APPLICATIONS OF ATOMIC FORCE MICROSCOPY IN THE BIOSCIENCES

GUEST SPEAKER:

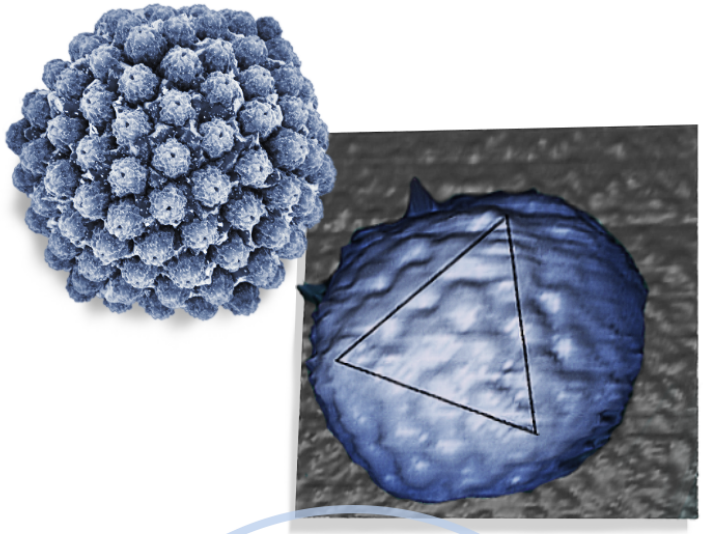
Sophia Hohlbauch
Senior Biological Applications Scientist, Asylum Research

May 21, 2020 11am PDT

COVALENT METROLOGY

OXFORD INSTRUMENTS

Asylum Research



COVALENT ACADEMY

Advancements in Instrumentation Series

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Covalent Metrology



- **Founded 2016**
- **Testing, measurement & characterization Platform**
- **30 team members (13 PhDs)**
- **9,500 ft² lab in Sunnyvale**
- **1-Stop-Shop Source for Answers**
  - 30 instruments in-house
  - 6 partnerships with instrument makers
  - 11 partner labs
  - 6 corporate "tool shares"
- **>275 customers** (80% repeat)

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Covalent Technical Groups and Organization

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hello@covalentmetrology.com

Electron & Scanning Probe Microscopy

(SEM/TEM, AFM, EDS, EELS, Nanoindent, acoustic microscopy)

Surface Analysis

(XPS, Auger, ToF-SIMS, dSIMS)

Optical Microscopy & Spectroscopy

(Laser Confocal, White Light, Chromatic Aberration, Spectral Ellipsometry, UV-Vis-NIR Spectroscopy)

Materials/ PC Board FA

(Cross Sections, Inspections, Dye & Pry)

X-Ray Characterization

(XRD/XRR, XRF, µCT)

Chemical Analysis

(ICPMS, GCMS, FTIR, Raman, Solid & Solution NMR, 2D NMR)

Nanoparticle Analysis

(DLS, PSA, Particle Zeta Potential)

Bulk Properties Characterization

(DSC, TGA, DMA, Rheometry, Tensile Test, Pencil Test, Surface Zeta Potential)

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COVALENT METROLOGY

- Partnership established May 2020
- Collaboration will focus on:
  - Biological Measurements
    - Structural and nanomechanical measurements on biological samples including cells, proteins, bacteria, DNA, RNA, lipids.
  - Nanoelectrical Measurements
    - Pierzoresponse Force Microscopy (PFM)
    - Conductive AFM (CAFM)
    - Scanning Capacitance Microscopy (SCM)
  - Nanomechanical Measurements
    - Stiffness/modulus mapping
    - Viscoelastic mapping
    - Indentation Measurements

Other Partners

Rigaku

PVA TePla

J.A. Woollam

Anton Paar

KEYENCE

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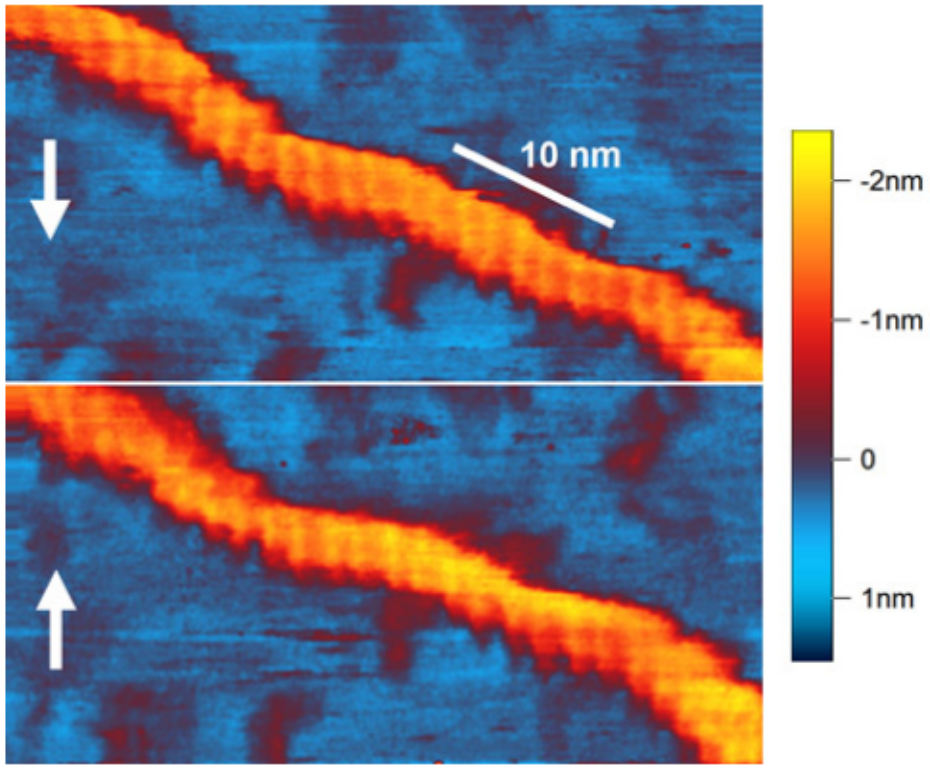
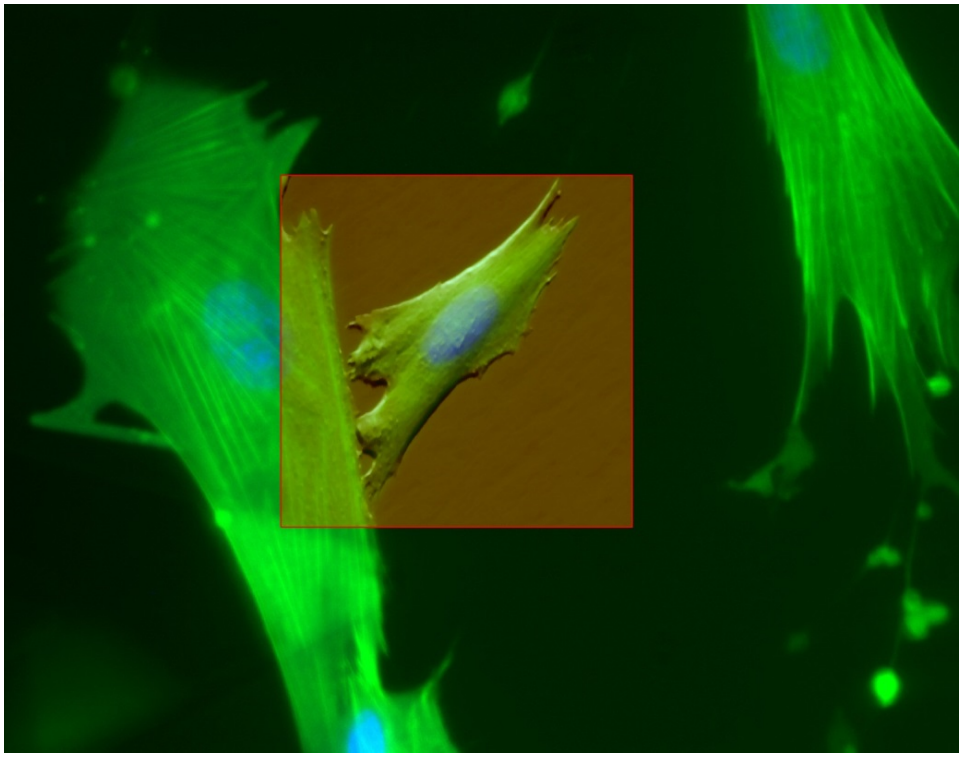
- Sophia Hohlbauch has over 20 years of AFM experience specializing in the life sciences. She is currently a Senior Biological Applications Scientist at Asylum Research and previously held a similar position at Digital Instruments / Veeco. She has presented on AFM topics at numerous biology and AFM meetings worldwide.

Atomic Force Microscopy (AFM) in Biology: From Molecules to Cells





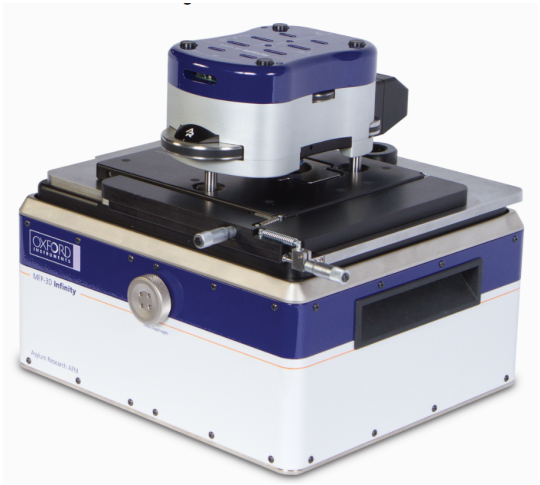
Outline

- Applications, Advantages
- Principles of AFM
- Probe Selection
- Sample Prep
- Examples

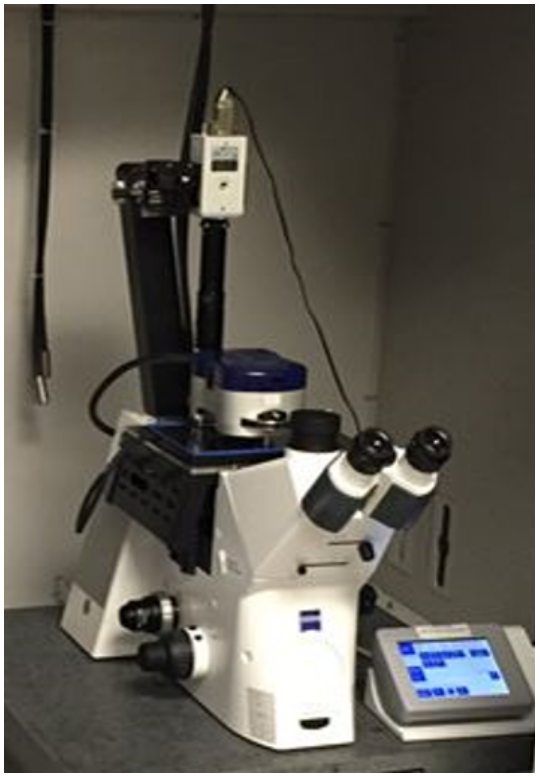
Asylum Research Products

Power and Flexibility in Our Systems









Broad Spectrum of Applications

Imaging techniques

- Molecules: proteins, nucleic acids,…
- Molecular assemblies: LB films, membranes, fibrils…
- Molecular interactions: proteins/proteins, proteins/DNA…
- Cells, virus, bacteria
- Tissues (bones, teeth, cornea…)

