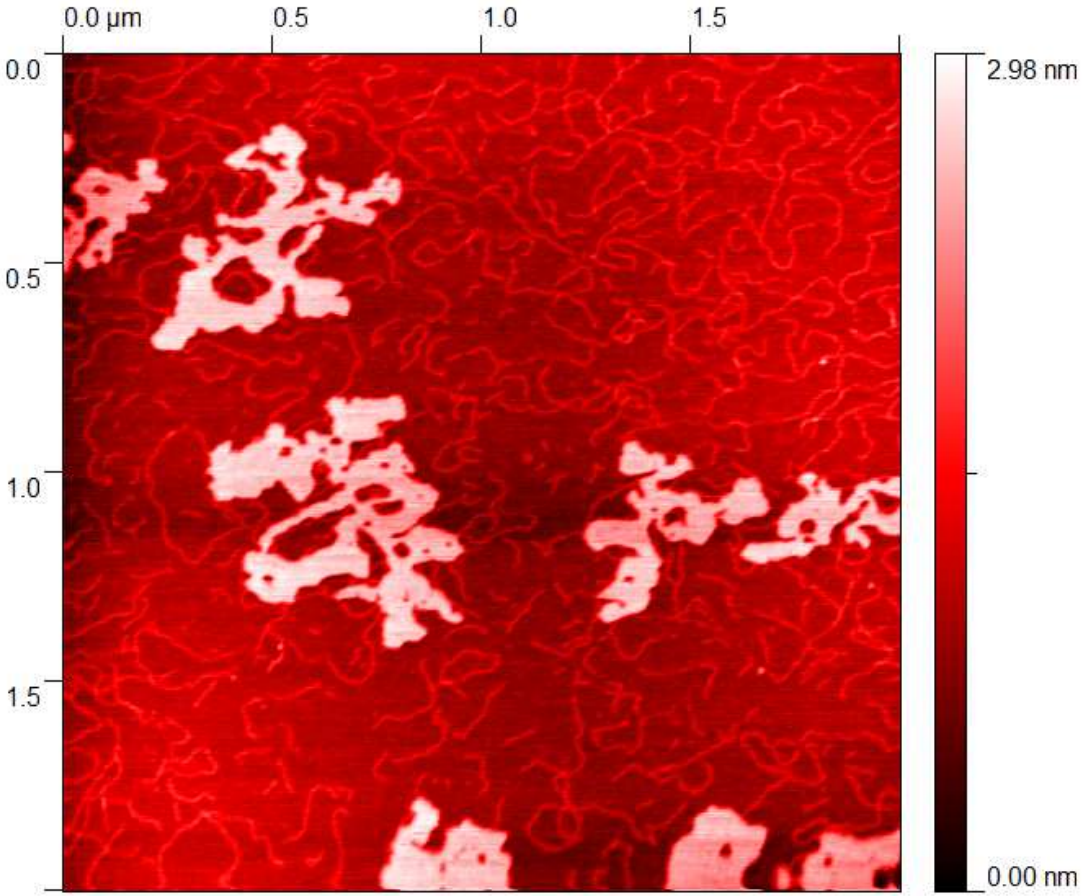


3-(Ethoxydimethylsilyl)propylamine
APDMES



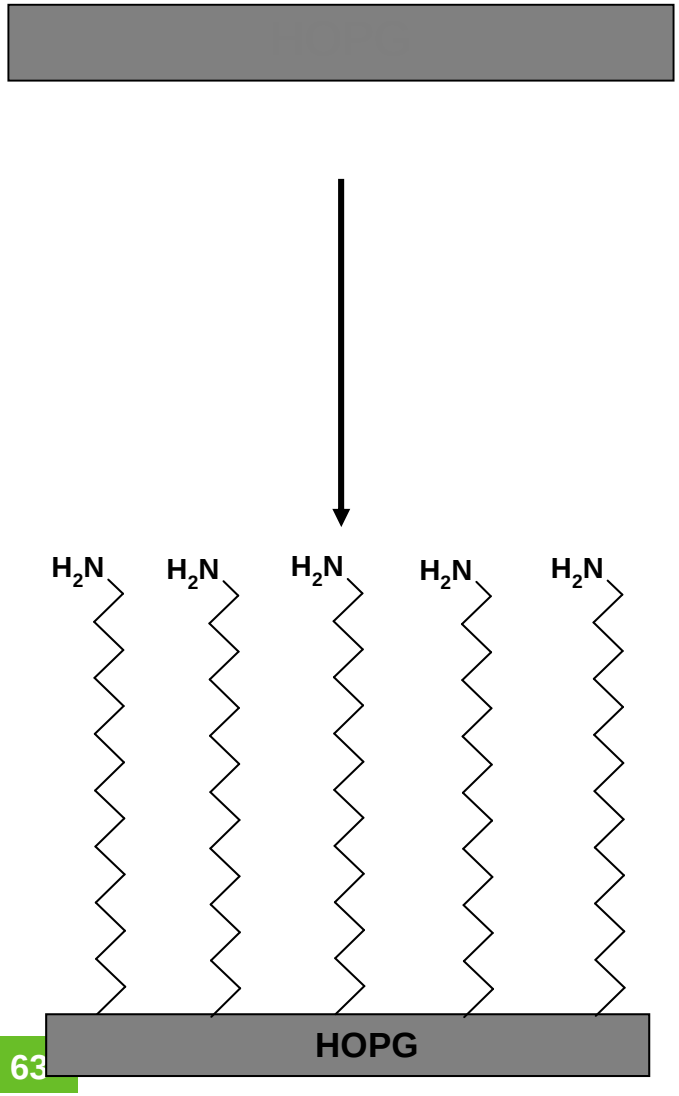
Self-polymerization

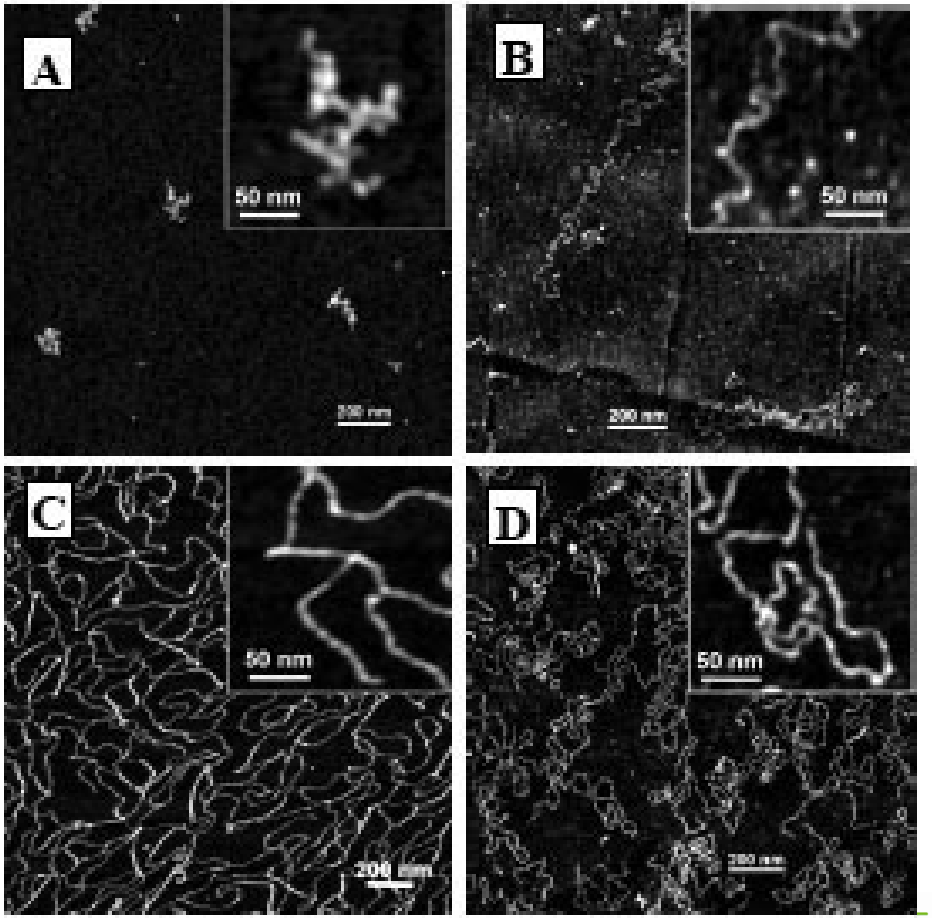
examples



B. DNA on HOPG

Adsorption of long chain double-sided ions (C_{16}/C_{18})

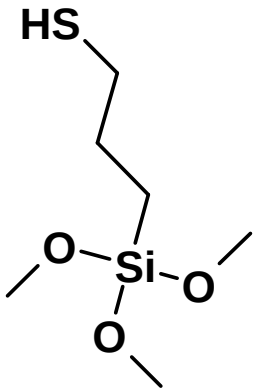




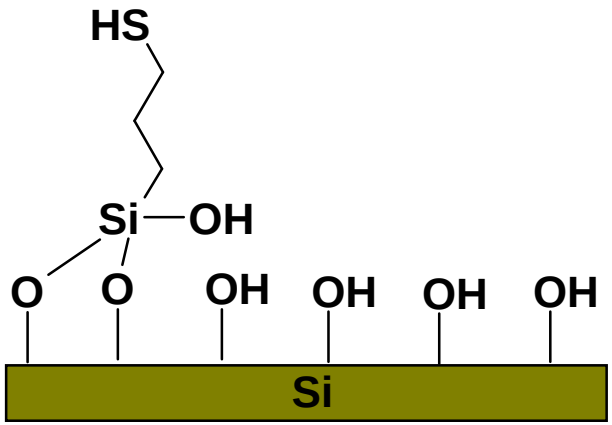
3. Nanoparticles

Substrates for immobilization: **mica** / **HOPG** (smooth surfaces), also gold, glass in selected cases.

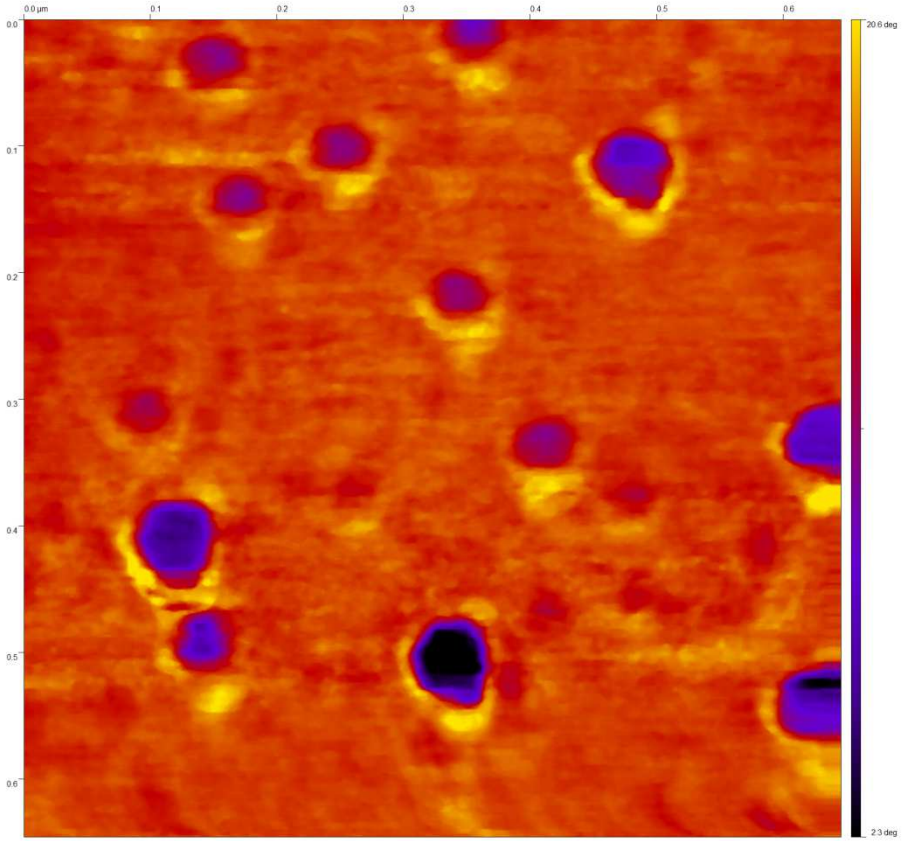
Example: gold nanoparticles (AuNP) mercapto-silanized mica (SH-mica):