Non-imaging techniques

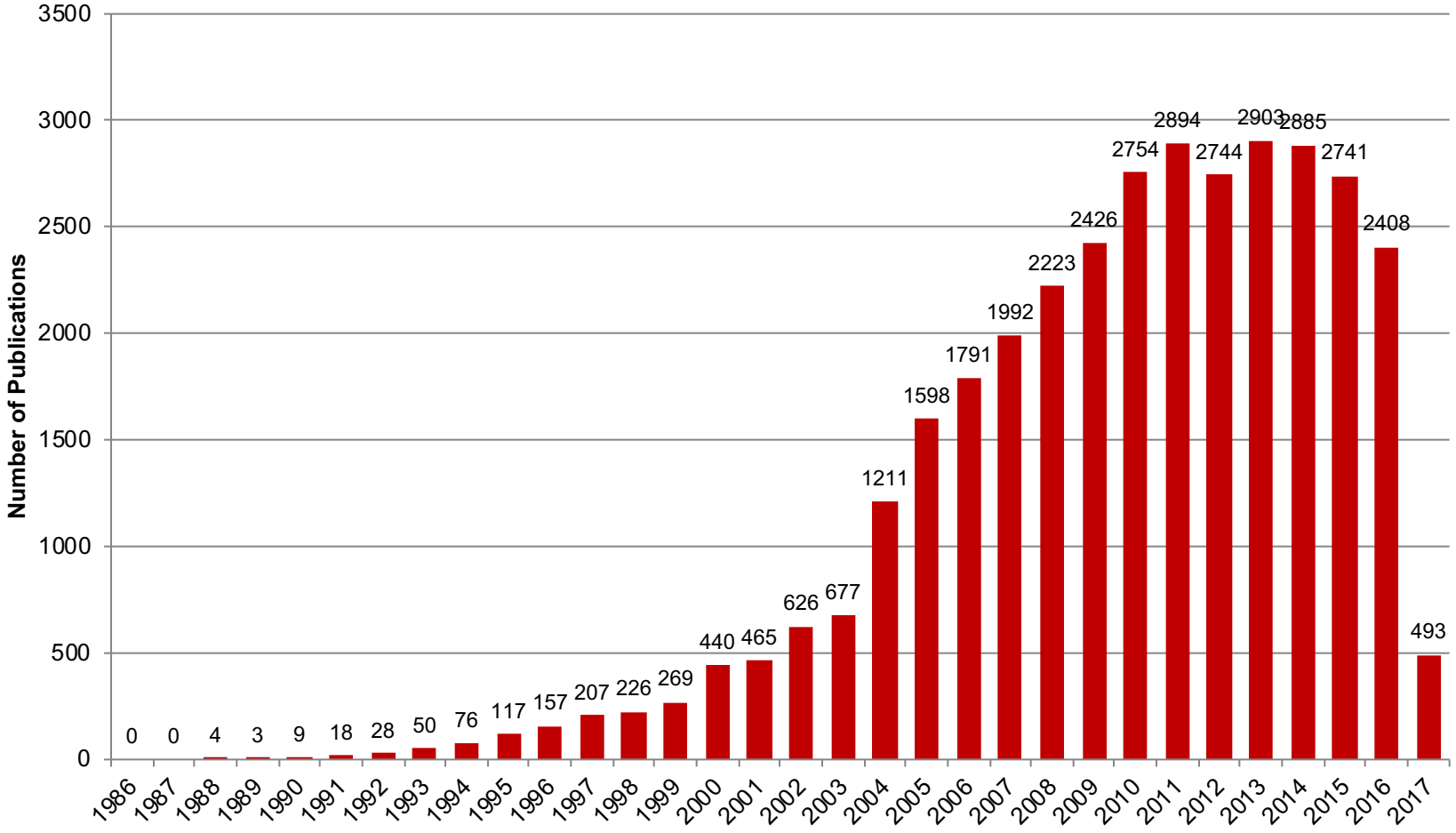
- Force measurements: pushing and pulling, elasticity
- Lithography

Advantages of AFM

- Works in liquid, at biologically relevant temperatures
- Relatively non-destructive: dynamic imaging
- Images in 3 dimensions
- No fixing, dehydrating, staining, freezing, coating, labeling…
- High resolution (0.6 nm in Z, ~nm in X and Y)
- Assessment of samples mechanical properties (force measurements, phase imaging…)
- Small instrument, with no maintenance

AFM Research

PubMed Citations per Year
Query: “atomic force microscopy”, limited by year
* 2017 publications through 2/22/17



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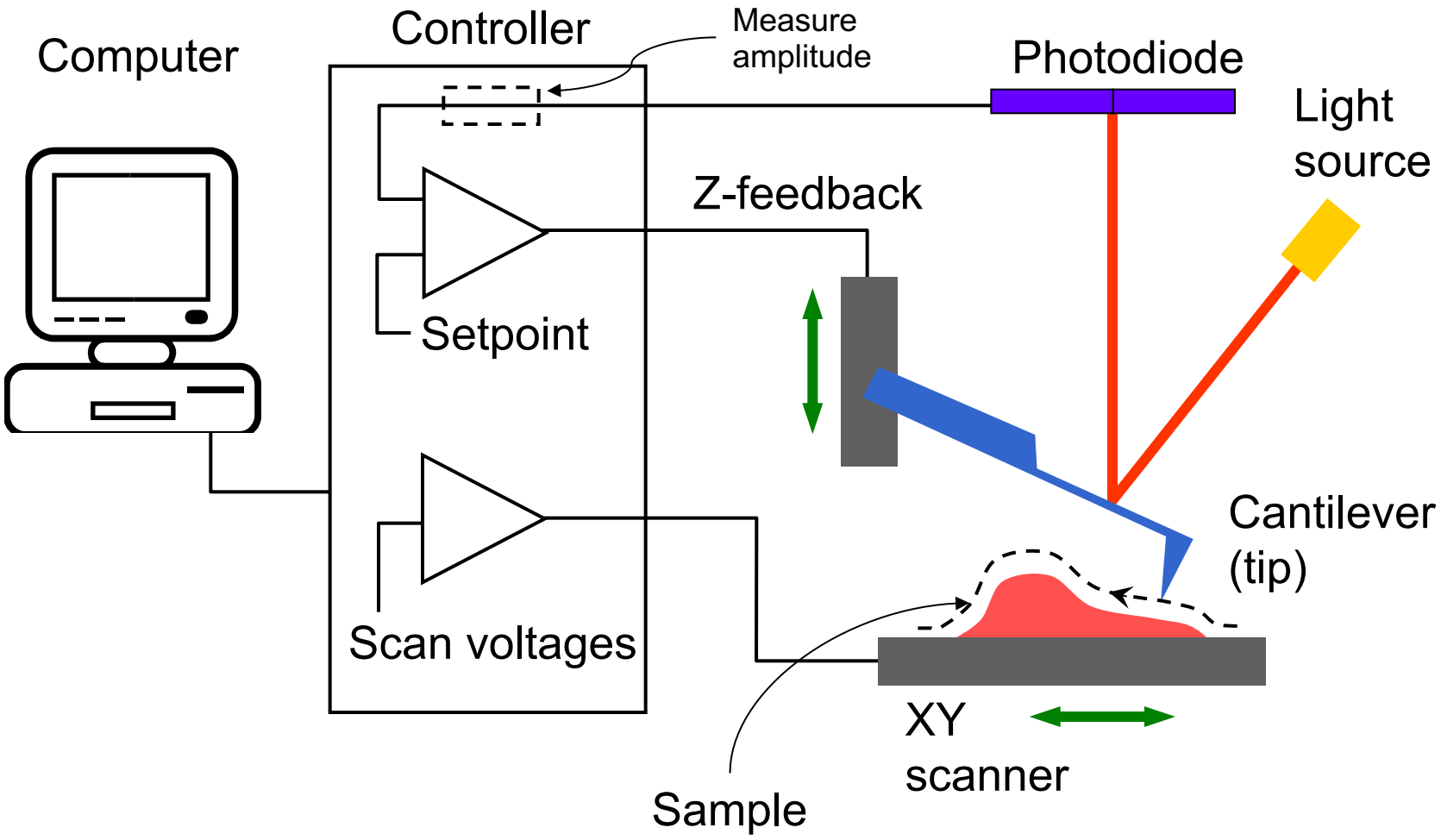
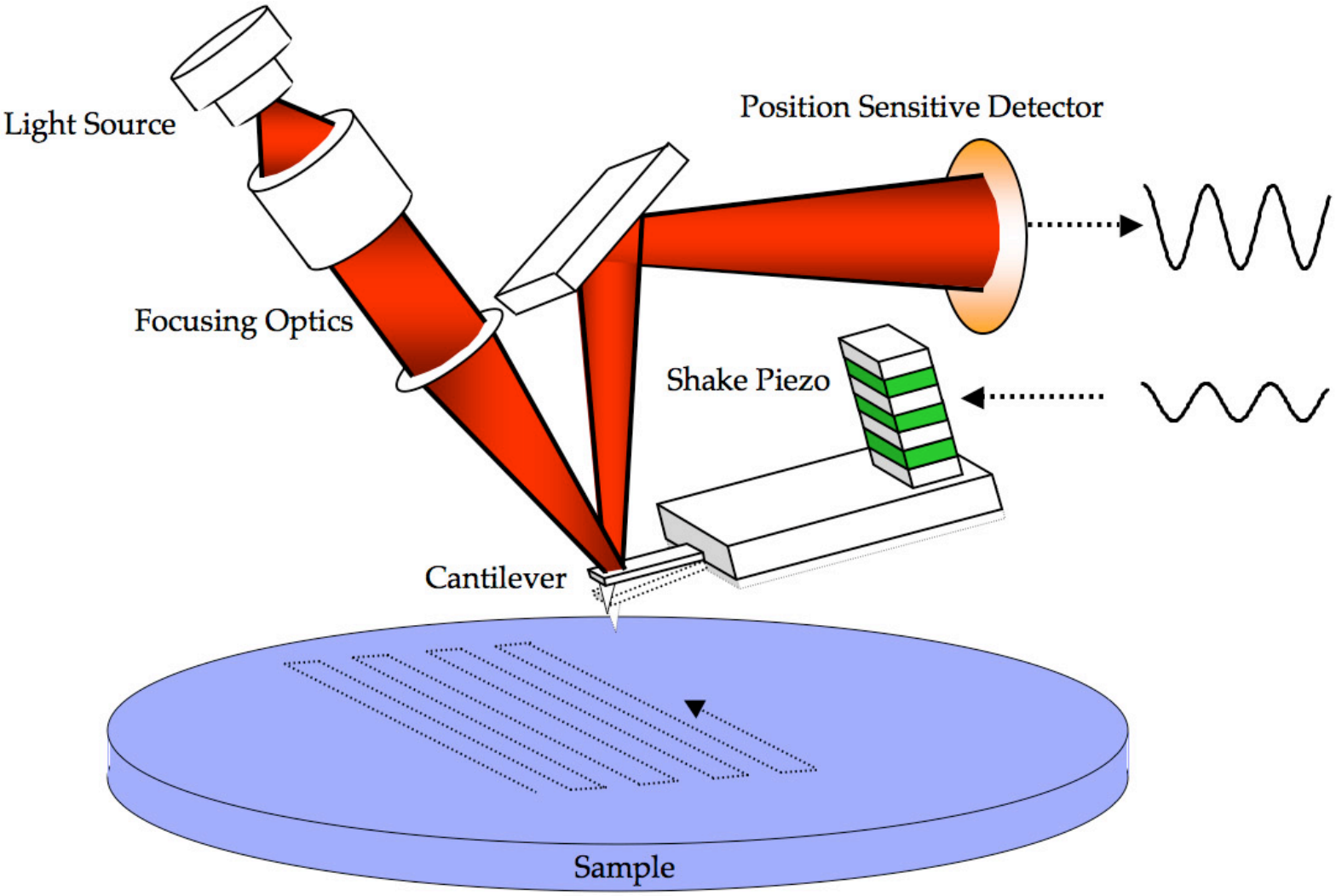
Principles of AFM