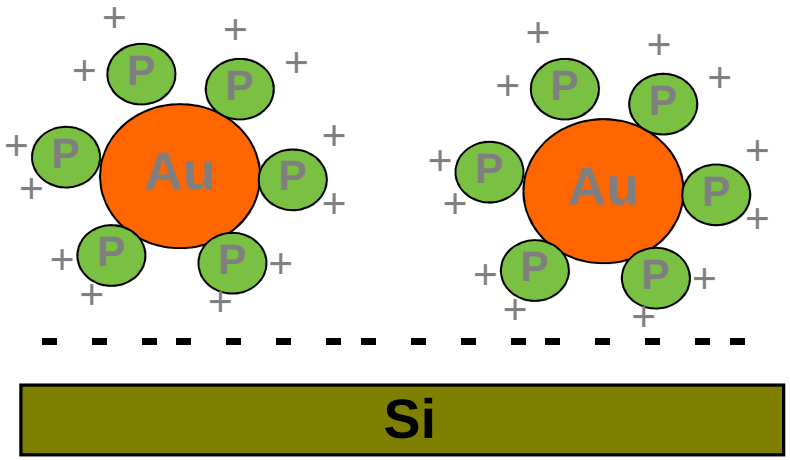


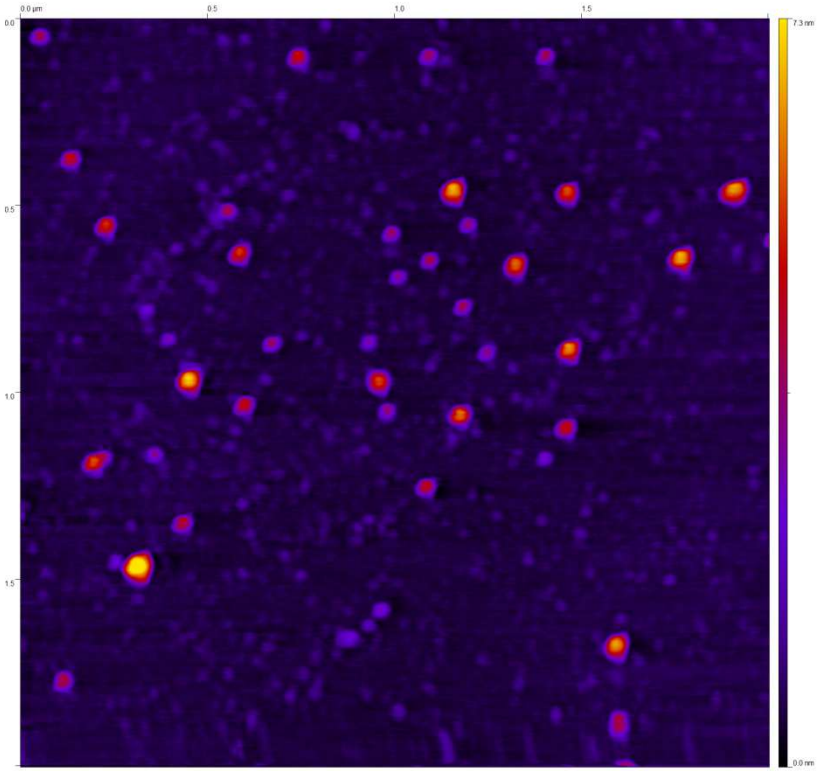
(3-Mercaptopropyl)trimethoxysilane
MPTS



SH-mica



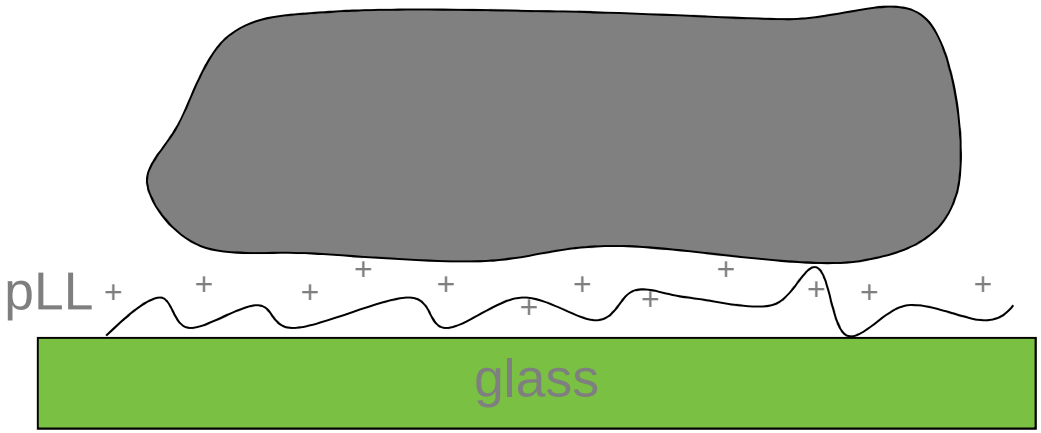


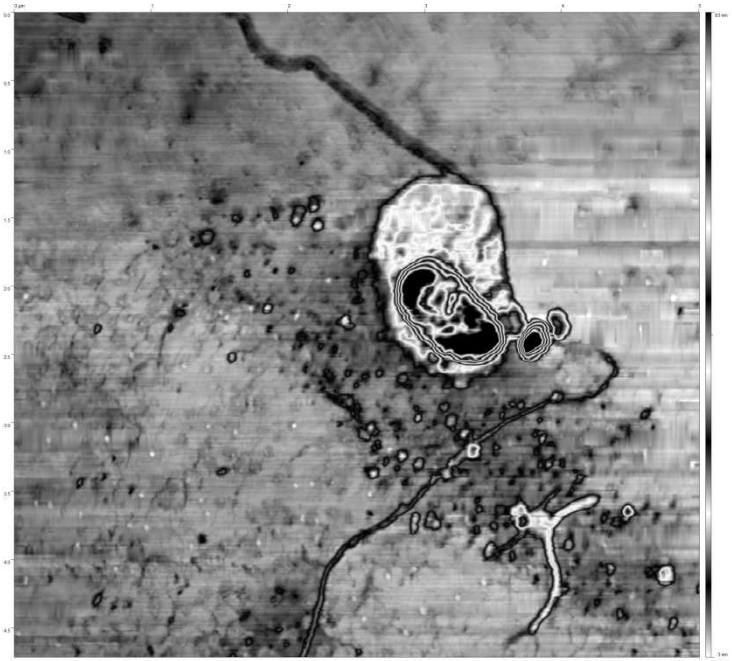


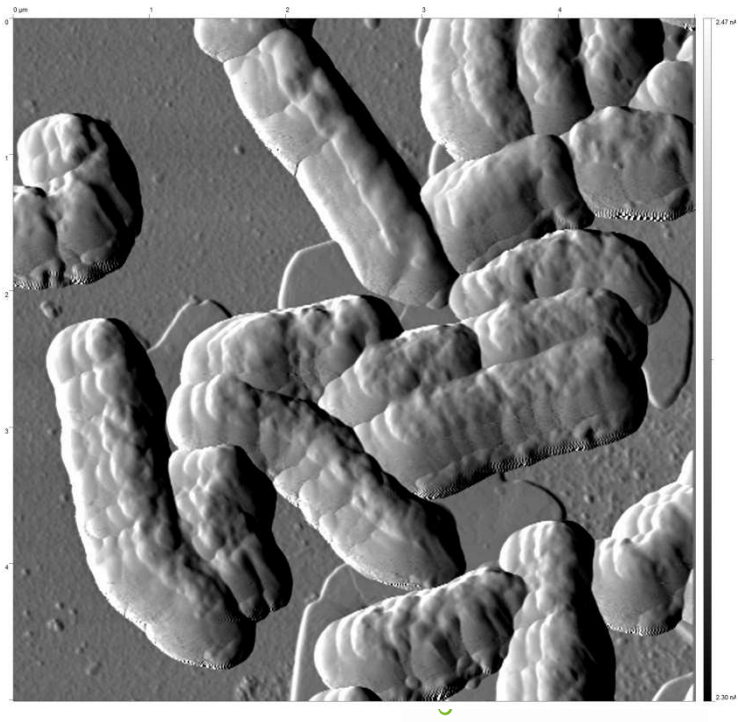
Gold nanoparticles (**AuNP**) conjugated with **protein** molecules:
protein = immobilization bridge

3. Bacteria, spores

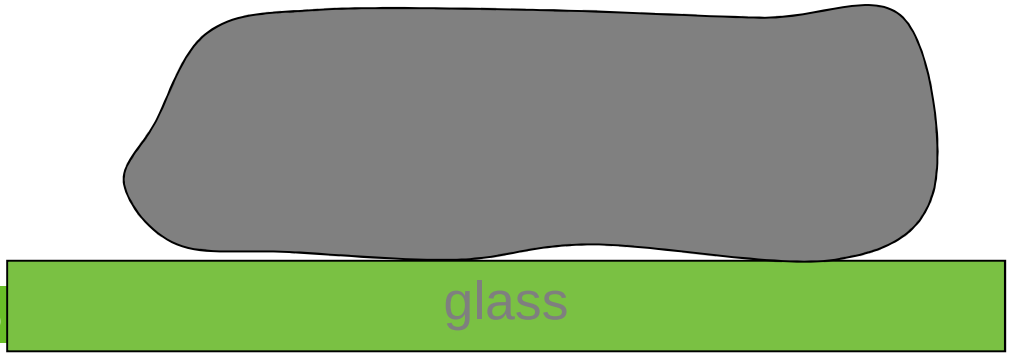
Protein adhesive layer, i.e. pLL
(poly-L-lysine → introducing positive charge)







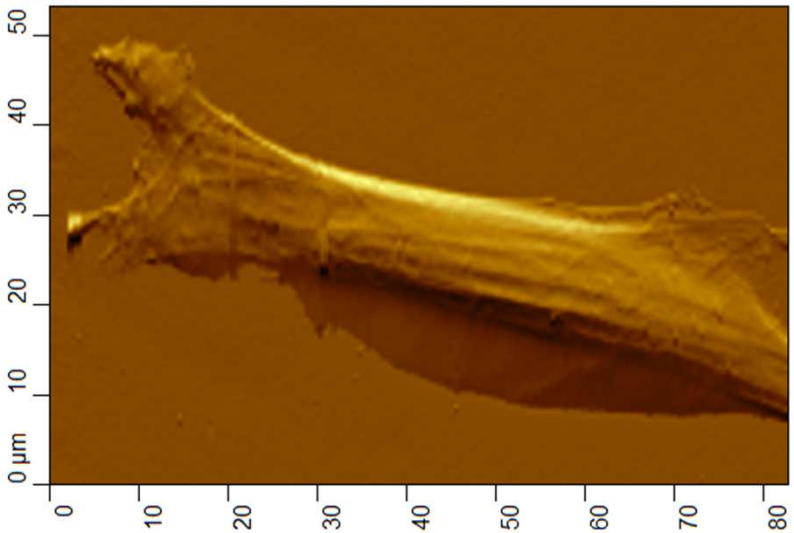
Standard coating on glass

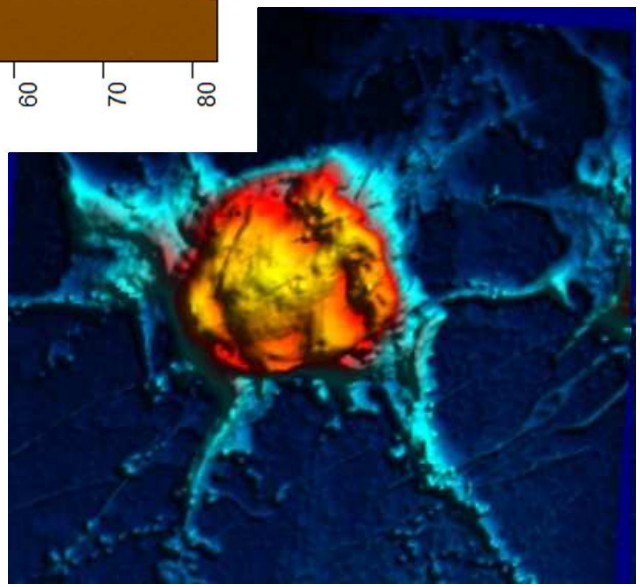


5. Eukaryotic cells

A. Standard culturing on polystyrene dishes

Adhesive protein layers usually takes place (i.e. pLL, RGD adhesion factors, fibronectin, etc.)



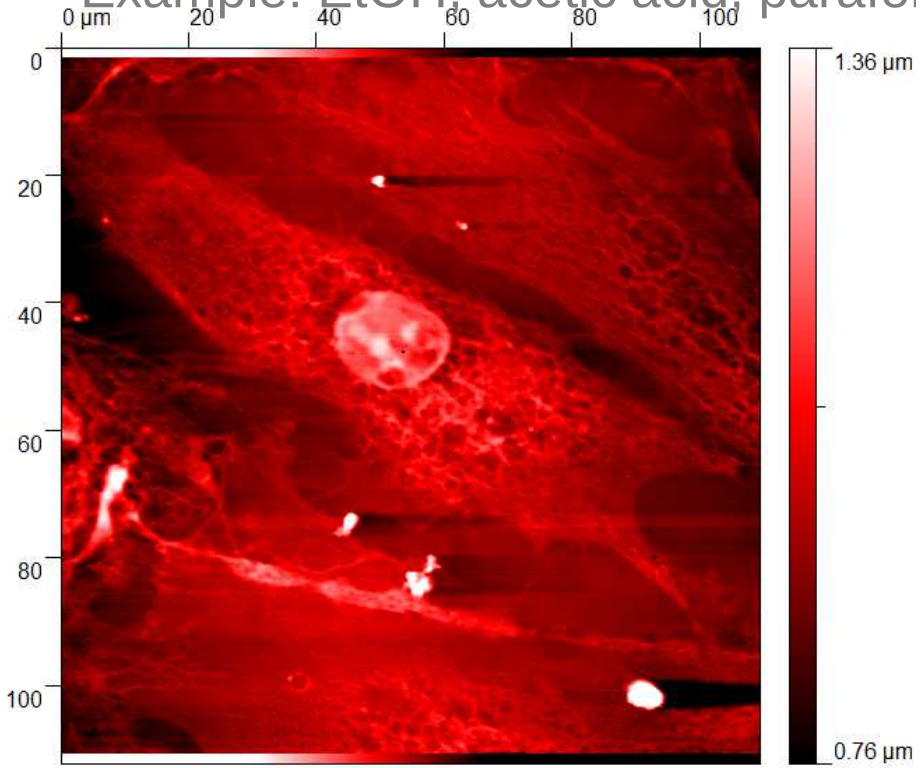


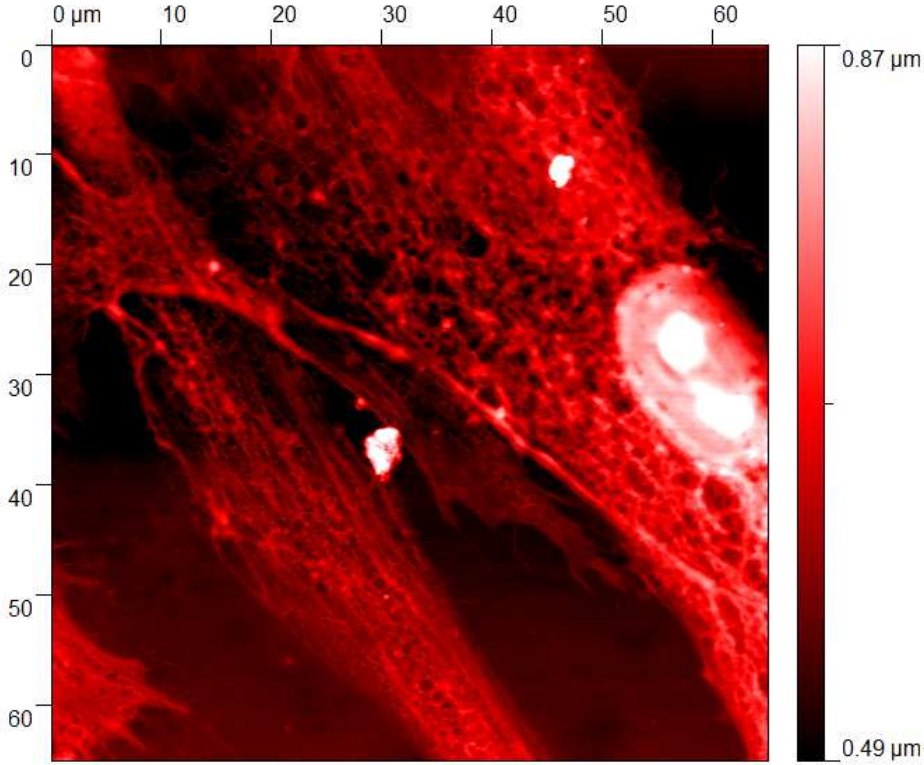
Cell culturing equipm

BioAFM incl. Petri dish **heater** for in-

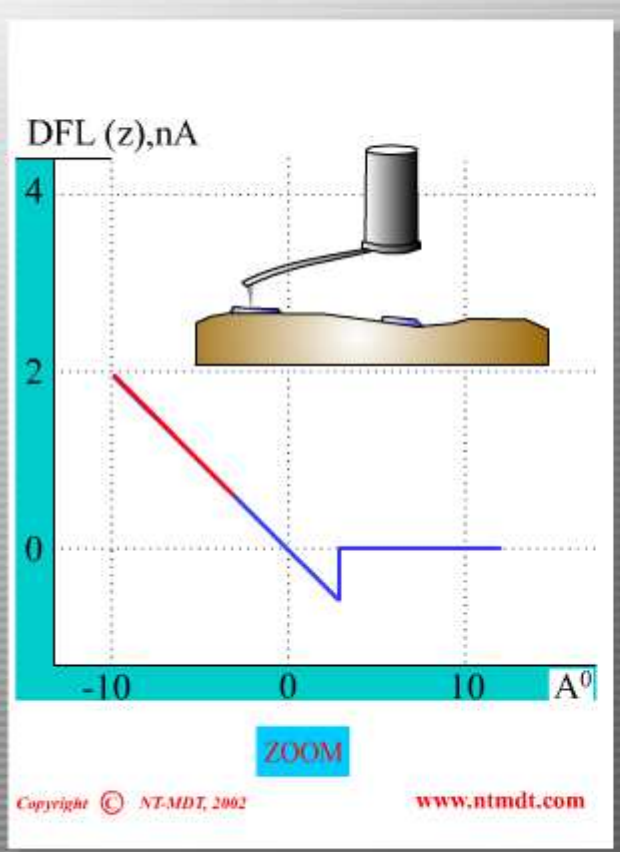
B. Fixation agents

- Adhesion of cells out of incubator (37ºC, 5% CO_2) is mostly problematic
- Allows study of cells in long term periods after removal from incubator
- Cell wall destruction
- Example: EtOH, acetic acid, parafo





AFM spectroscopy



Examples

Force-distance curves

Force is measured in an **SFM** by collecting a force curve, which is a plot of cantilever deflection, d_c, as a function of sample position along the **z**-axis (i.e. towards or away from the probe tip; the **z**-piezo position). It assumes a simple relationship (i.e. **Hooke's Law**) between the force, **F**, and the cantilever deflection:

$$F = - k d_c$$

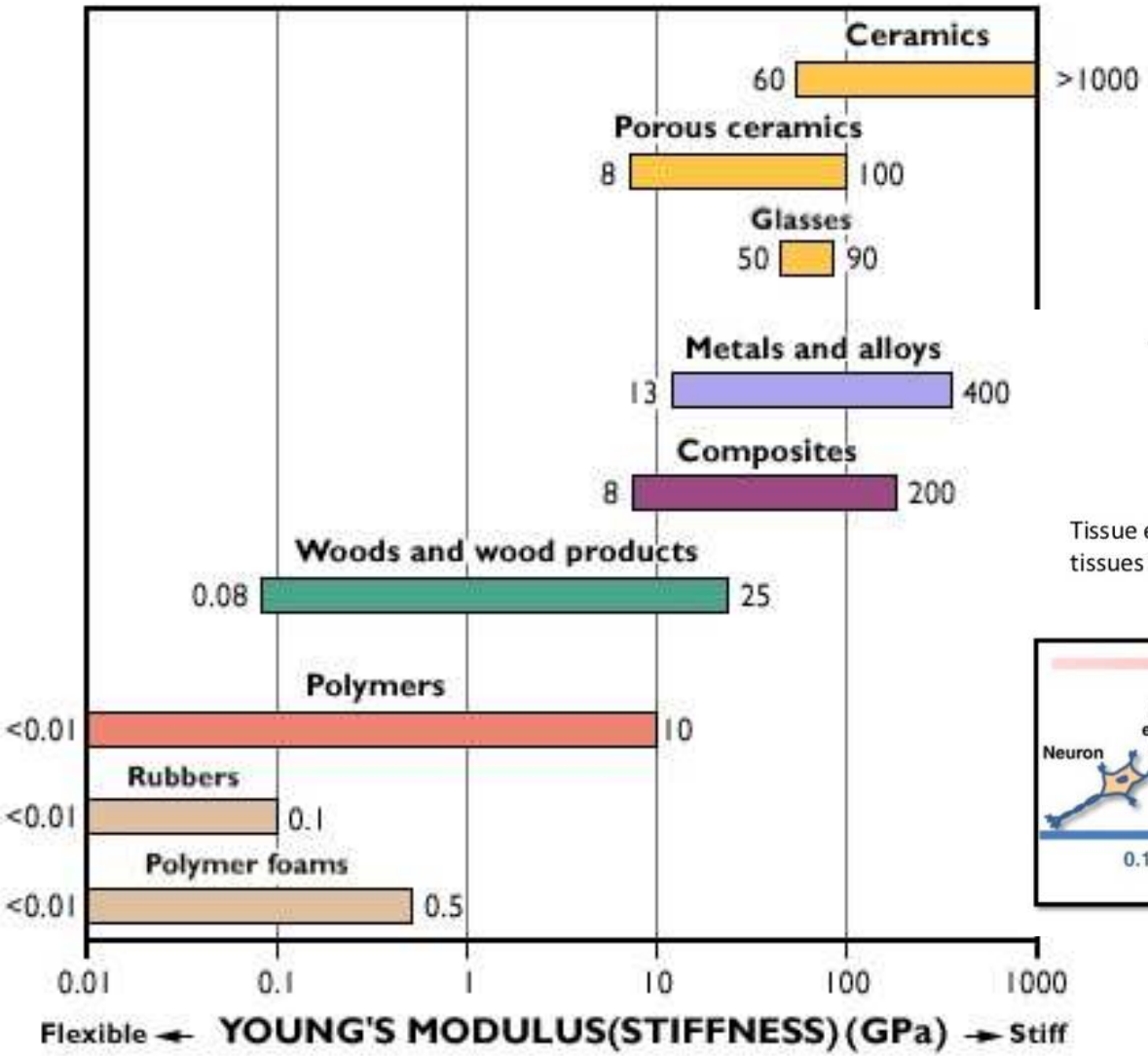
where **k** is the spring constant of the cantilever.

Motivation

Why to quantify elasticity of (living) objects?

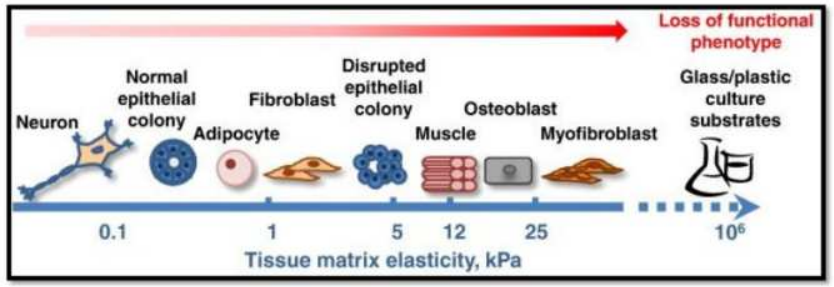
- **Stiffness** (Young's modulus) **mapping**
  → stiffness = basic parameter of any material
- **Elasticity-phenotype** relation ship
- **Mechanobiological** characterization
- **Driving of instrument** properties (QNM, QI)

Young's modulus of materials



Tissue's Young Modulus

Tissue elastic modulus (E) is given by the resistance offered by the tissues to deformation effects, i.e. the tissue stiffness.



http://www-materials.eng.cam.ac.uk/

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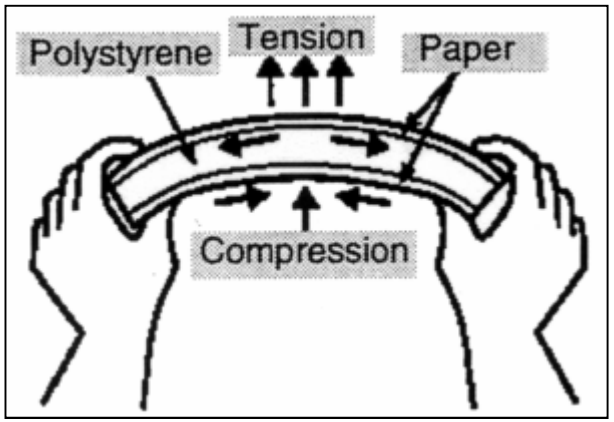
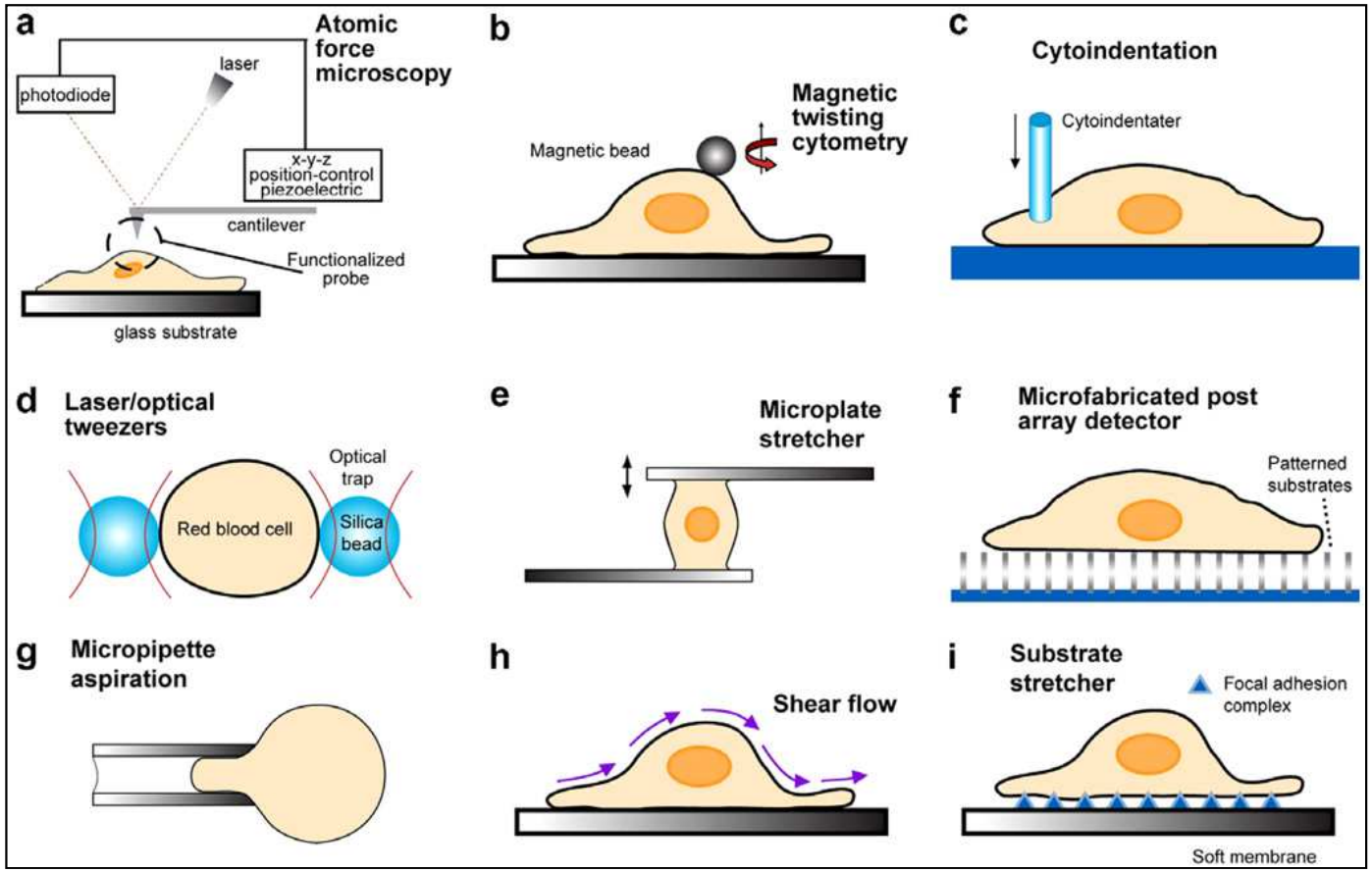
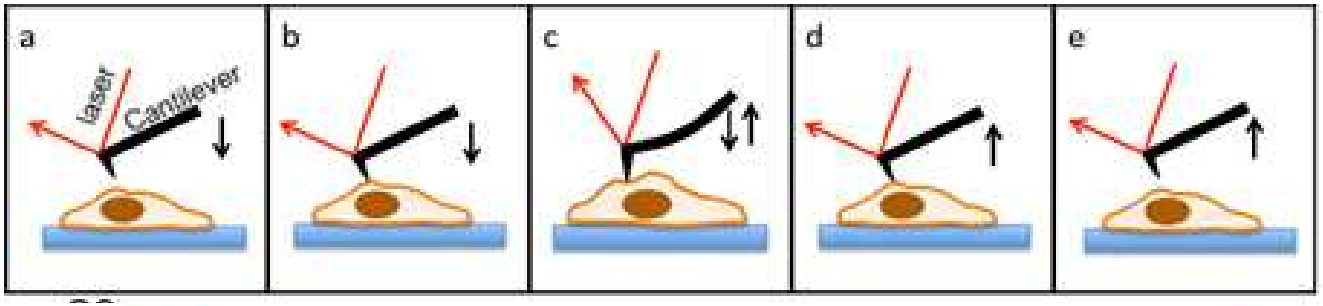
Methods to measure Young's modulus

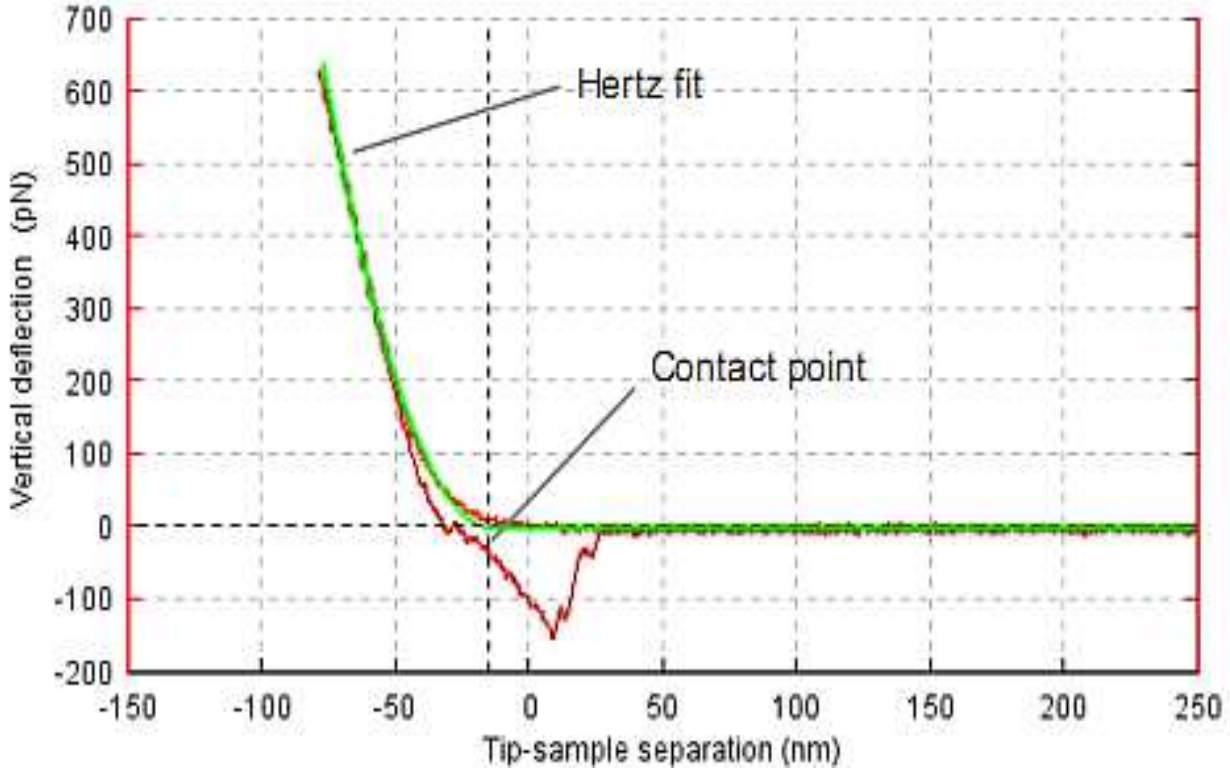


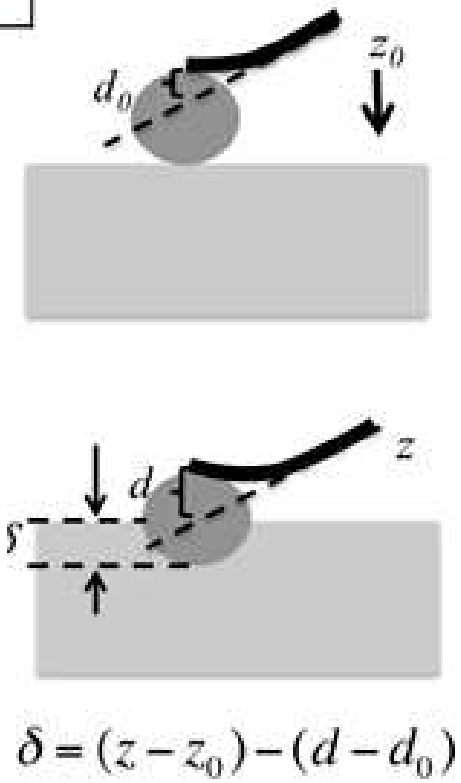


Acta Biomater. 2007 Jul; 3(4): 413–438.