AC Imaging


Light Source
Position Sensitive Detector
Focusing Optics
Shake Piezo
Cantilever
Sample

Force Measurements

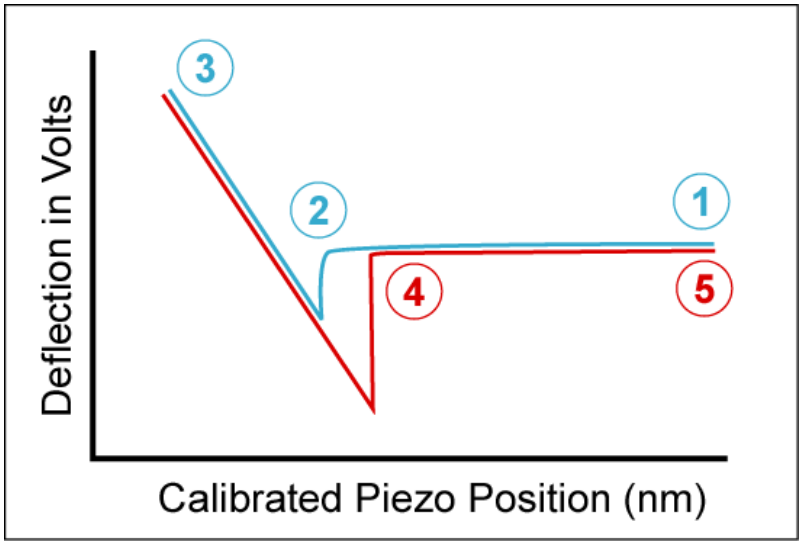
Hooke's Law:

$$F = k * x$$

F = force

k = spring constant/stiffness

x = displacement of the spring



Applications

Inter and intra molecular forces

e.g. unfolding forces of biomolecules, molecular interactions

Hardness assessment of surface

e.g. elasticity of living cells

Acquiring the Best Images: How to Do It?

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Appropriate substrate:

Should be atomically flat, such as gold, mica, graphite

+

Proper sample preparation:

varies depending upon type of sample

+

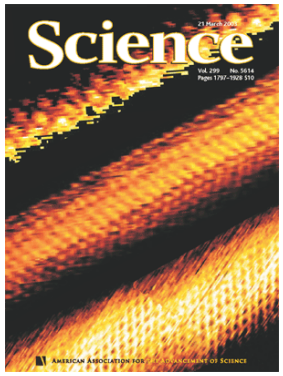
Appropriate tip selection:

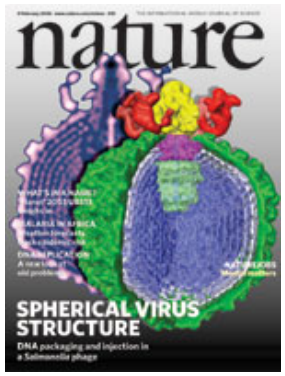
Fluid vs. air

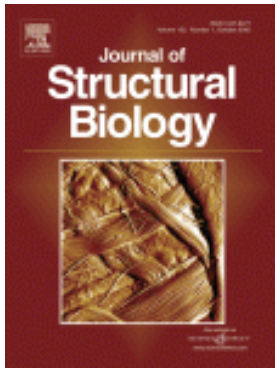
Contact Mode vs. AC Mode

=

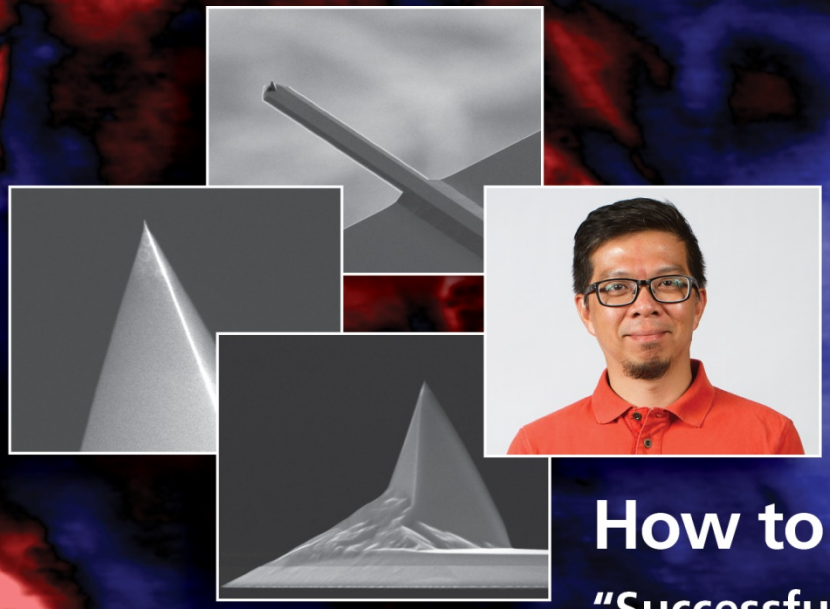
Publishable AFM data







AFM
Asylum Research



How to Choose the Right AFM Probe

"Successful AFM imaging starts with the probe."

PRESENTER: F. Ted Limpoco, Ph.D., Chemistry
Applications Scientist, Oxford Instruments Asylum Research

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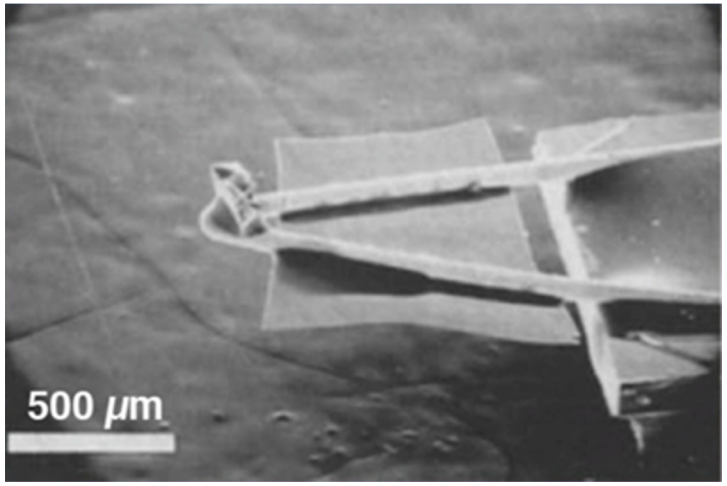
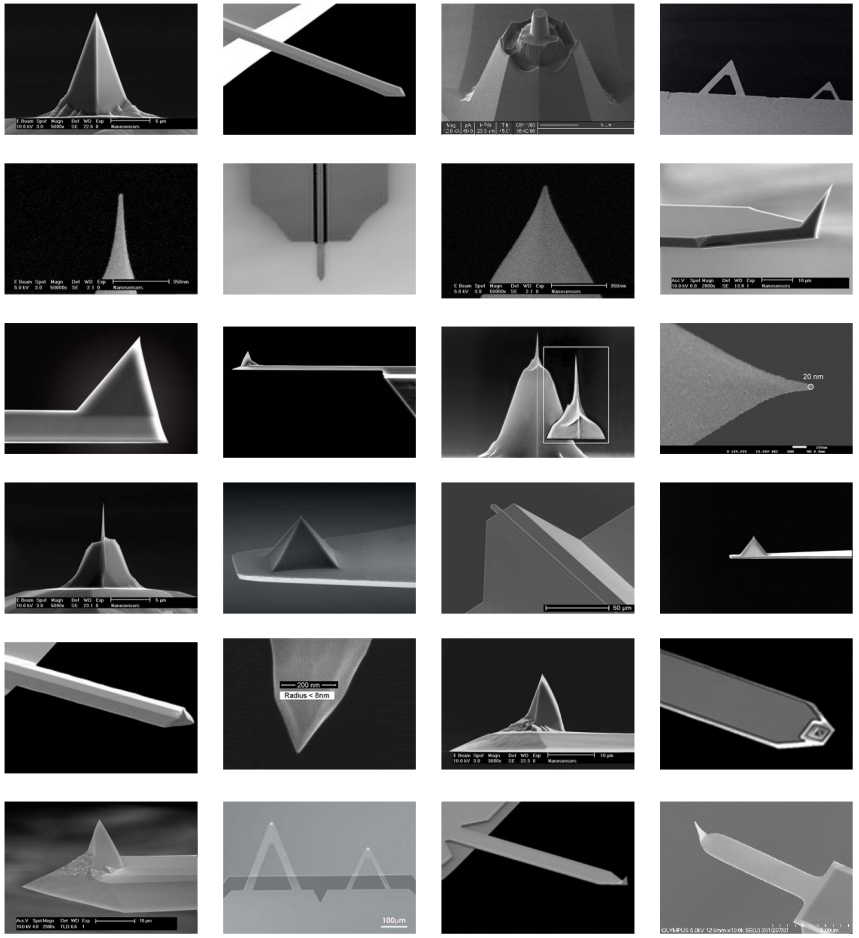
So many tips, so little time...



Very early AFM probe (1986-1990)

- Bent fine wire glued to a substrate
- Metallized glass mirror
- Crushed diamond shard glued to the end of lever



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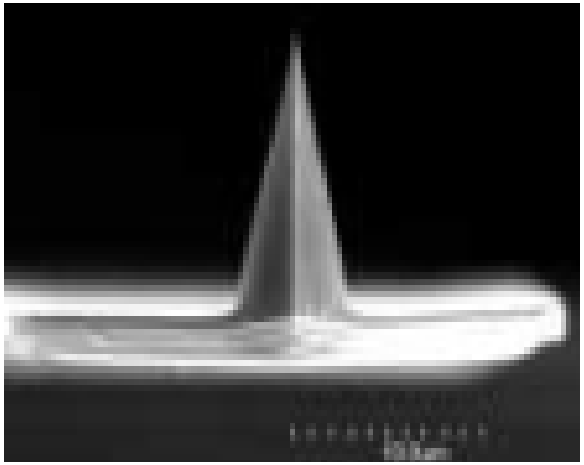
Cantilevers

Silicon

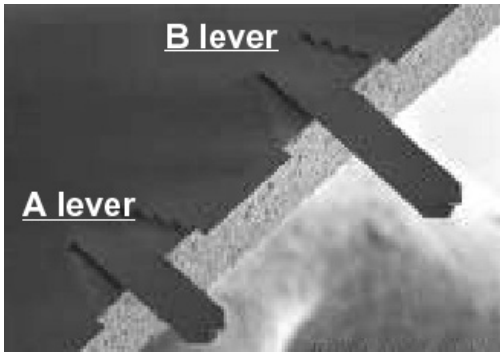
- For: AC mode air
- Typically are not problematic



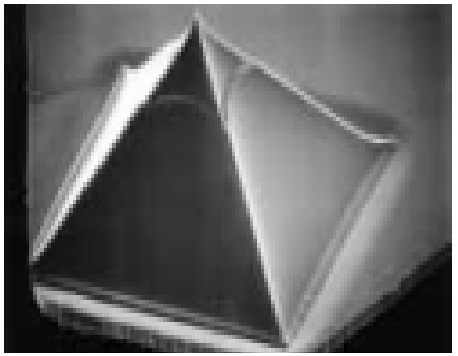
Olympus AC240TS

Silicon Nitride

- For: 1) AC mode in liquid
  2) Contact mode in air/liquid
- Vary a lot with vendors