Mechanical Properties of Living Cells Using Atomic Force Microscopy







J. Vis. Exp. (76), e50497, doi:10.3791/50497 (2013).

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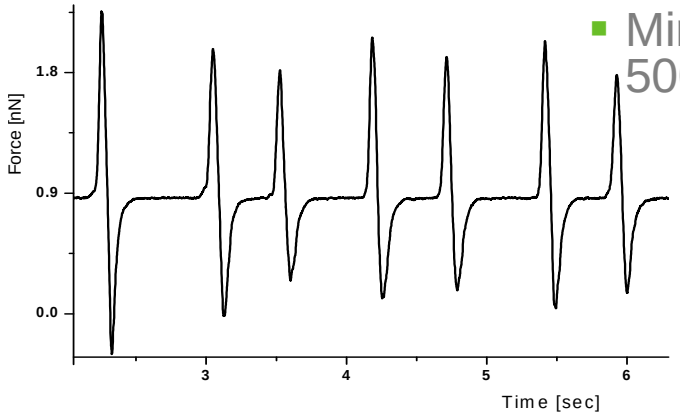
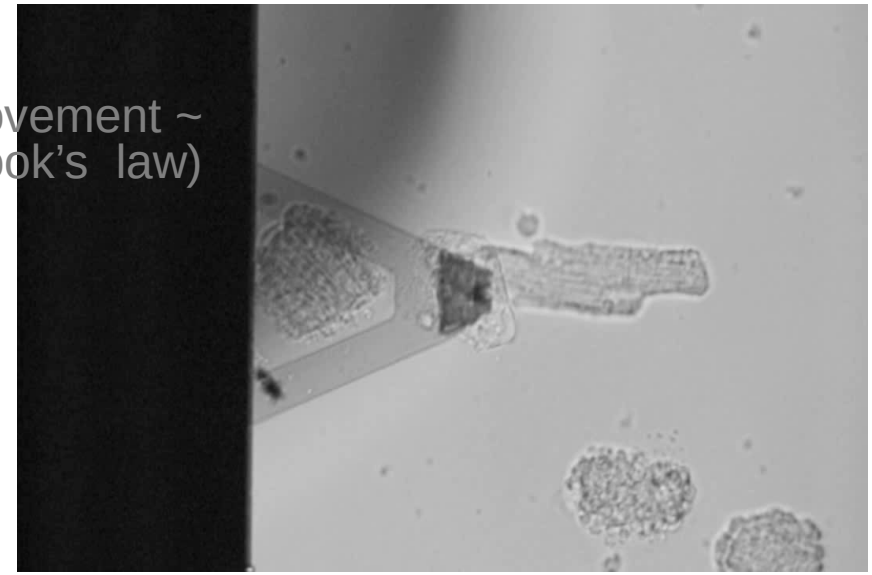
1. Biomechanical characterization

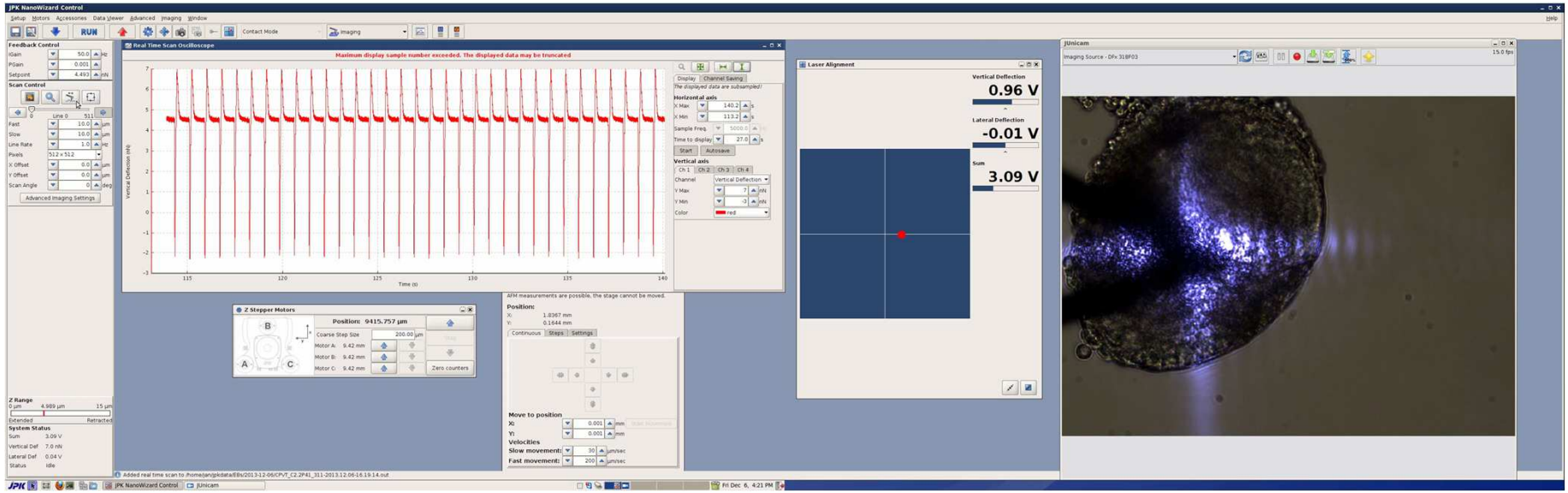
Primary CMCs

- Noise ~ 10pN
- Min. detectable movement ~ 500 pm (acc. to Hook's law)





Embryonic bodies - iPS cardiomyocytes



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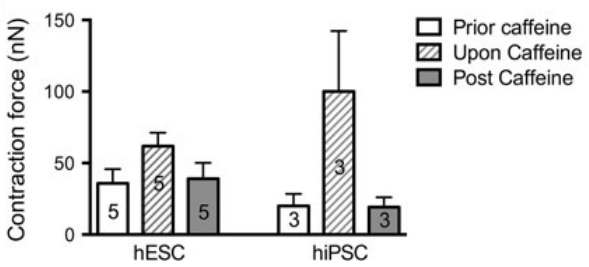
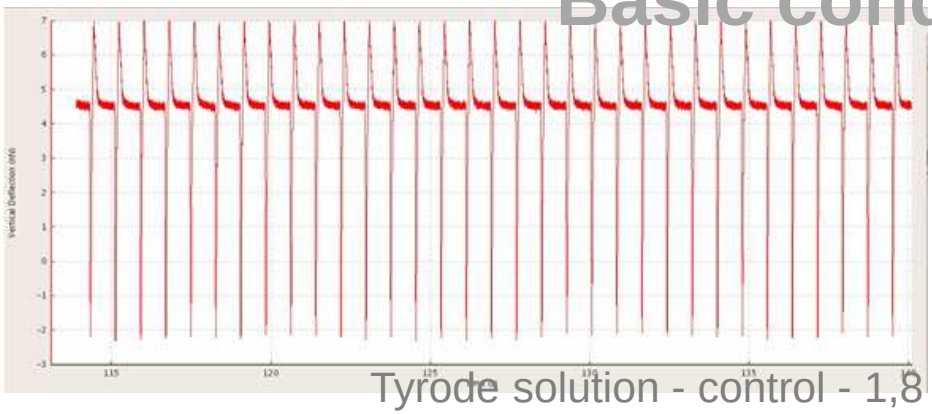
Drug testing studies

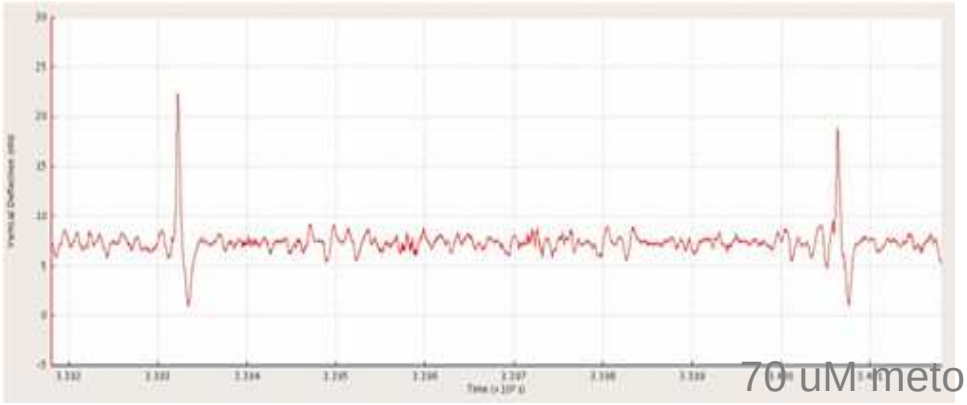


Basic conditions

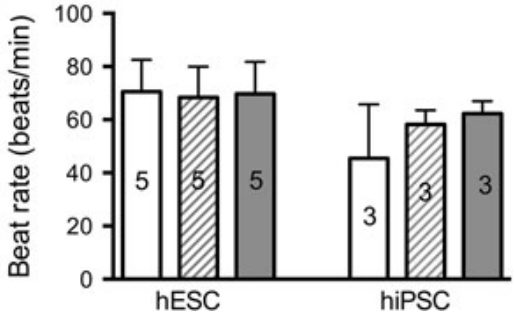


Tyrode solution - control - 1,8 mmol Ca 2+

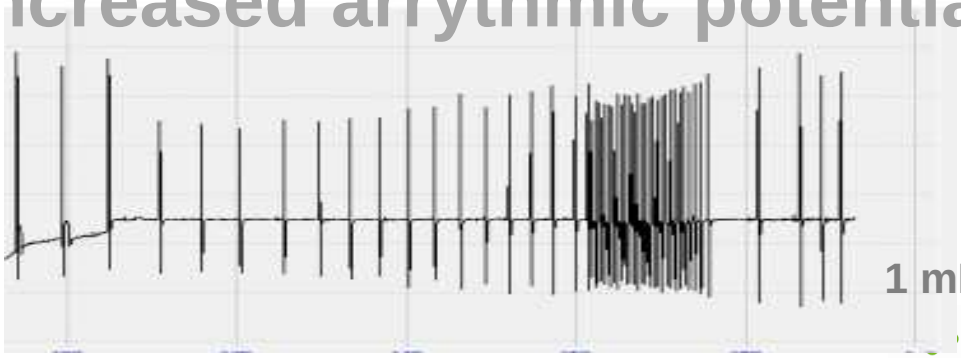
Adrenergic reactivity - Metoprolol



70 uM metoprolol



Increased arrythmic potential



1 mM caffeine

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2. Nanomechanical mapping

& relation to physical and phenotype properties

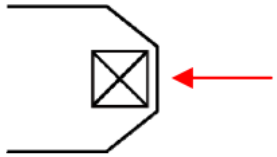
Hertzian fit

Measured curves were fitted to following function:

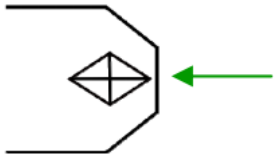
Four-sided pyramid

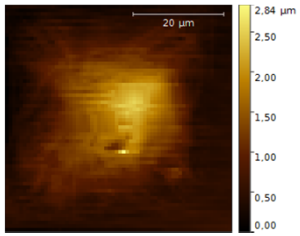
$$F = \frac{E}{1-\nu^2}\frac{\tan\alpha}{\sqrt{2}}\delta^2$$

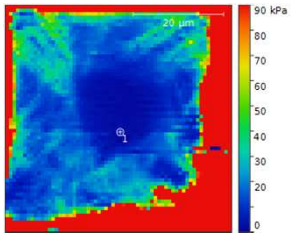
α = face angle, usually given for Si_3N_4-cantilevers

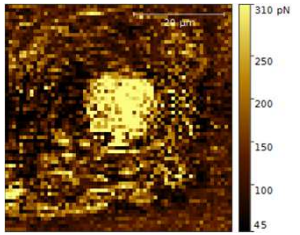


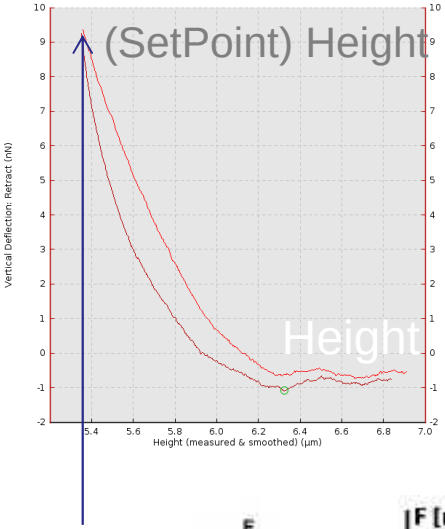
β = edge angle, usually given for Si-cantilevers

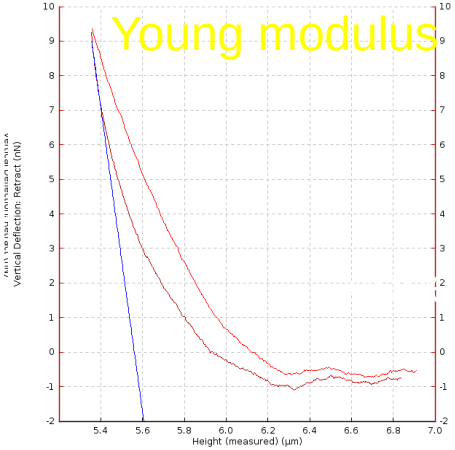


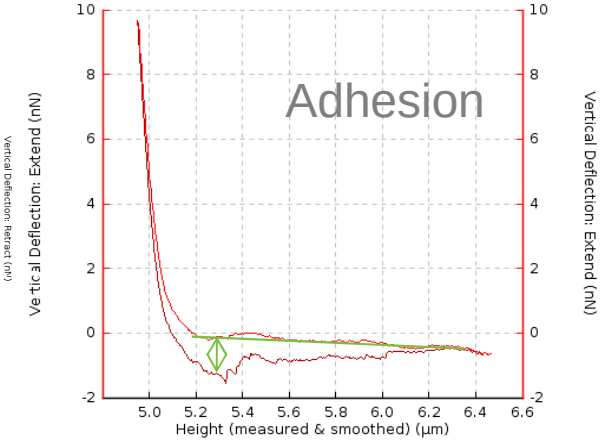


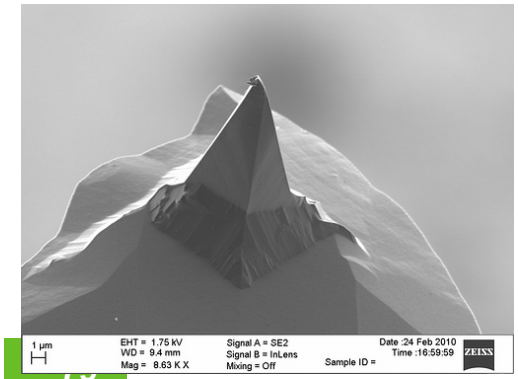


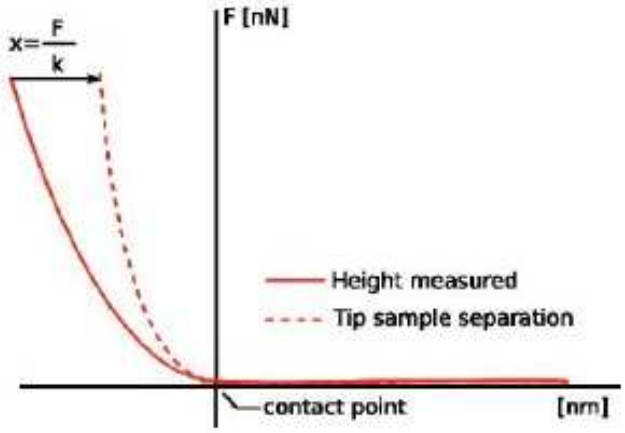


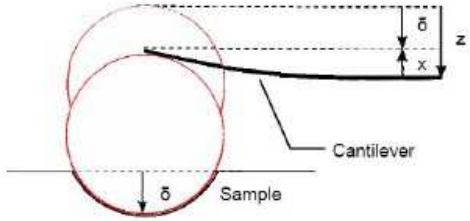












Tip-sample separation = correction of measured curve (height) for cantilever bending

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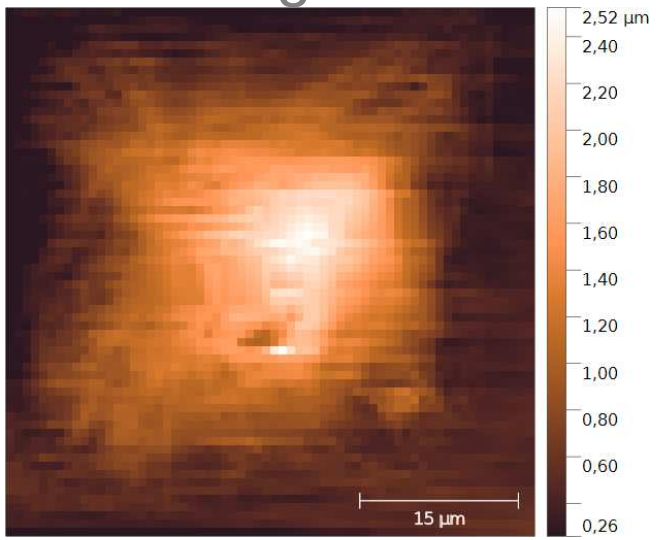
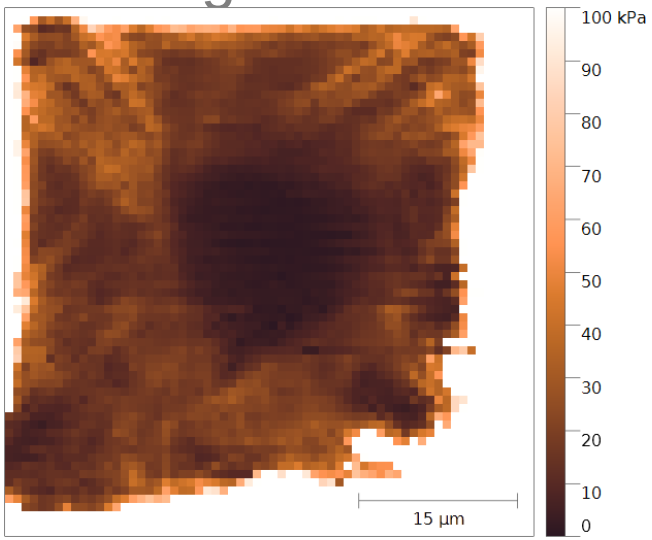
ADMSC cells

Height

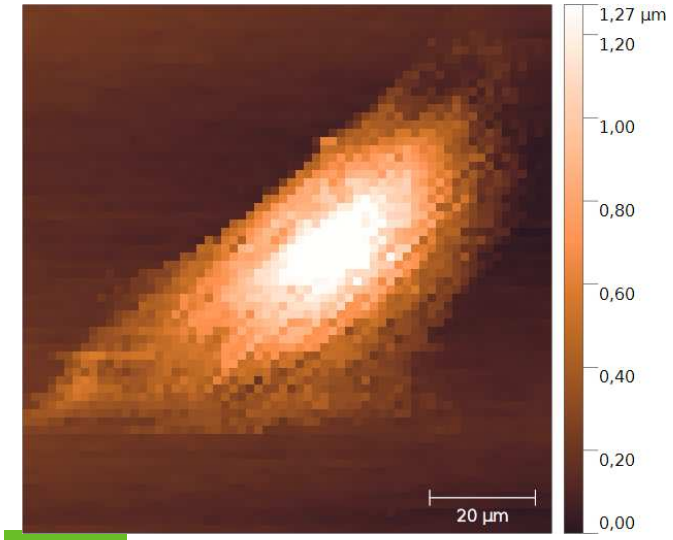


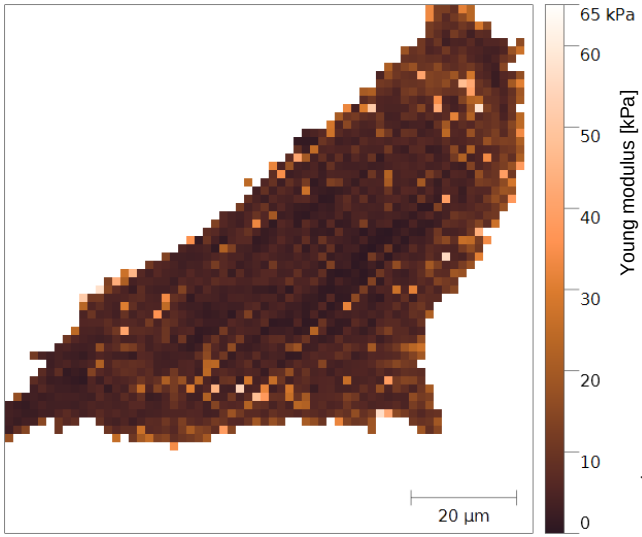
Young modulus

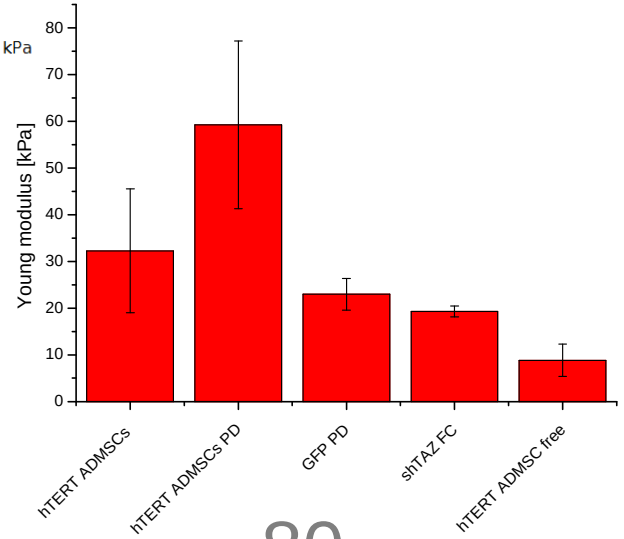


Mesenchymal stem cells (hTERT AD-MSCs) on micropatterned substrates

shTAZ cells







80

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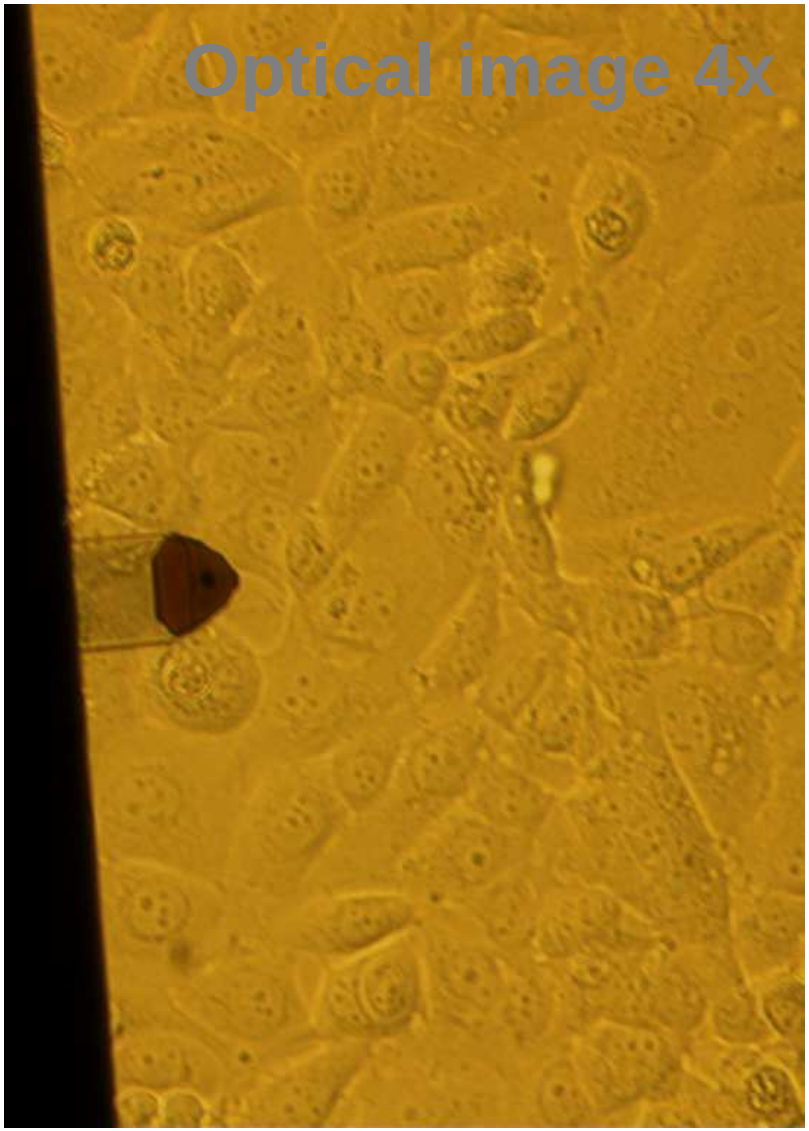
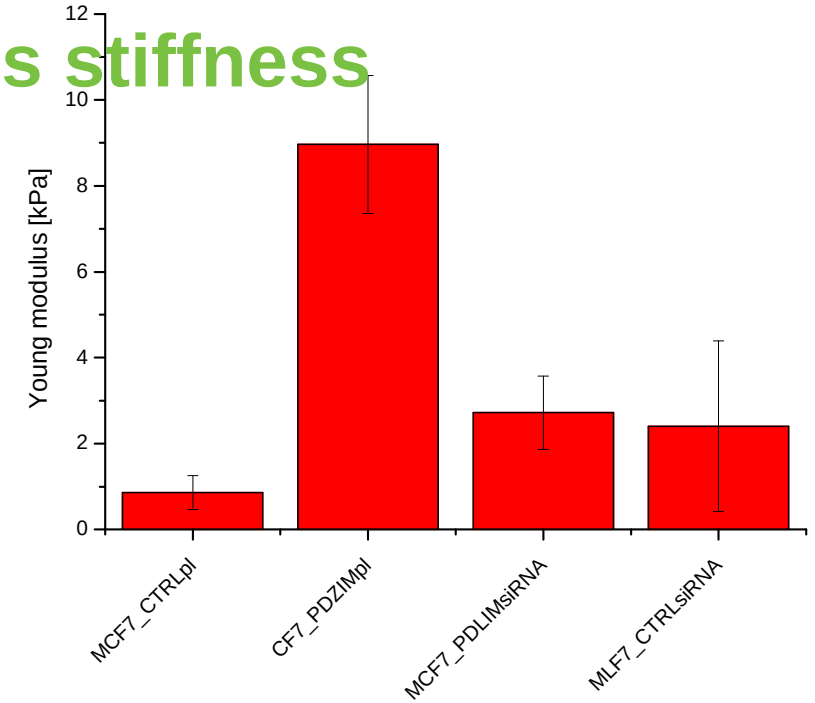
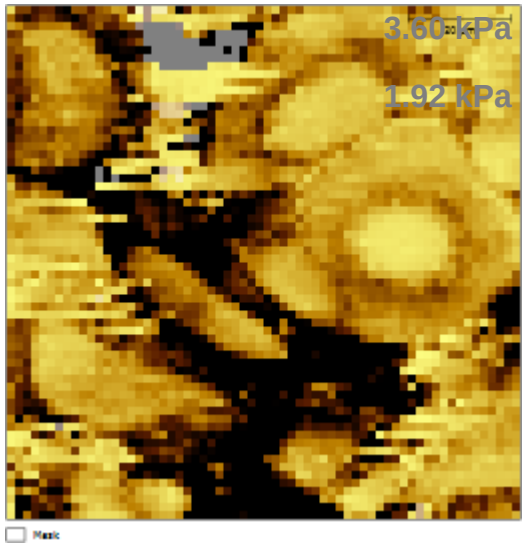
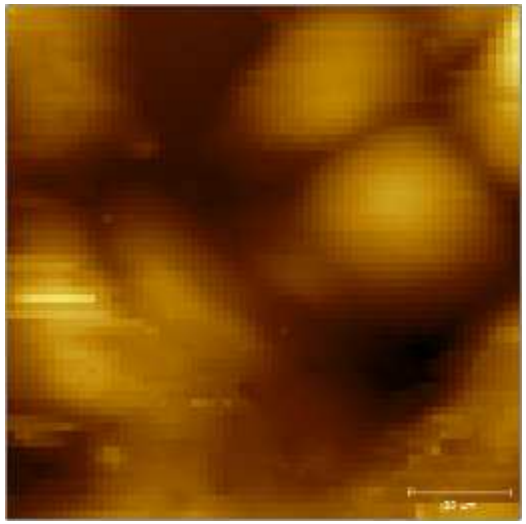
Cancer cells stiffness





Cells MCF7_PDLIMsiRNA





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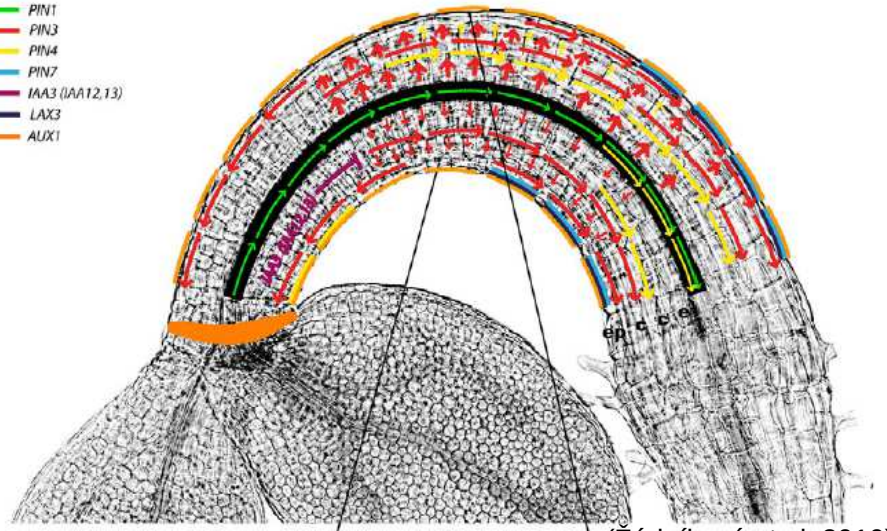


(Žádníková et al. 2010)

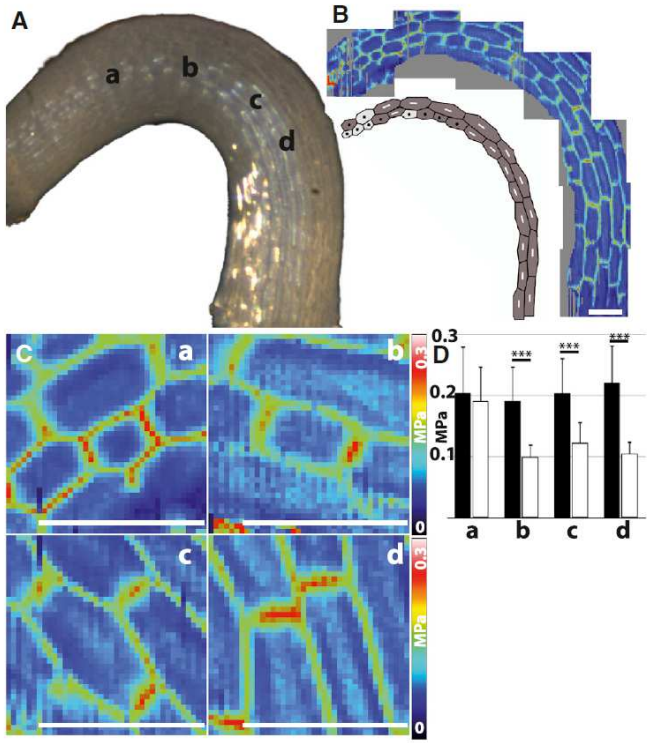
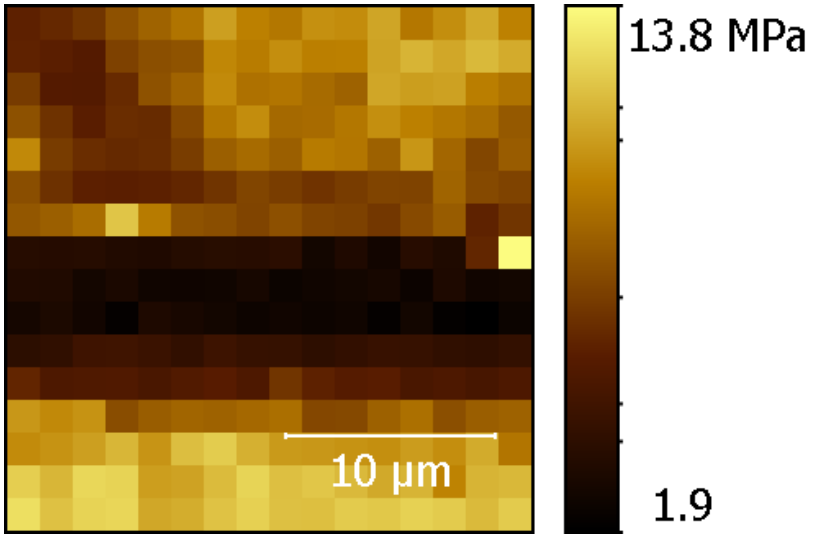
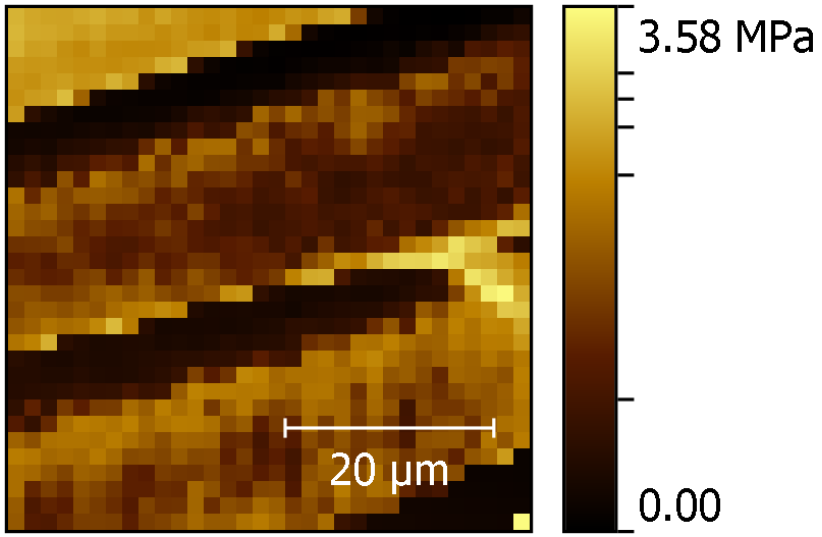