Olympus Biolevers



Olympus TR400PSA

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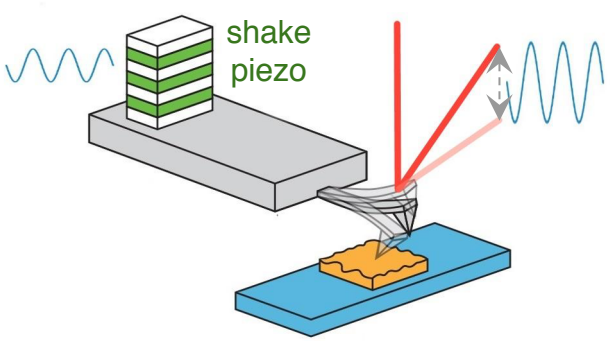
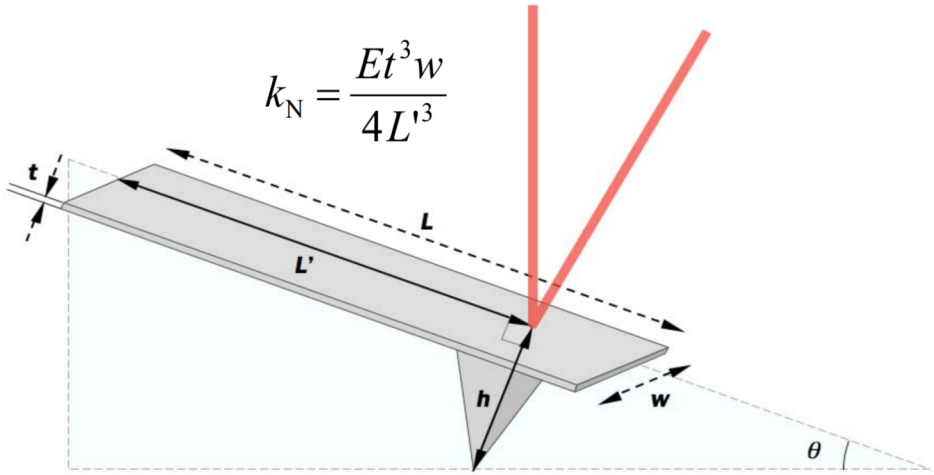
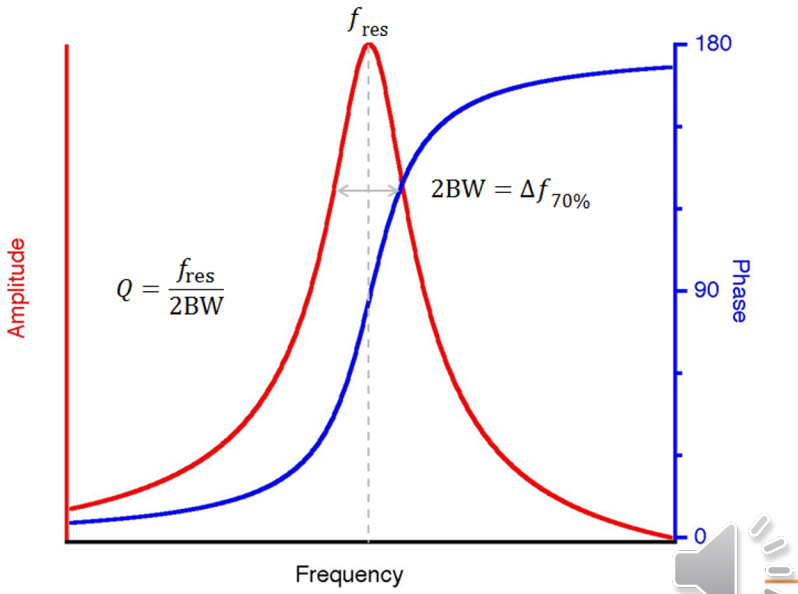
Key Probe Parameters

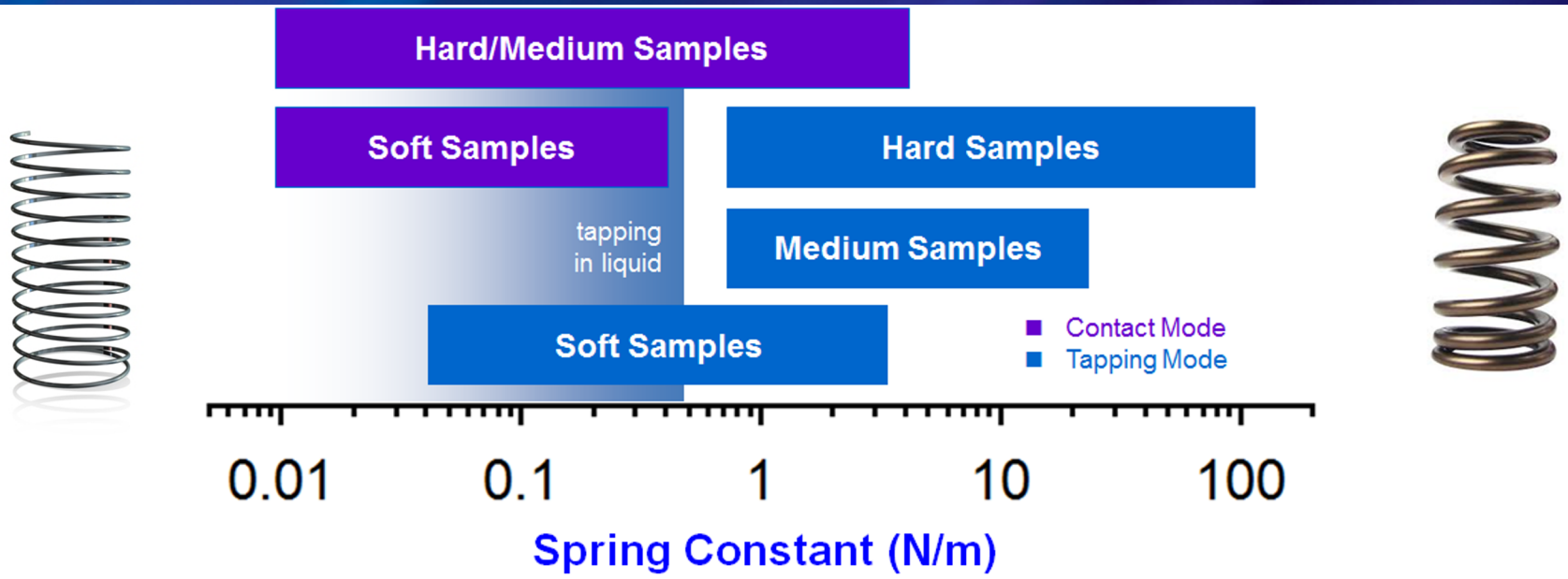
Spring Constant – k	Imaging force
Tip Sharpness – r	Lateral resolution
Resonance Frequency – f	Imaging speed
Quality Factor – Q	Imaging speed







Spring Constant (k)



- Match cantilever to sample stiffness, esp. for nanomechanics.
- The more compliant the cantilever, the less the tip will wear.
- Tapping in air: $k >$ ~0.5 N/m, to avoid tip getting trapped by adhesion with sample.
- If sample is sticky, increase k.
- If sample is loosely-bound or not well-attached, decrease k.

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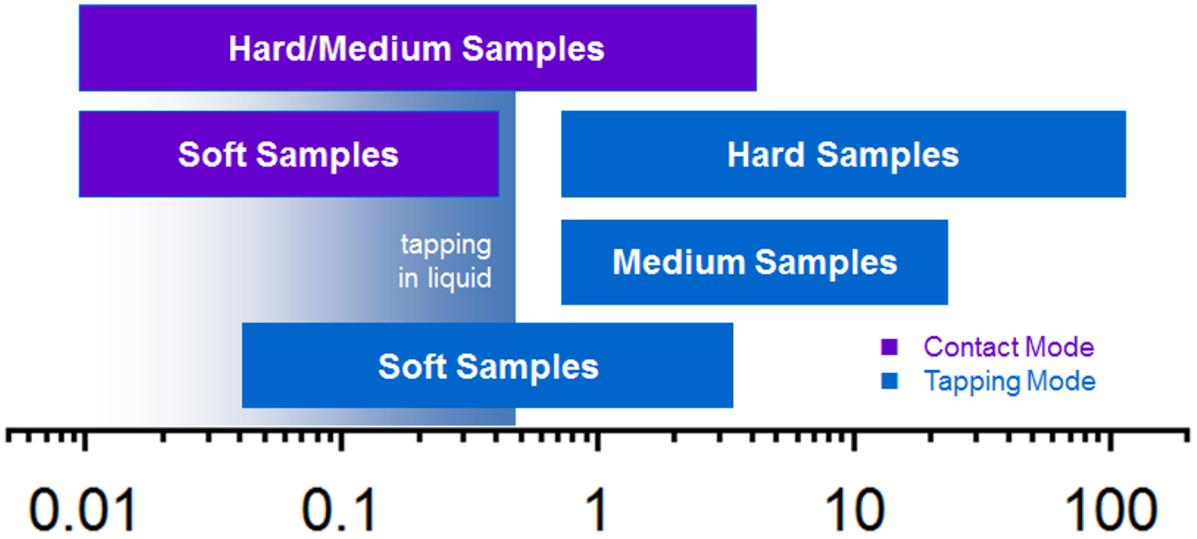
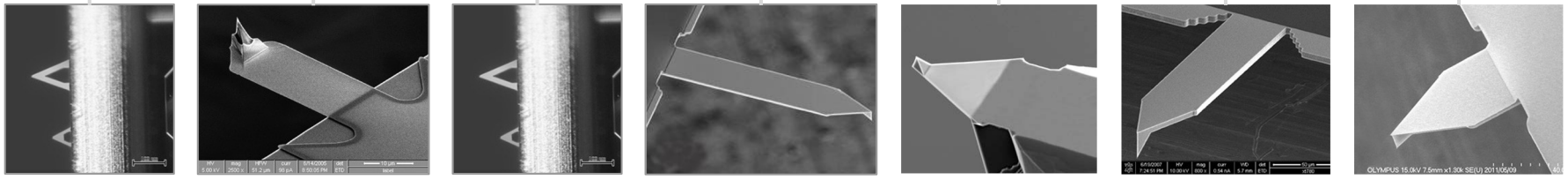
Spring Constant (k)









TR400 long	Biolever Mini (BL-AC40)	TR800 short	AC240	Arrow UHF	AC160	AC55
cells	**DNA, proteins**	**proteins**	**thin films polymers**	**calcite**	**thin films polymers**	**thin films polymers**