Figure 1. Cellular Asymmetry in Wall Stiffness in Dark-Grown Hypocotyl

(Peaucelle et al., CB 2015)

Plant samples under AFM spectroscopy investigation

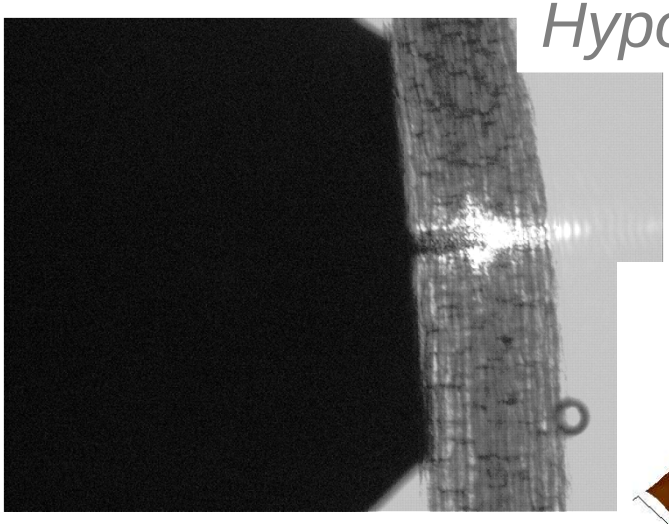


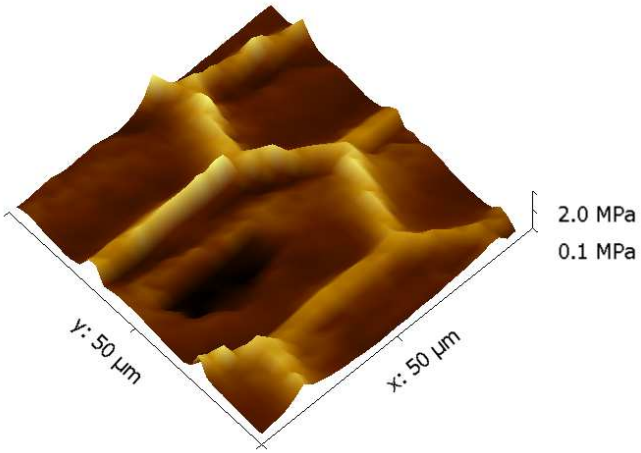


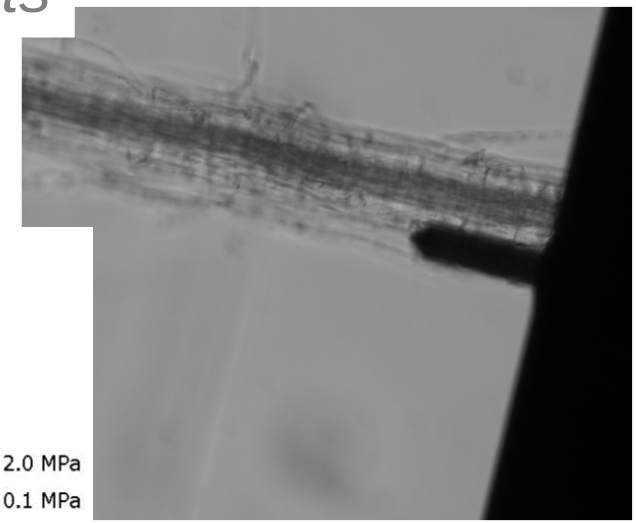
Water – YM 7.09 MPa

Manitol YM 0.69 MPa

Hypocotyl and root parts







by Marçal Gallemí

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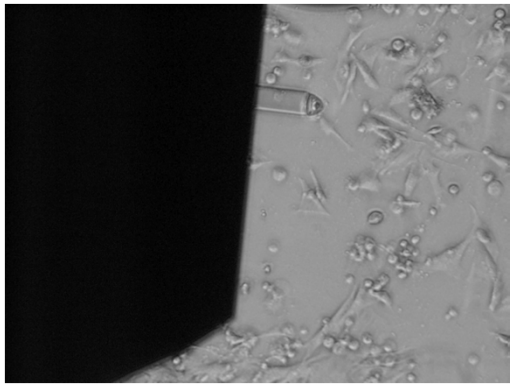
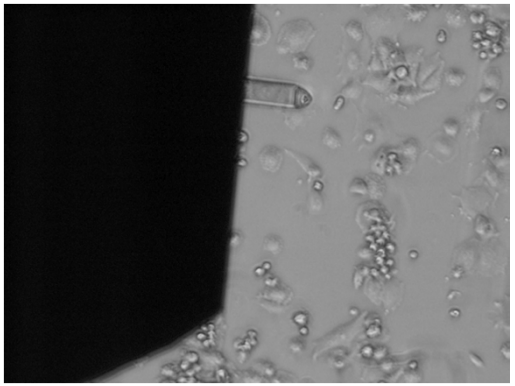
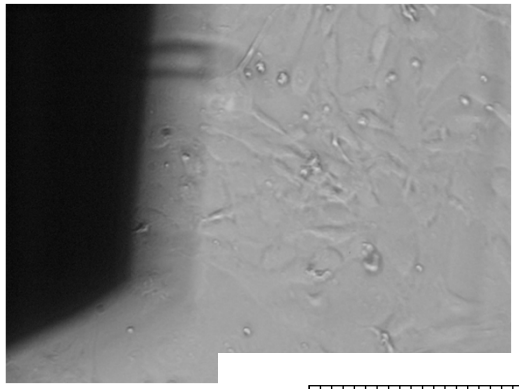
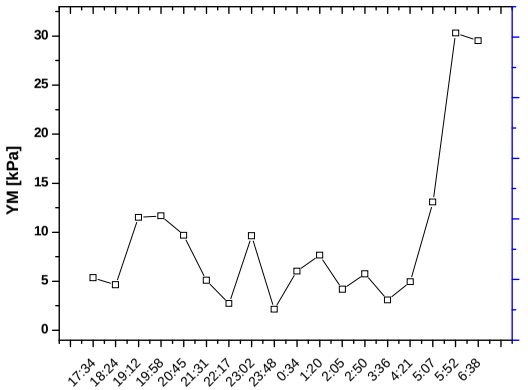
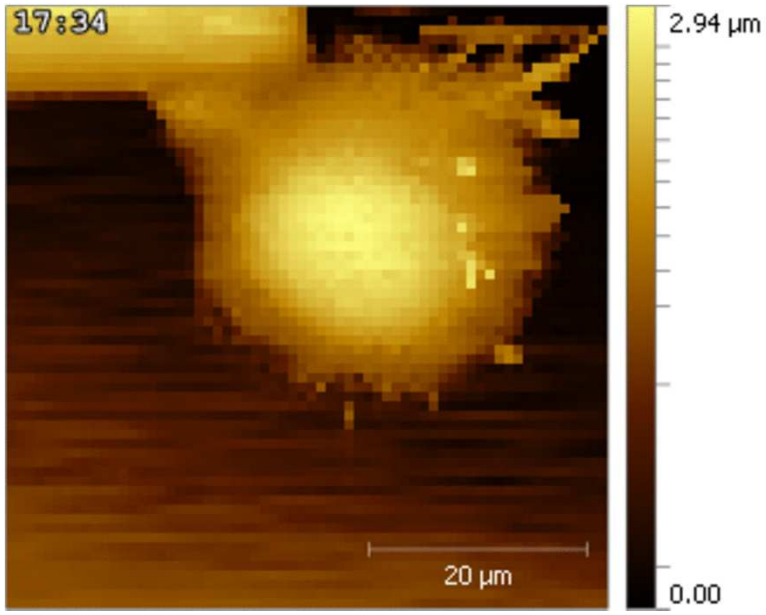
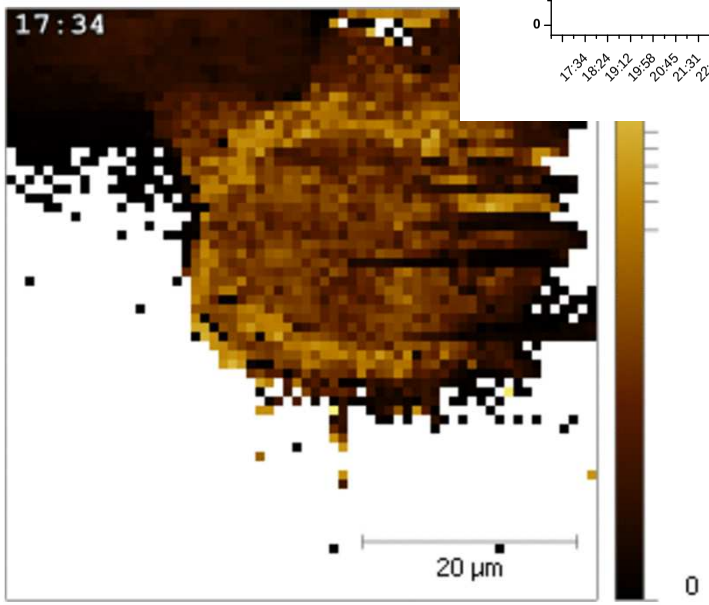




Fibroblasts thawing process







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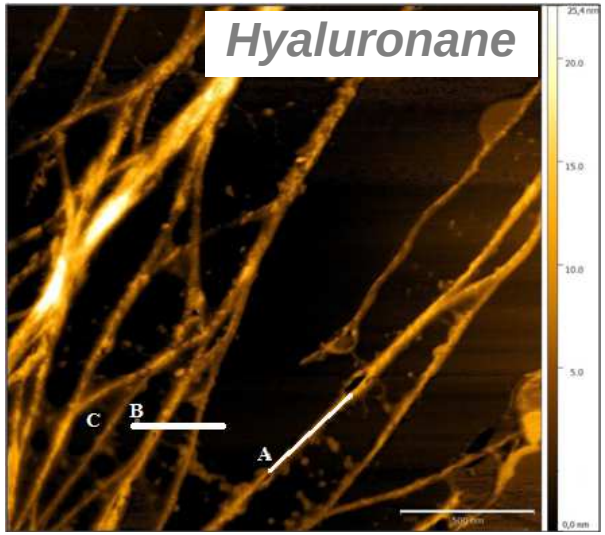
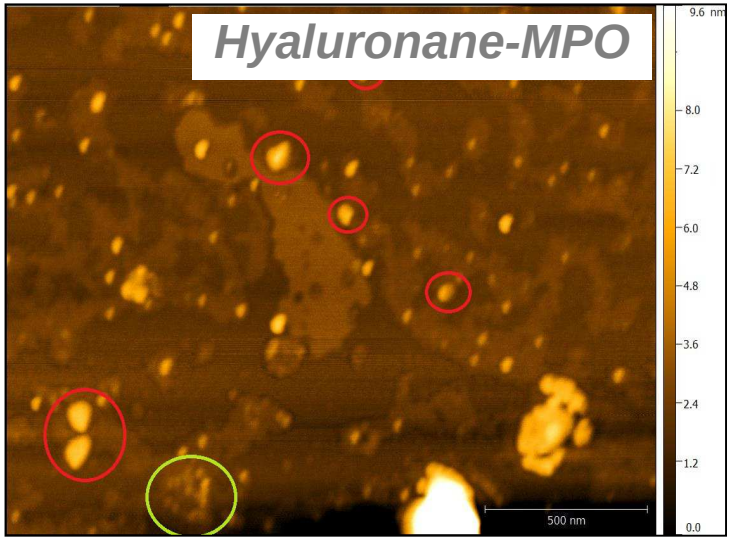
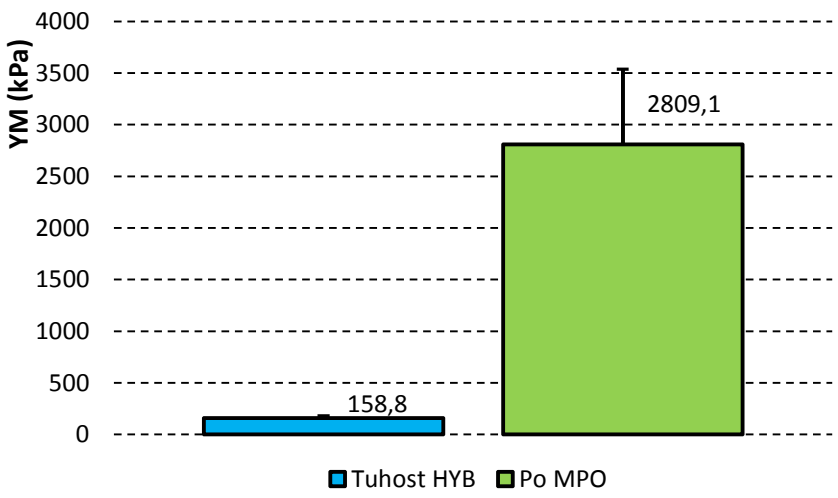
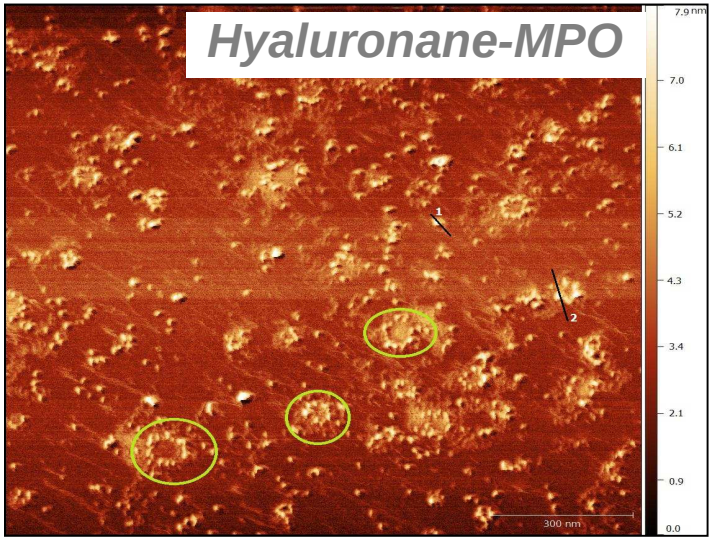
Glycoaminoglycanes

structure & interaction with myeloperoxidase





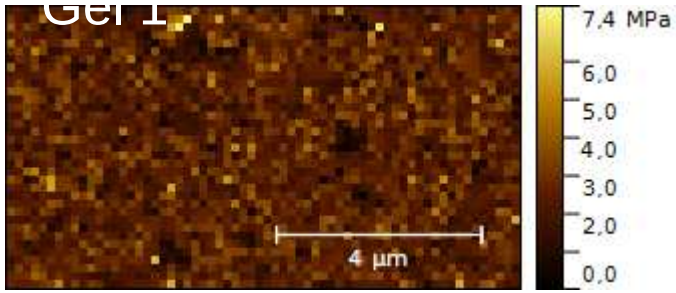


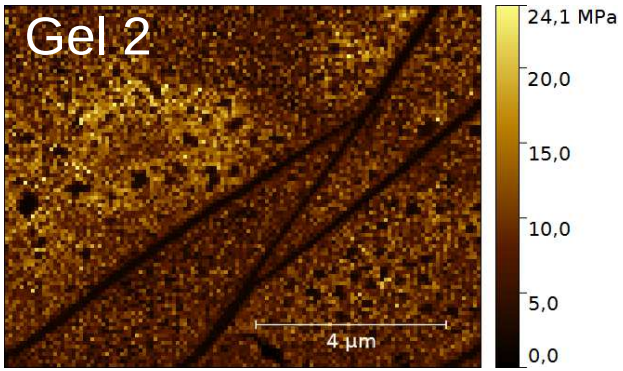


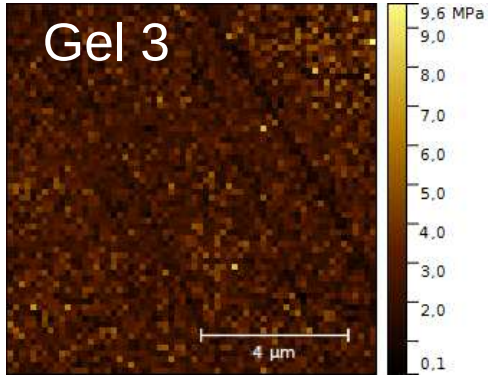
Flexible surfaces (gels) as support for single CMs

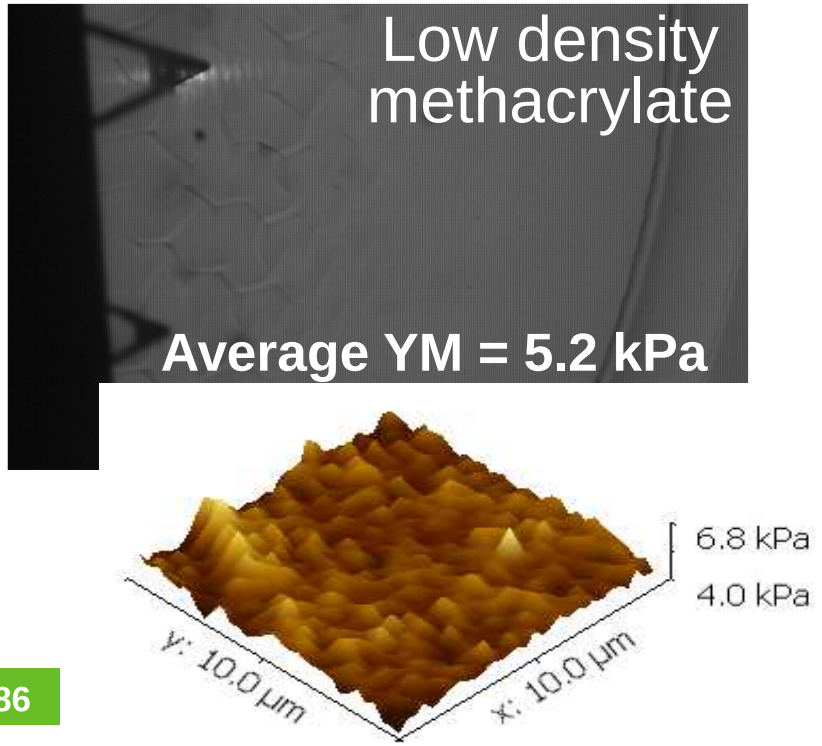
*With **Vladimir Vinarsky, Giorgia Nardone, Giancarlo Forte (ICRC, FNUSA, Brno)***

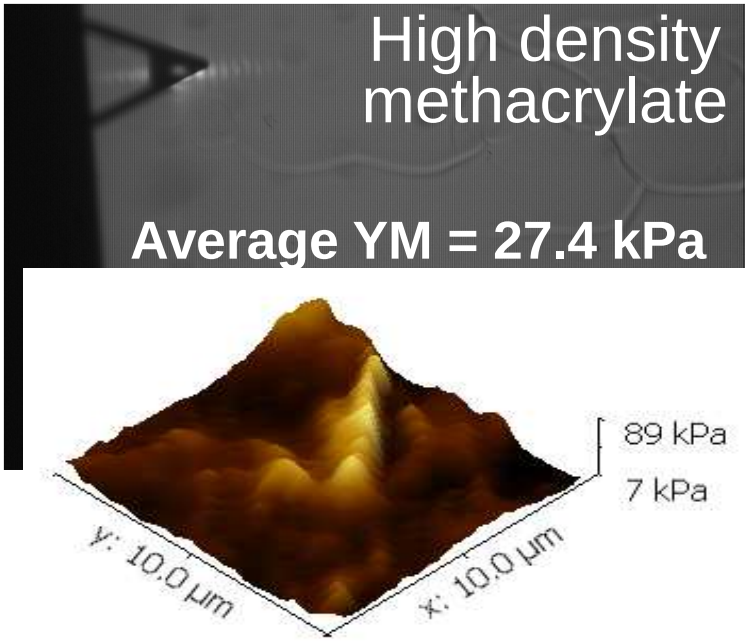
PDMS based gels











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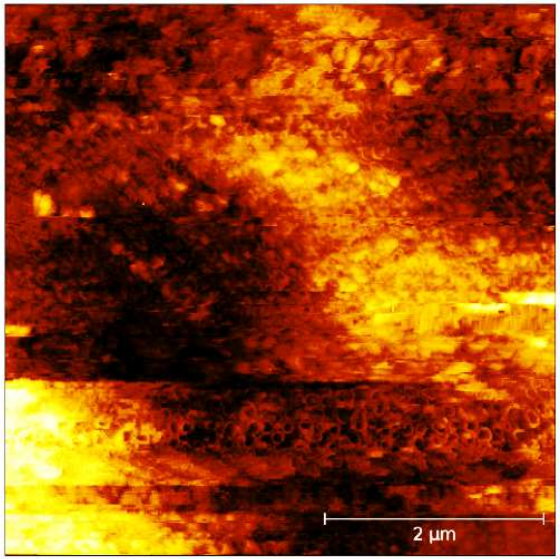
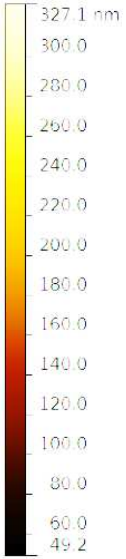
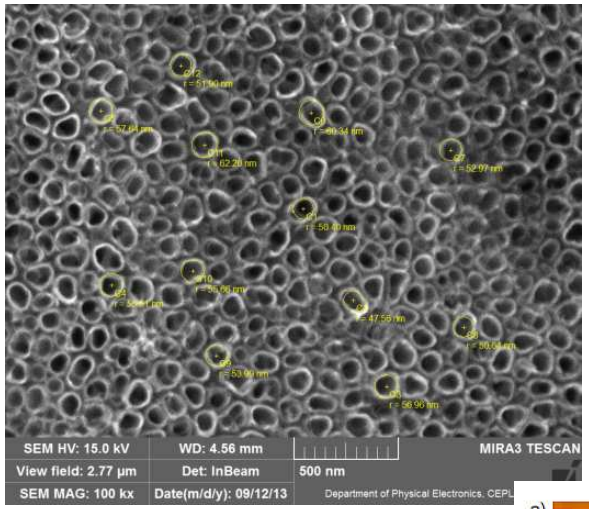
3. (Semi)automatic driving of AFM

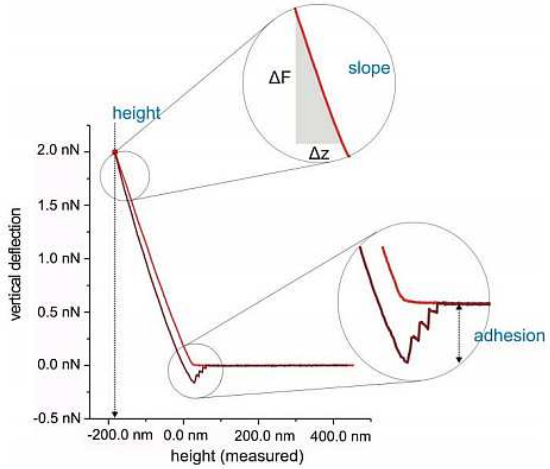
Quantitative imaging (QI mode)

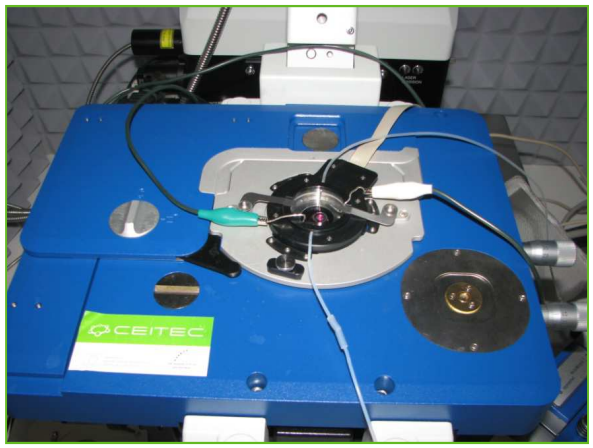
TiO_2 NT

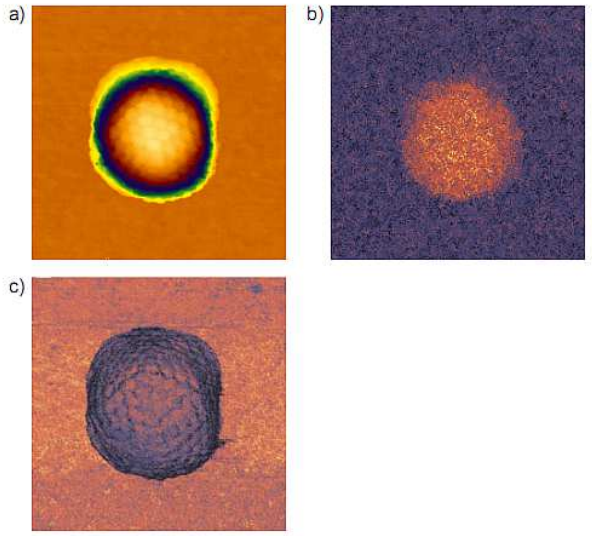








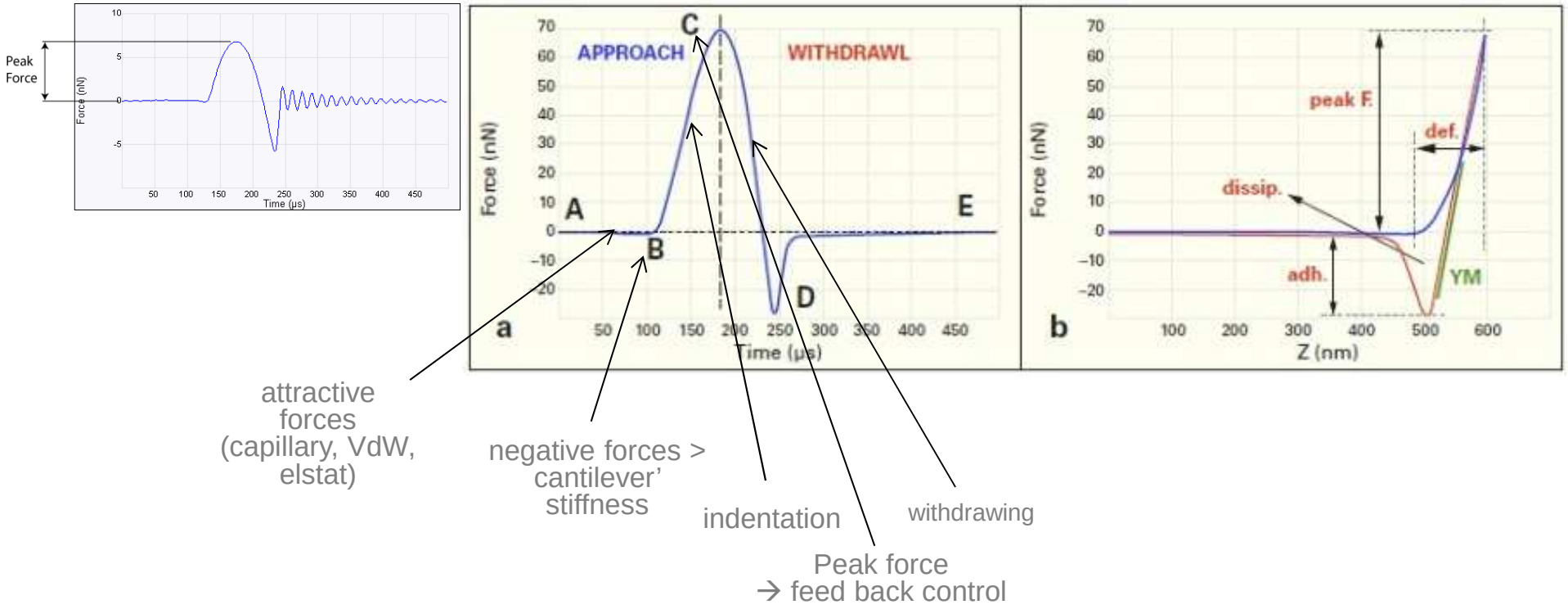




JPK NanoWizard 3

Fig. 10: *Herpes Simplex Virus capsid imaged in liquid, scan size 300 nm x 300 nm. a) Height image (z-range: 100 nm) shows substructure of the virus. b) In the adhesion image it is possible to detect the sticky virus (data range: 200 pN). c) the substructures can be also recognized in the elasticity image.*

Quantitative NanoMechanics (QNM)



PeakForce QNM = quantitative nanomechanical information (biological samples without damaging)

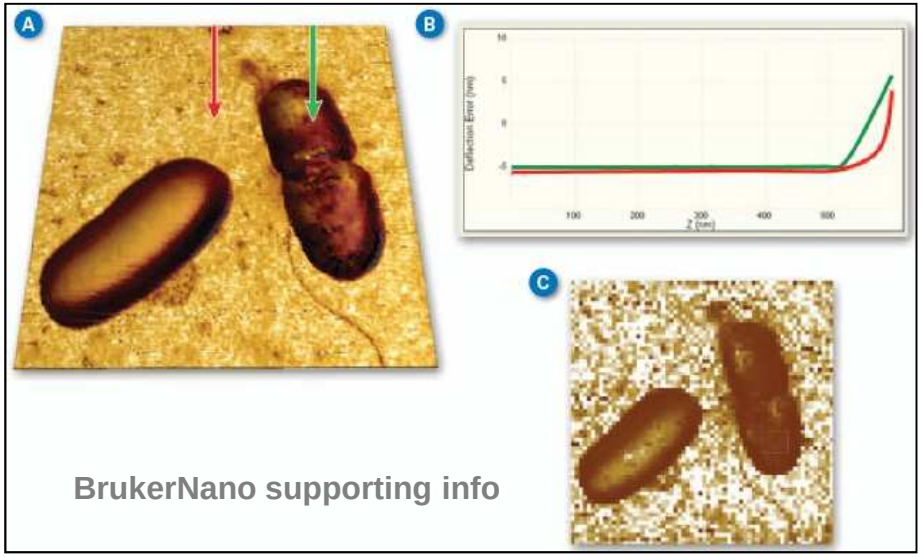
Based on **Peak Force Tapping technology** - probe is oscillated (~TappingMode), res. freq 1 - 8 kHz (=sampling rate) depending on the tool).