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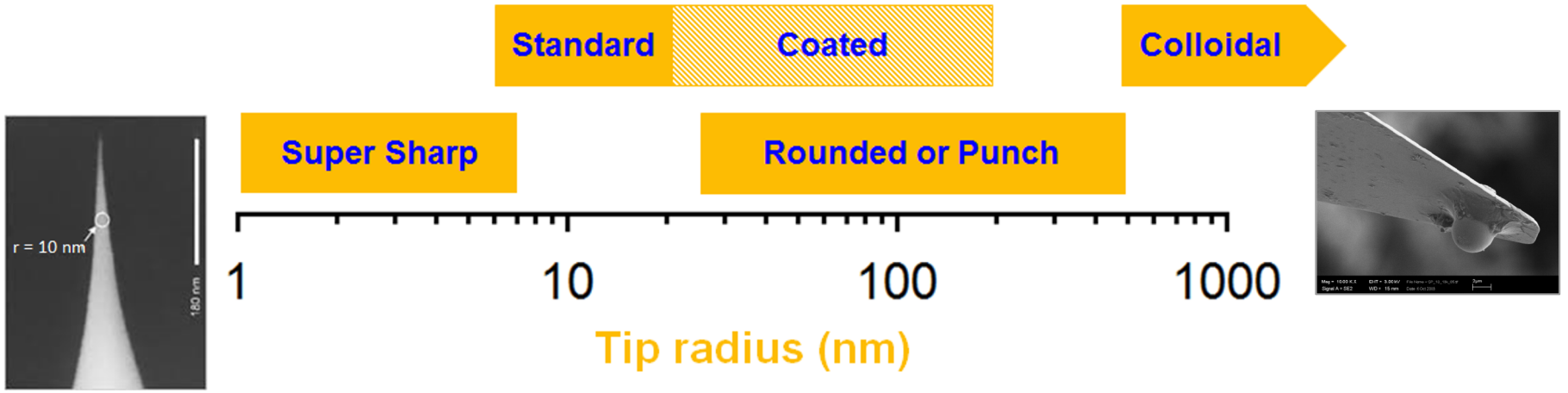
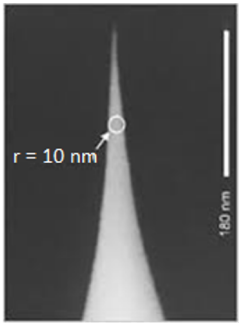
Tip Radius (r)



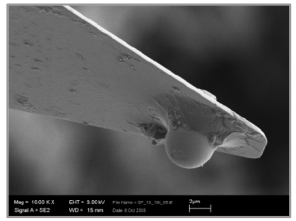
- Tip sharpness sets the lateral resolution of the image.
- Use super sharp tips ($r \approx$ 1-2nm) only when needed – $$$.
- Molecular and atomic point-defect resolution is possible with an atomic asperity at the end of the tip.
- Larger tips ($r >$ 20nm) may be preferred for some soft biological samples (e.g. cell membranes) to prevent tearing.
- Larger tips of “known geometry” may be preferred for nanomechanics.
- Avoid using cantilevers with aluminum coatings when imaging in fluid.

Tip Radius (r)

Standard | Coated | Colloidal

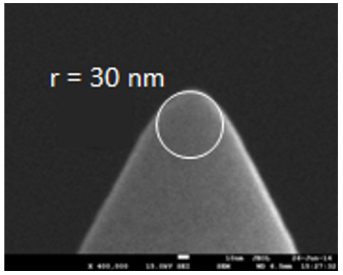


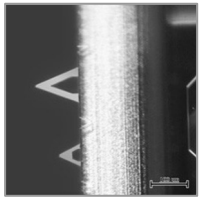
Super Sharp | Rounded or Punch



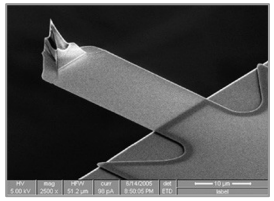
1 10 100 1000

Tip radius (nm)

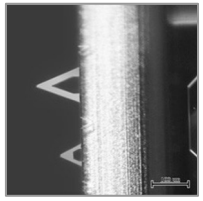




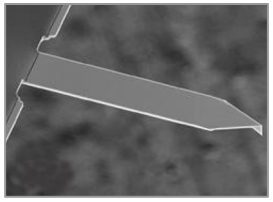
TR400 long



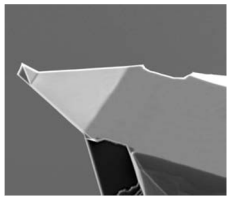
Biolever Mini (BL-AC40)



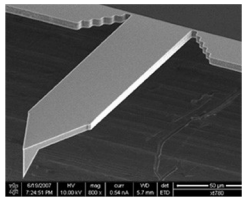
TR800 short



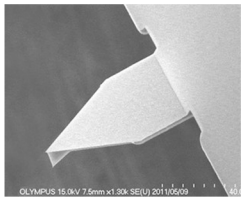
AC240



Arrow UHF



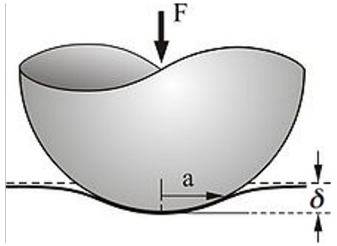
AC160



AC55

consider contact pressure!

100 nN



10 nm = ~GPa

1 μm = ~MPa

(for a glass sphere on a glass flat)

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Tip Radius

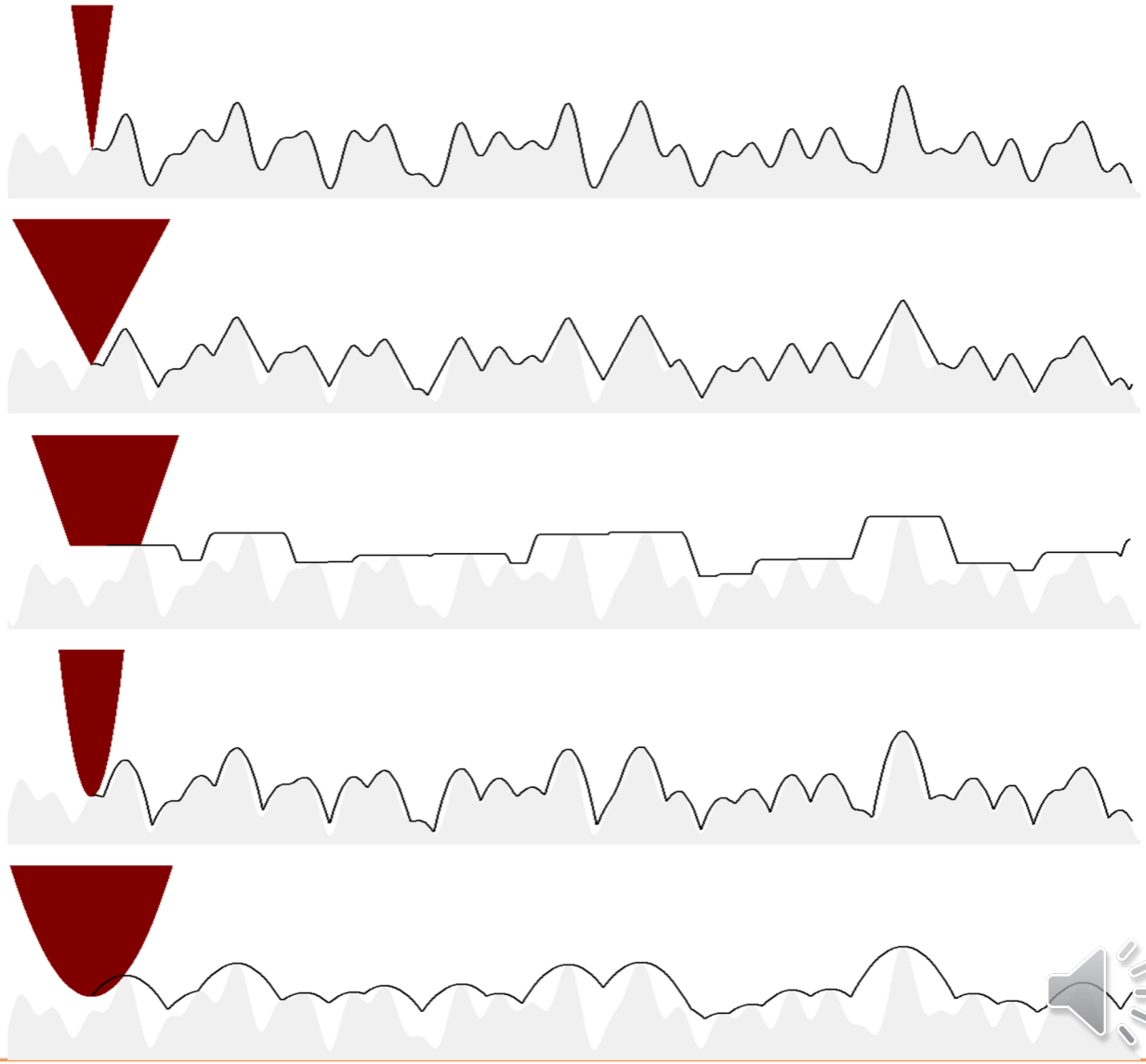
Narrow Pyramid
("super sharp tip")

Wide Pyramid
("typical tip")

Truncated Pyramid
("broken tip")

Narrow Parabola
("typical tip")

Wide Parabola
("rounded or blunt tip")



Courtesy of Joe Griffith

Sample Preparations

- Problem: Functionality of adsorbed molecules?
- Techniques derived mostly from EM and optical microscopy
- Most of the time: Molecules are dissolved in aqueous buffers and deposited onto the substrate.
- But almost every sample requires a unique approach.

Summary: Substrates + Samples

- Requirements:
  - Atomically flat surface (exception: cell imaging)
  - Surface charge: positive, negative
- Substrates:
  - mica (negatively-charged) – DNA, proteins
  - AP-Mica (Modified with Aminopropyltriethoxysilane, positively-charged): more rough surface – DNA, proteins
  - Graphite: hydrophobic – proteins
  - Gold – molecular pulling experiments
  - Glass coverslips – cells
  - Polystyrene petri dishes – cells

Examples



Nucleic Acids



Measurements on Nucleic Acids

- Length
- Conformation
  - Persistence length
- Protein binding
  - Location
  - Bending induced by bound factor
- Force measurements
- Measurements are individual vs. the statistical measurements in a gel

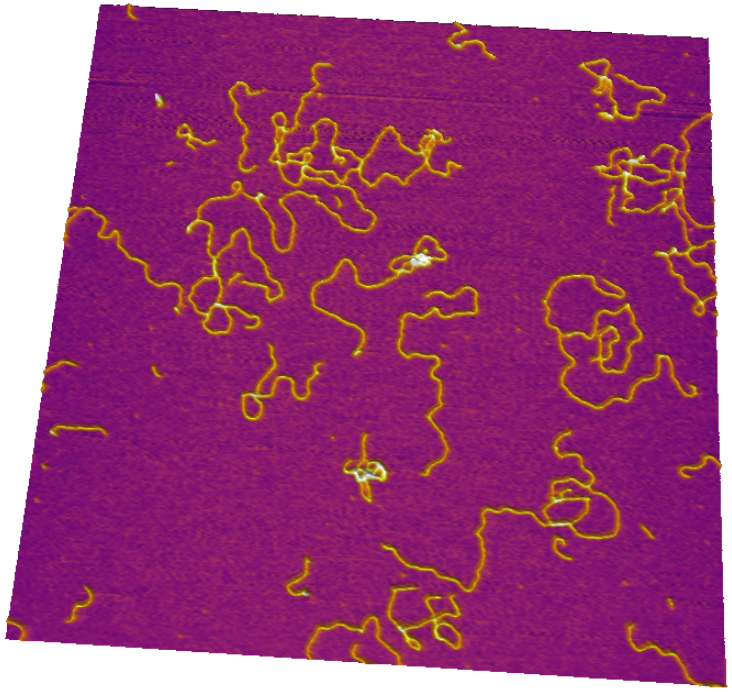
Sample Prep: DNA (Expt #1)

Imaging Conditions:

- Concentration: 1-5 µg/ml
- Substrate:
  - mica (negatively-charged)
  - AP-Mica (positively-charged)
- Buffer: contains divalent cations

  ie- 40mM HEPES-HCl, 5mM $NiCl_2$,

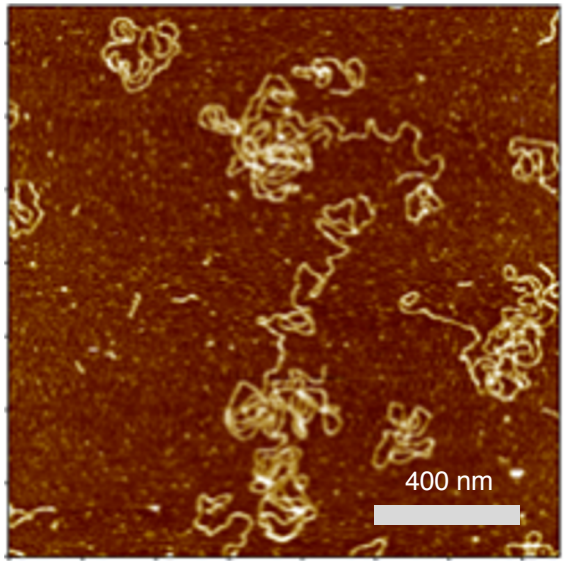
  pH 6.6-6.8
- AC Mode in air or fluid

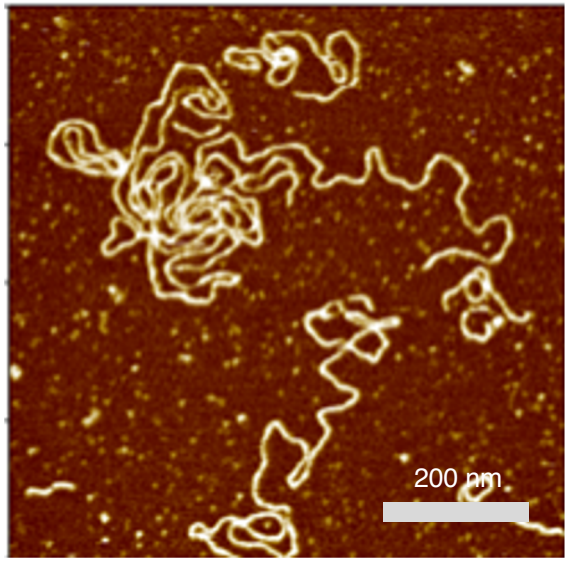


Protocol:

- Incubate the DNA solution on mica for 5-10 minutes, rinse with buffer to remove unbound molecules.

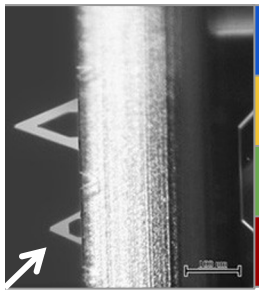
DNA in Liquid



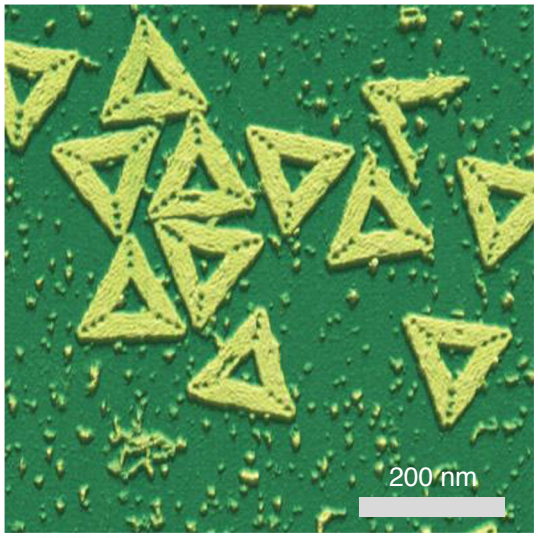


DNA molecules, 2 Hz.

MFP-3D-BIO, TR400PB



k	0.09 N/m
r	32 nm
f	32 kHz
Q	41

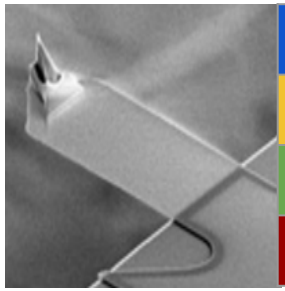


DNA Origami





MFP-3D-BIO Biolever Mini (BL-AC40TS)



k	0.09 N/m
r	8 nm
f	110 kHz
Q	

Low spring constant silicon nitride probe for **tapping mode** in fluid.

Caveat: tricky to use on MFP-3D-BIO

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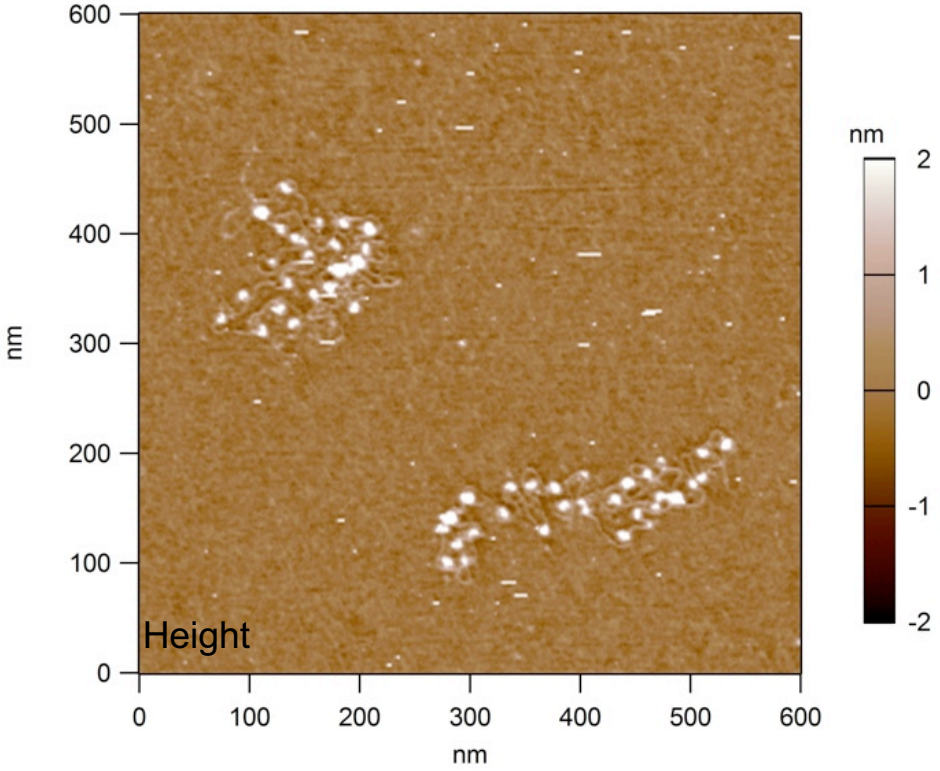
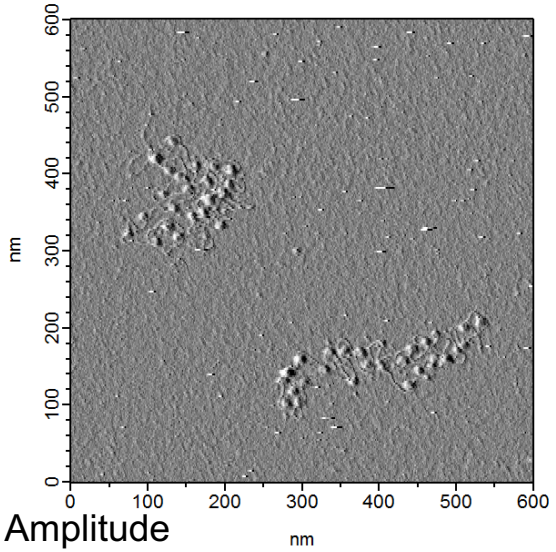
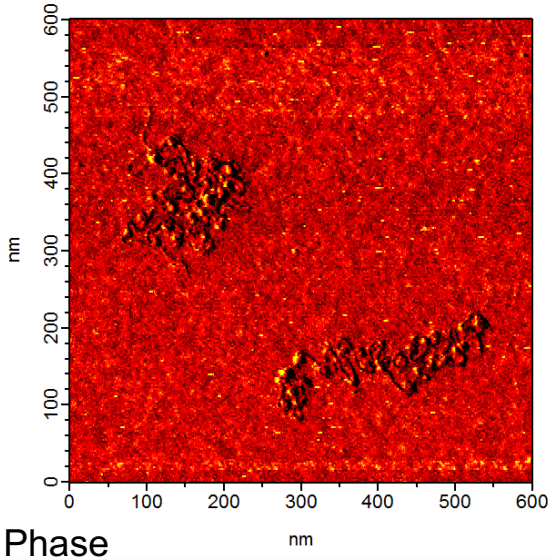
Chromatin

DNA with attached Nucleosomes







Chromatin in buffer, immobilized on polylysine imaged with a scan rate of 2.4Hz with the Biolever mini

Sample courtesy Prof. Jens Michaelis

DNase Cleavage of Lambda Digest DNA

625 Hz with 320×64 pixels using an Olympus AC10 probe for a frame rate of 8.7 fps

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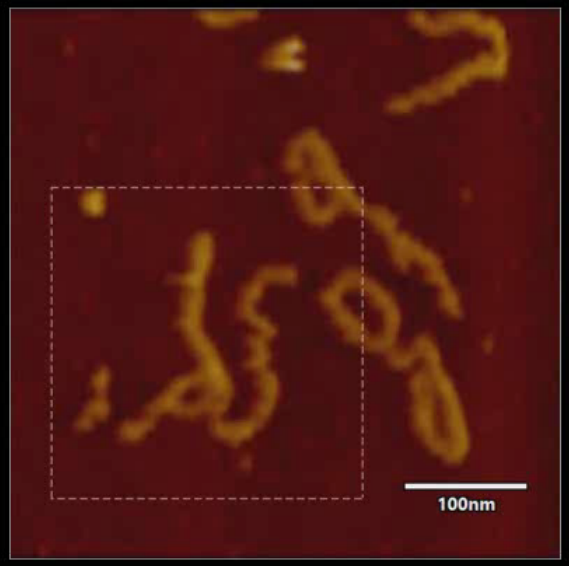
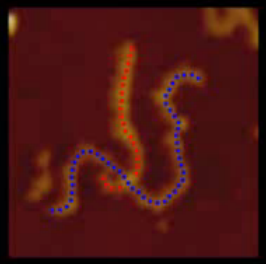
Cypher VRS

1) DNA is loosely adsorbed onto mica
2) DNase1 is a non-specific DNA cleaving enzyme
   - DNase is perfused into the imaging chamber
   - DNase attaches to DNA and cleaves it