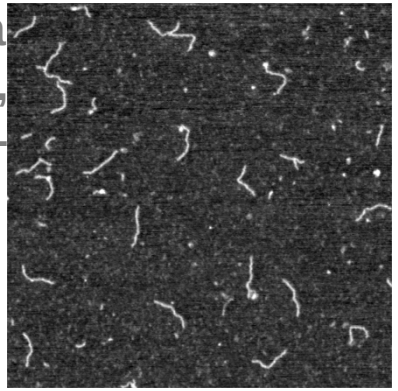
Difference:
Tapping Mode – const. amplitude,
Peak Force Tapping maximum peak force on the probe (much lower comparing to contact mode – biological samples)

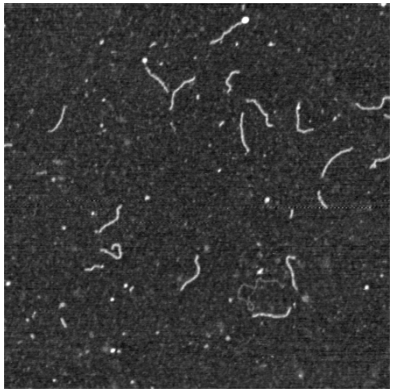
Bruker Dimension Icon/FastScan

DNA on mica

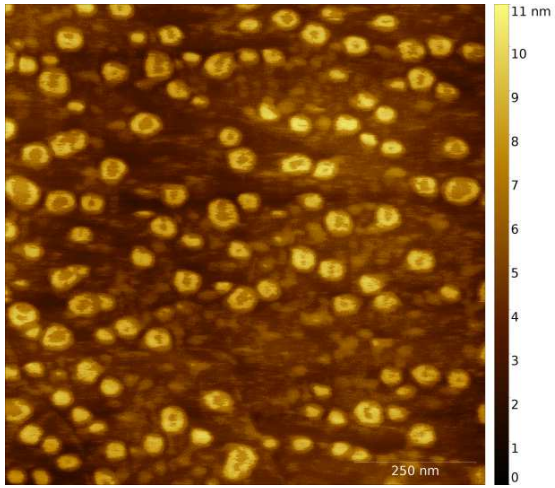
With **H. Kolarova, H. Zapletalova, UPOL**

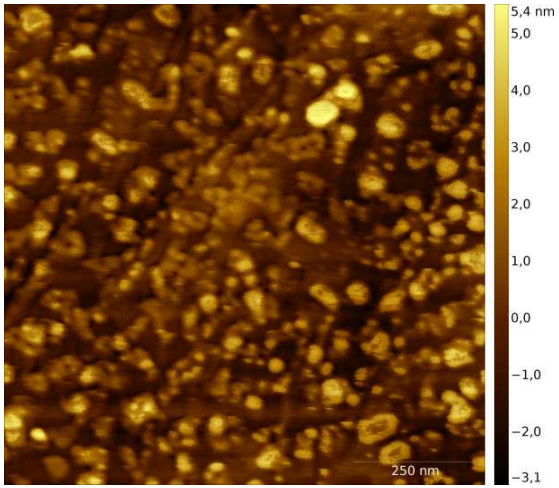


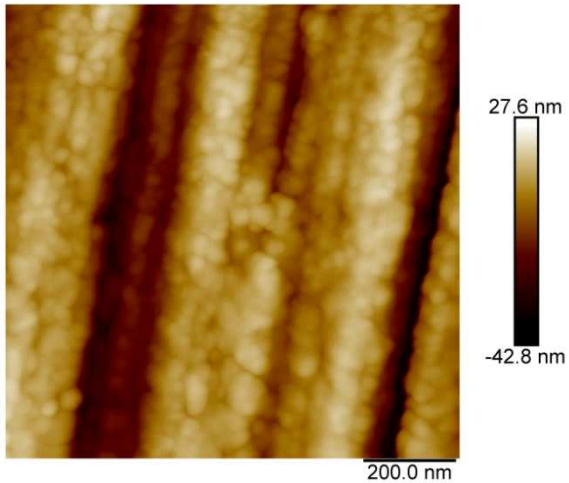




Lipozomes on graphite electrode







With **J. Vacek, UPOL**

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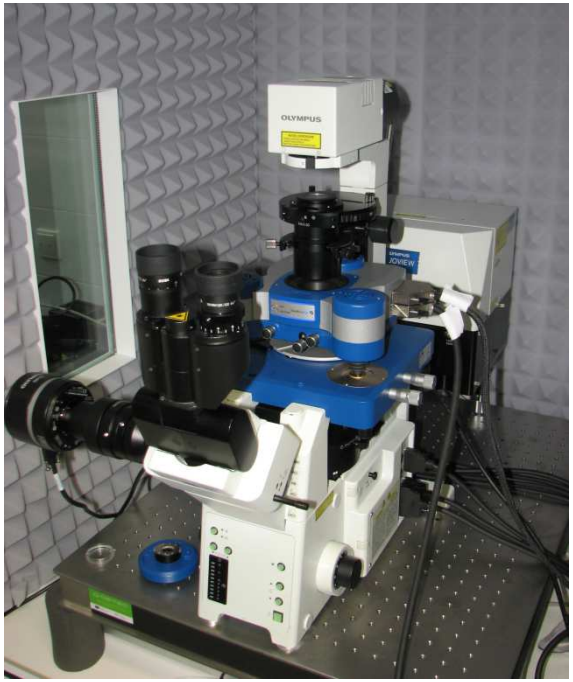
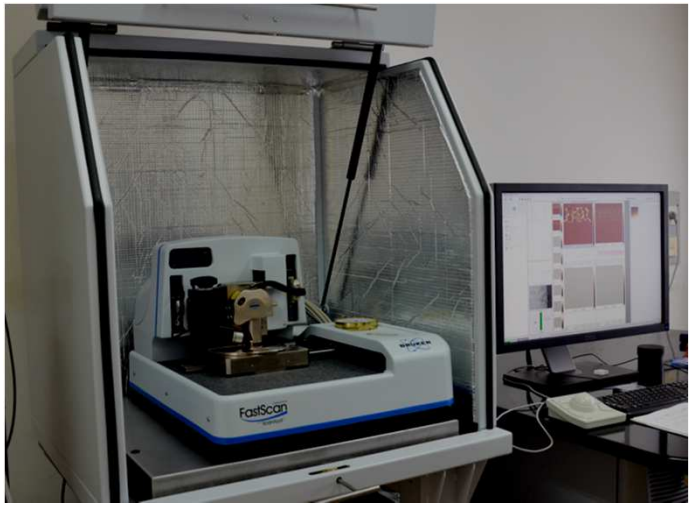
AFM CoreFacility
CEITEC MU

CEITEC AFM CoreFacility

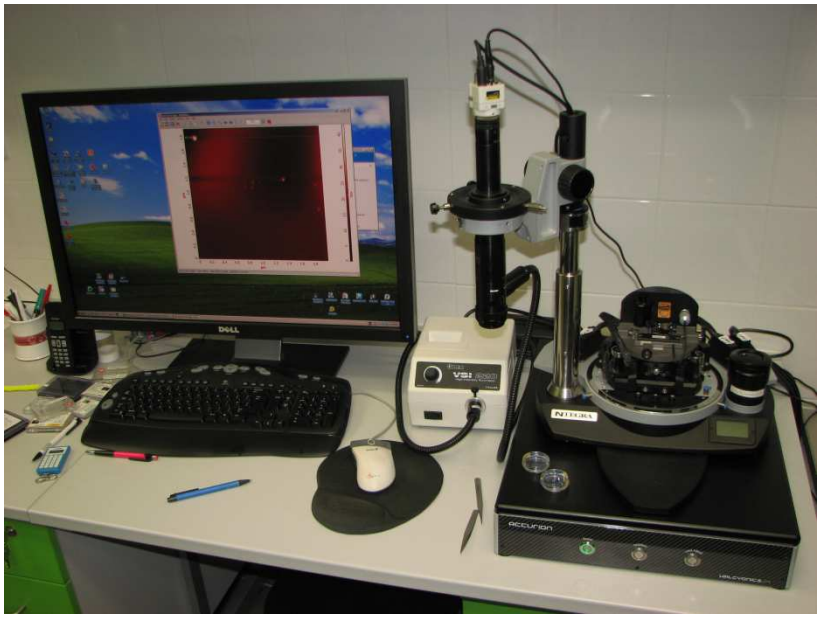
JPK NanoWizard3

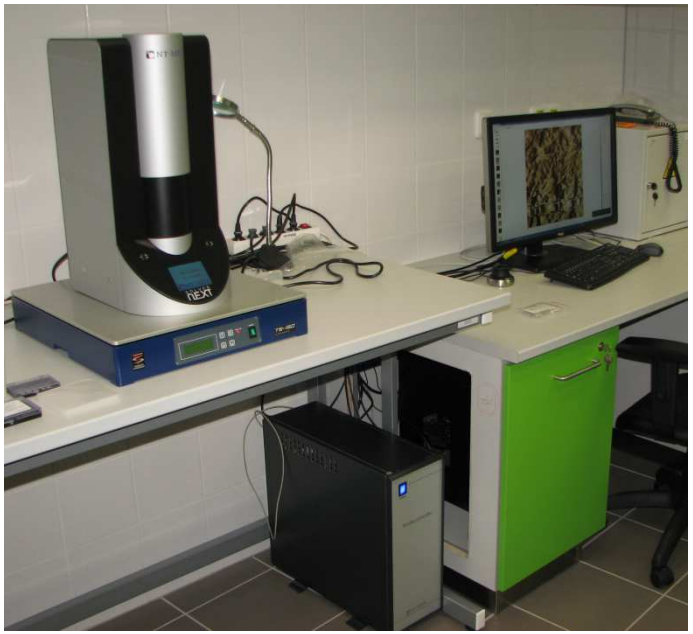




Bruker FastScan Bio

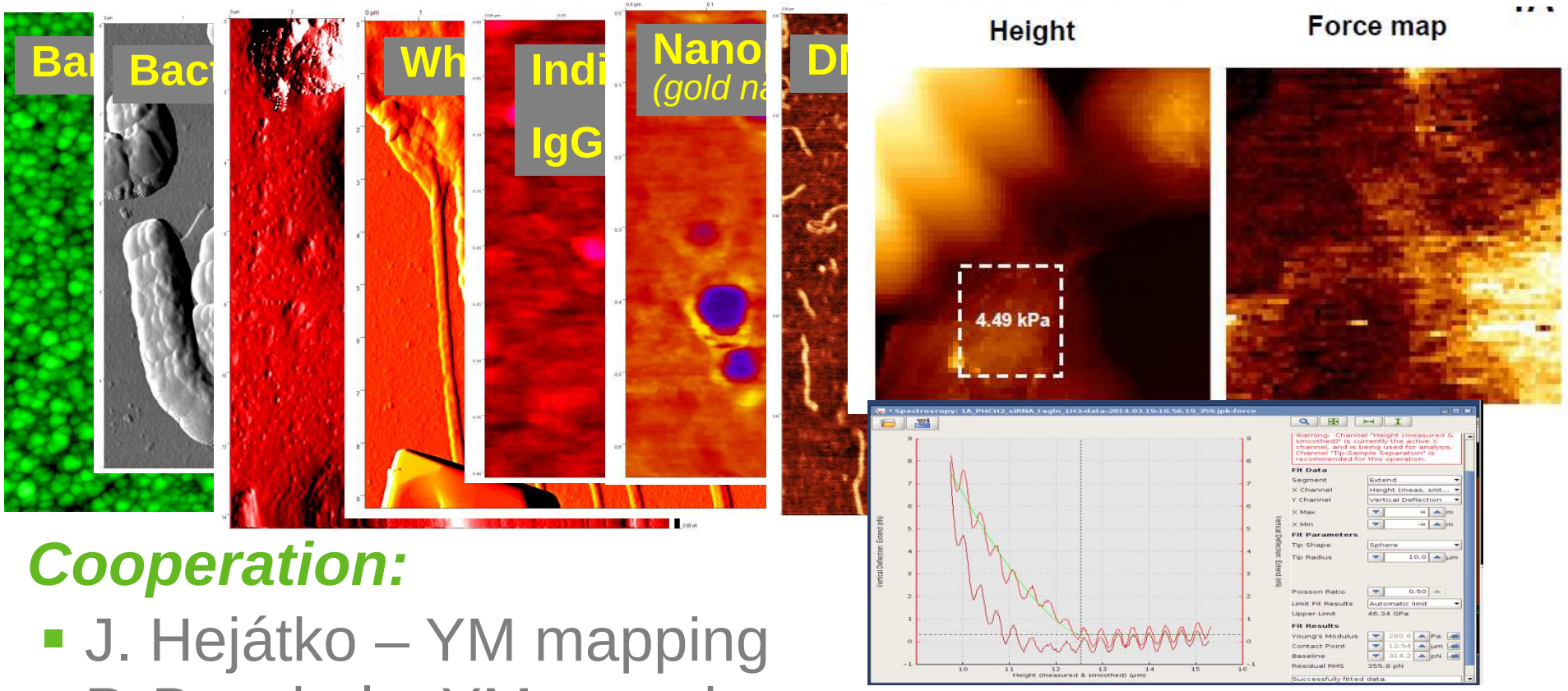
NTMDT NTgra Vita





NTMDT Solver Next

AFM visualization of biomolecules and bioobjects



Cooperation:

- J. Hejátko – YM mapping
- P. Bouchal – YM mapping
- J. Paleček - DNA
- M. Pešl, V. Rotrekl CMCs
- J. Sládková – CMCs
- A. Meli - CMC
- M. Kalbáčová – TiO2 NT
- H. Kolářová - DNA
- I. Crha - sperms

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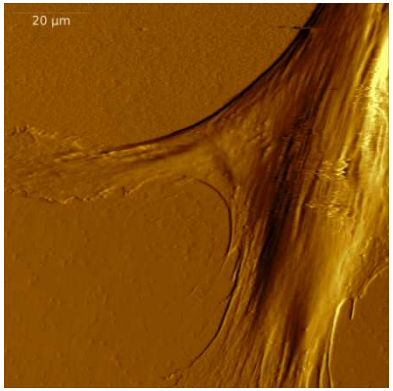
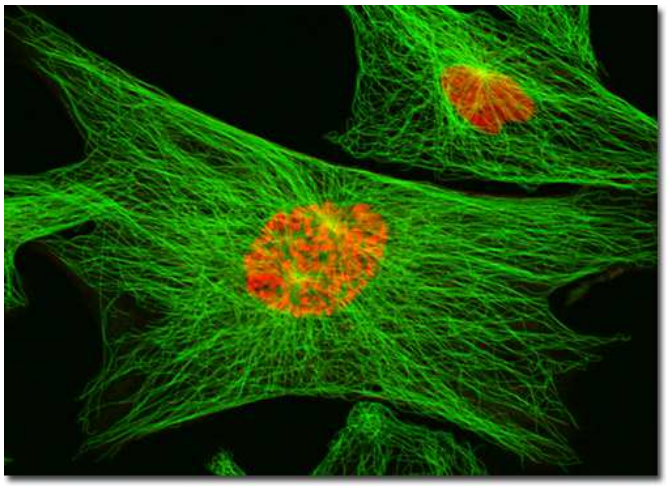
Optical microscopy

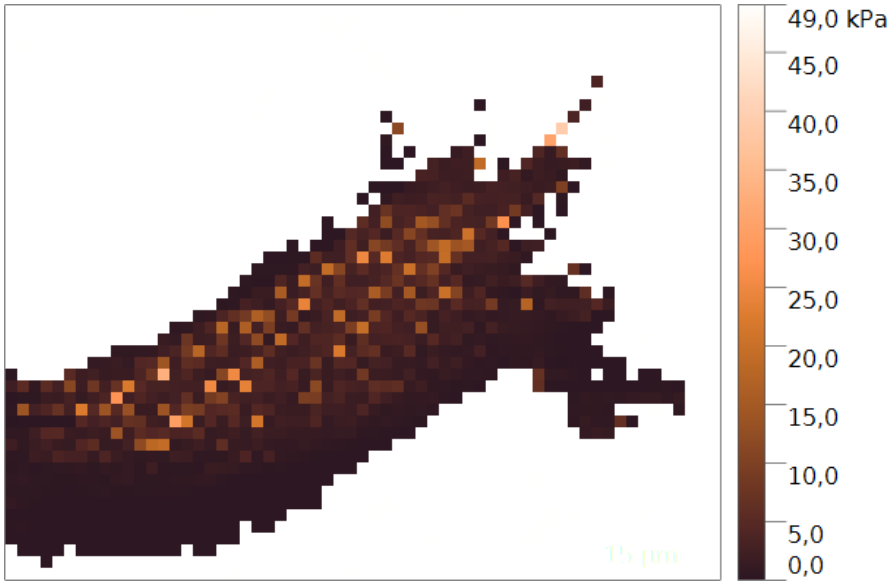
AFM



Confocal microscopy



Young modulus



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Děkuji za pozornost