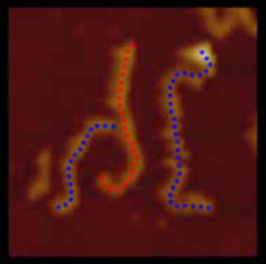




Time = 5.4 s
Two overlapping strands of linear DNA



Time= 6.7 s
DNase1 enzyme binds to one of the strands



Time= 7.9 s
DNA strand is cleaved at the site of binding



Now playing in real-time @ 8.7 frames per second

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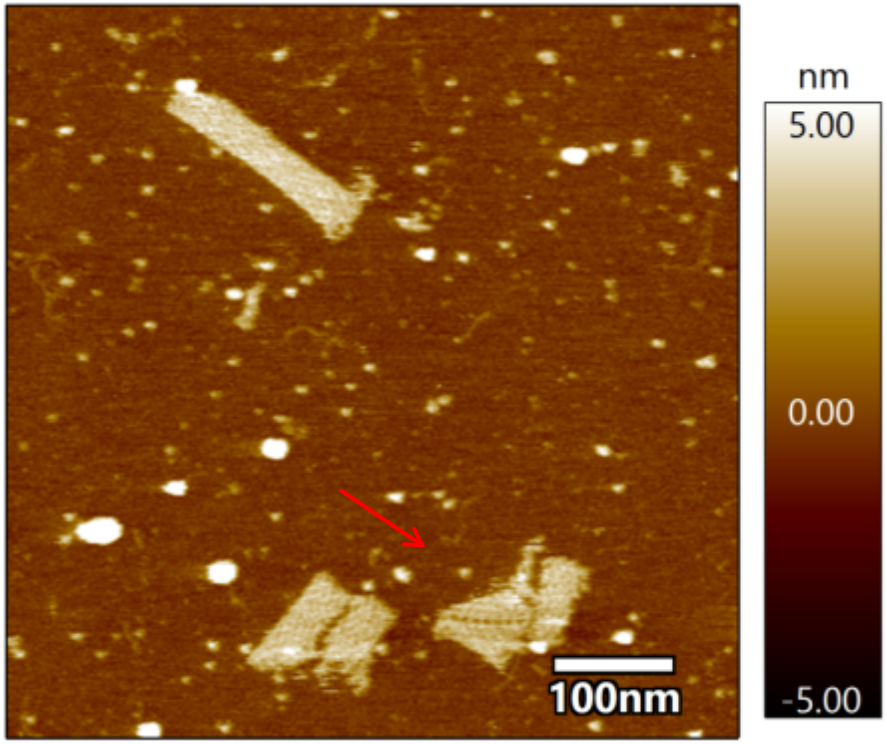
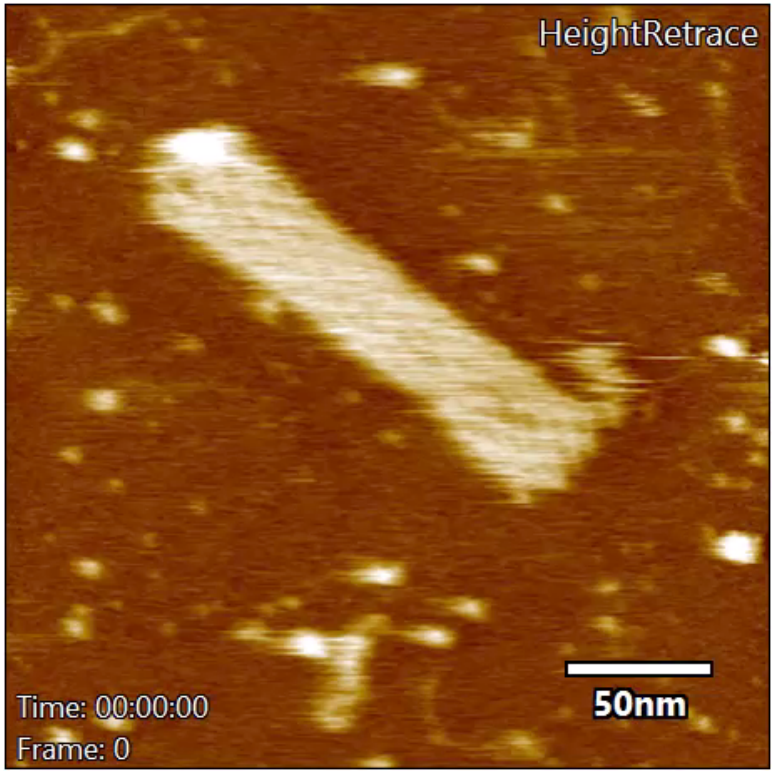
Cypher VRS

620nm scan, 512 x 384, 100 Hz, 0.26FPS



270nm scan, 512 x 384, 400 Hz, 1FPS
Playback: 15X



Olympus AC10 Cantilever

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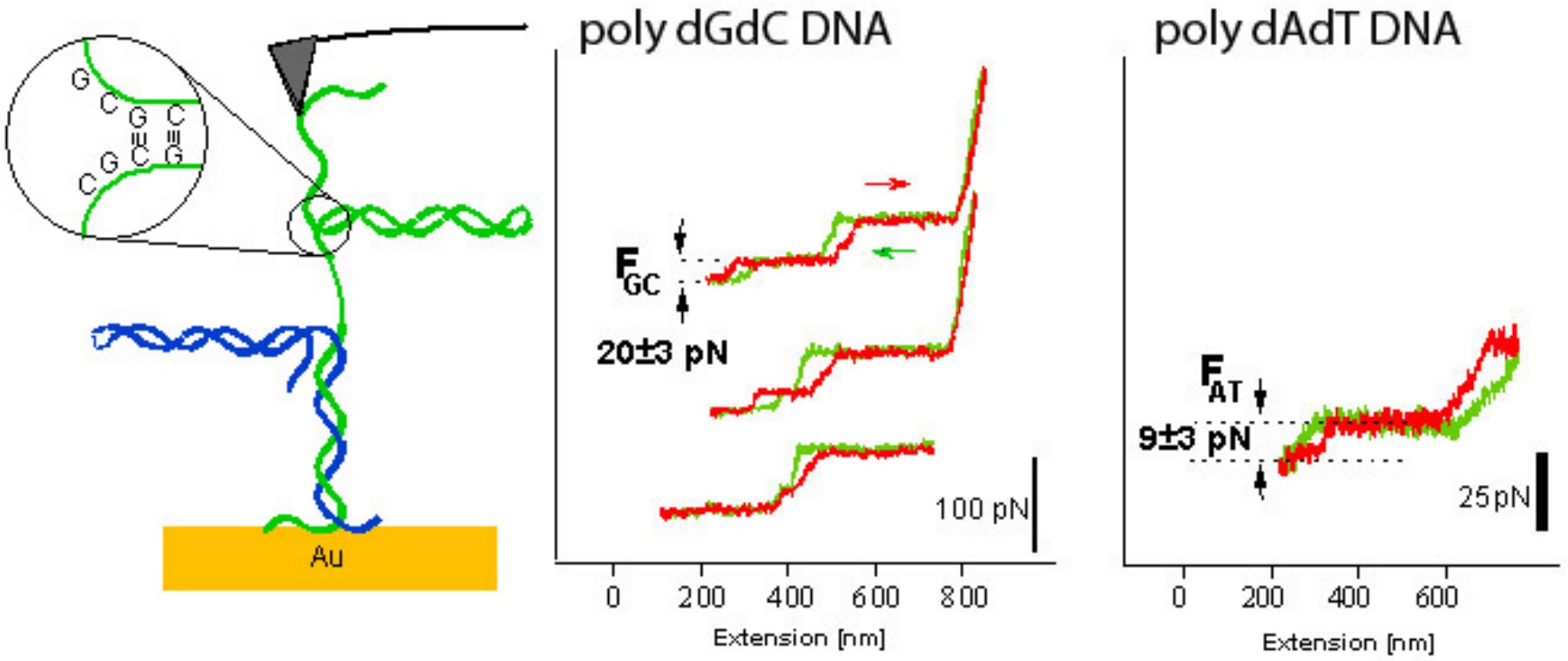
Sample courtesy of MD Dong, Aarhus University

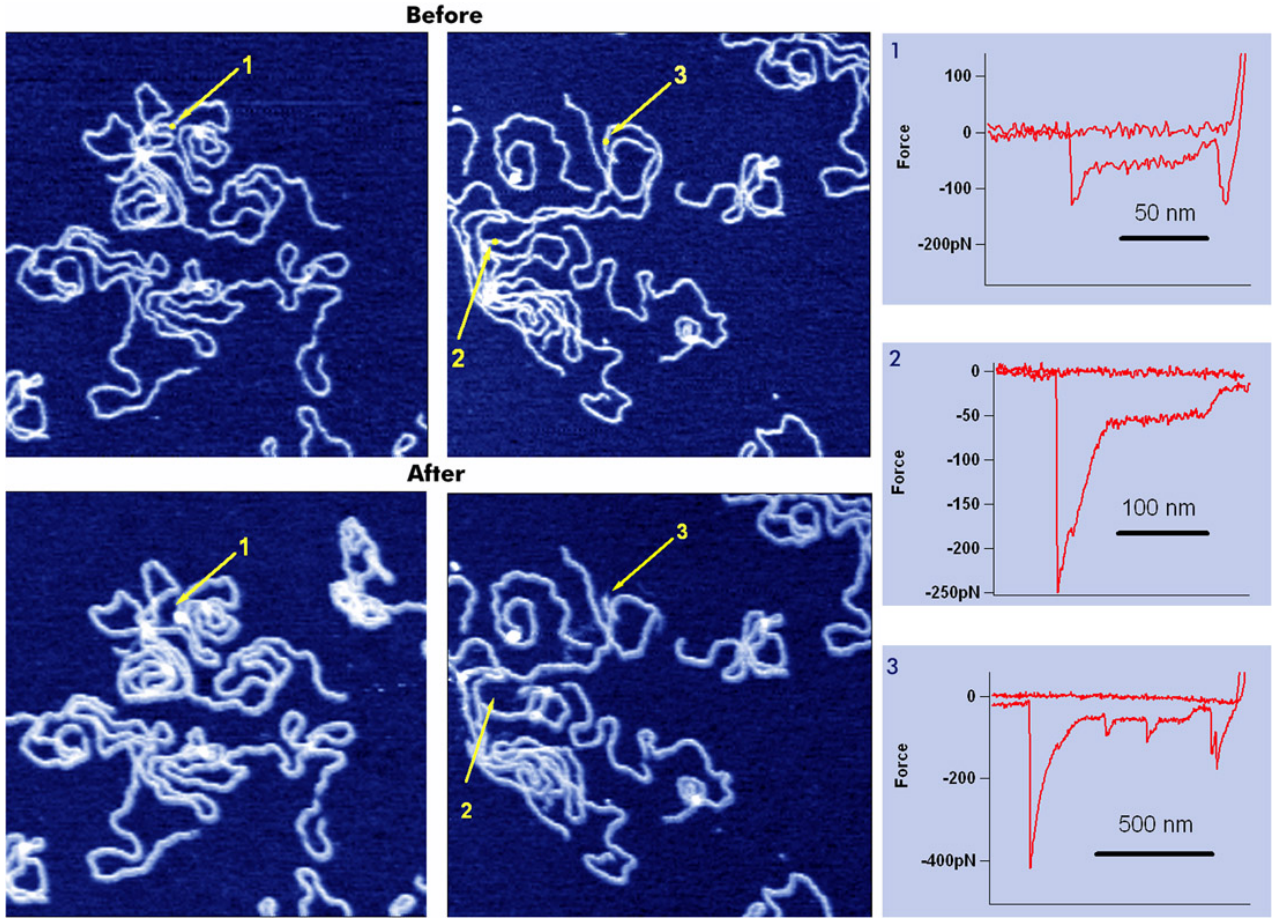
Measuring DNA Base Pair Binding



Rief et al., Nature Struct. Biol. 6, 346 (1999).

Directed Force Measurements

High precision and accuracy



Lambda Digest DNA image and subsequent pulls. 2µm scan.

Proteins

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Atomic Force Microscopes

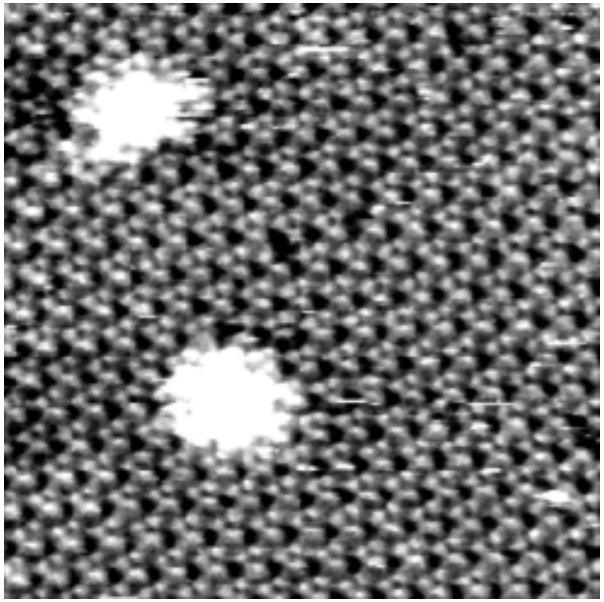
Measurements on Proteins

- 2D lattice
- Number of subunits
- Forces
  - Ligand binding
  - Unfolding
  - Membrane

Sample Prep: Proteins

Imaging Conditions:

- Liquid is preferable. In air, salts crystallize.
- Concentration: 1-100 µg/ml
- Substrate:
  - HOPG or mica for imaging
  - Gold for pulling
- Buffer: pH<pI to promote positive charges
- AC Mode (Contact mode for pulling)
- Highly individualized depending on proteins

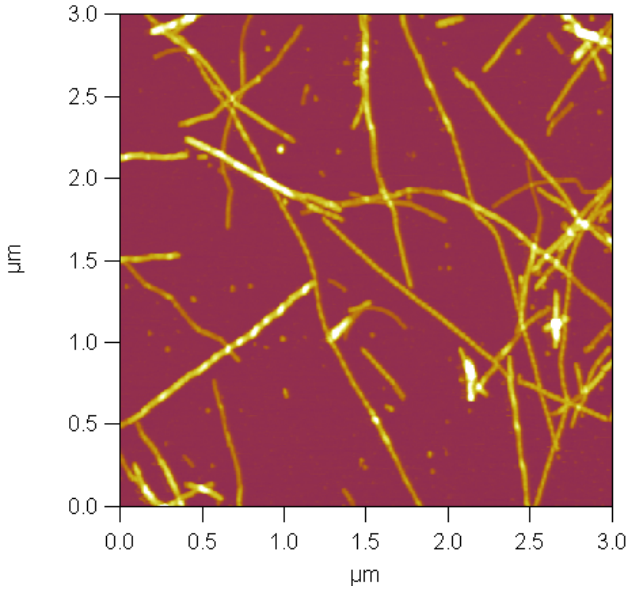


Protocol:

- Incubate protein solution on substrate for at least 15 min (even up to 24 hours), rinse with buffer.
- Play with pH, salt concentration to optimize the results.

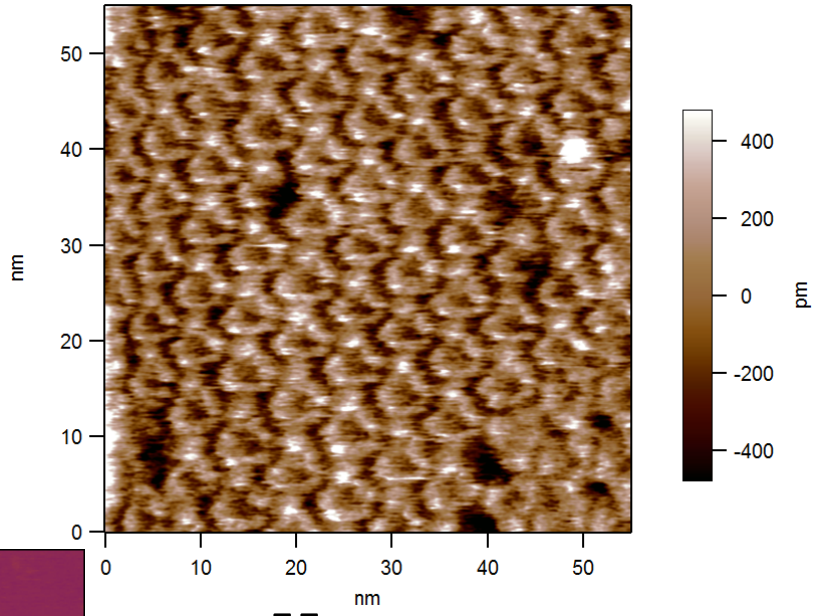
Topography

Insulin

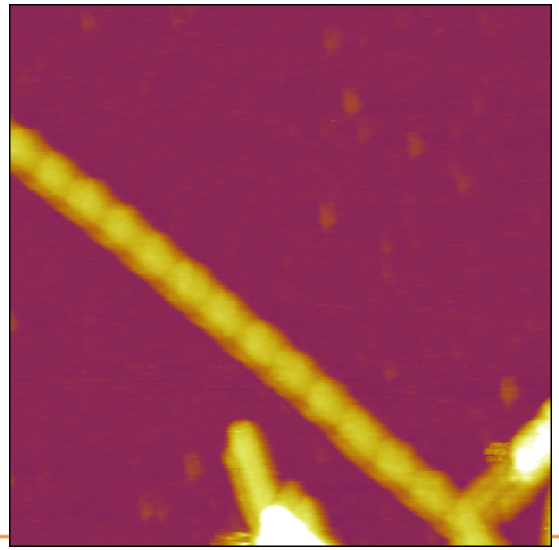


3µm scan

Bacteriorhodopsin

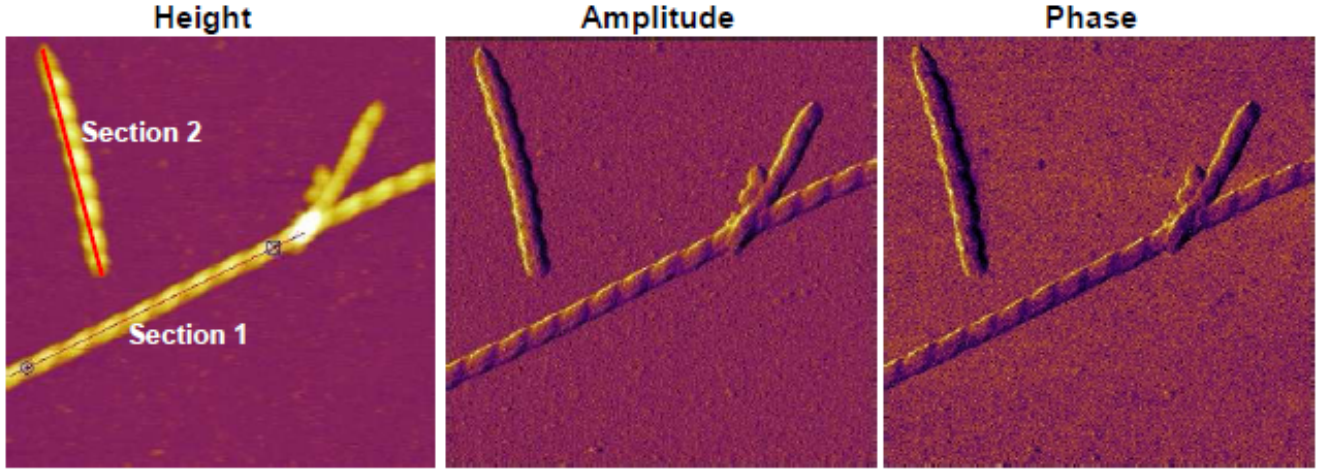


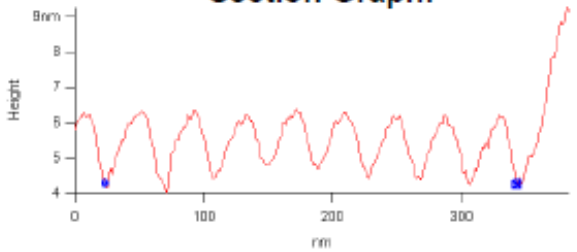
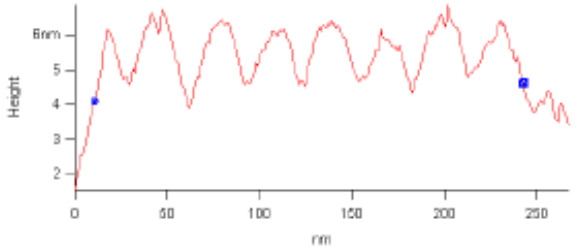
55nm scan



420nm scan

Amyloid Beta 42 Fibers

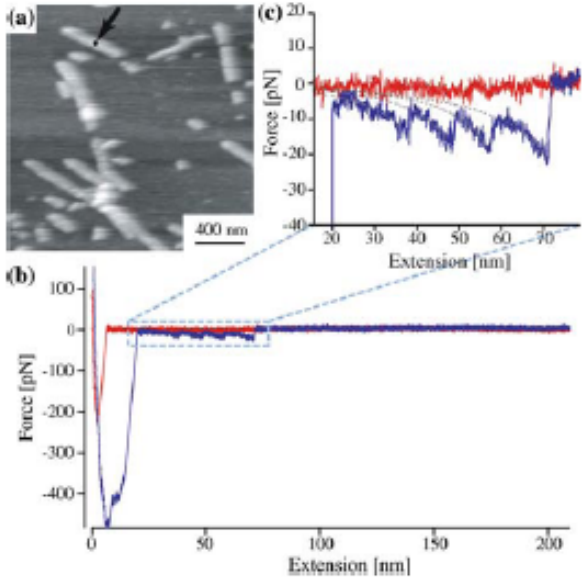


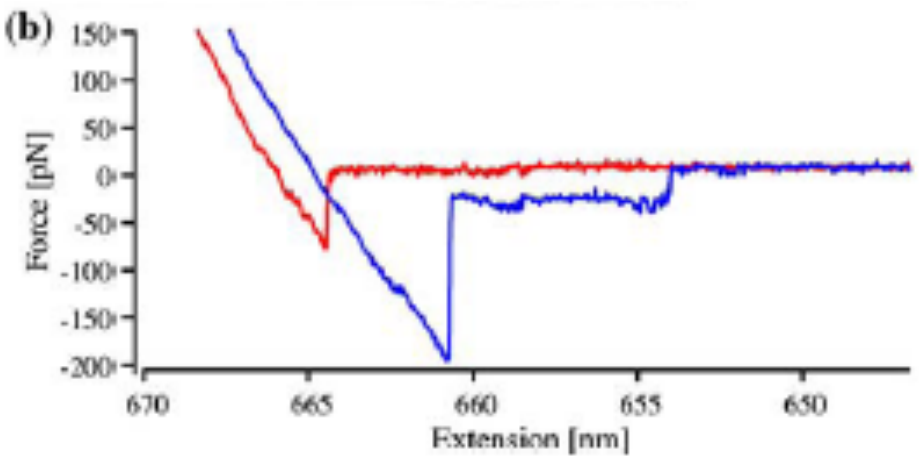
Section:	Section Graph:	Periodicity:
1		319.50 / 8 = 39.94
2		232.36 / 8 = 29.05

Sample courtesy of J.-M. Lee and X. Hu, Washington Univ.

Pulling Amyloid Fibers

Dr. Suzi Jarvis' Research, Trinity College Dublin, Ireland





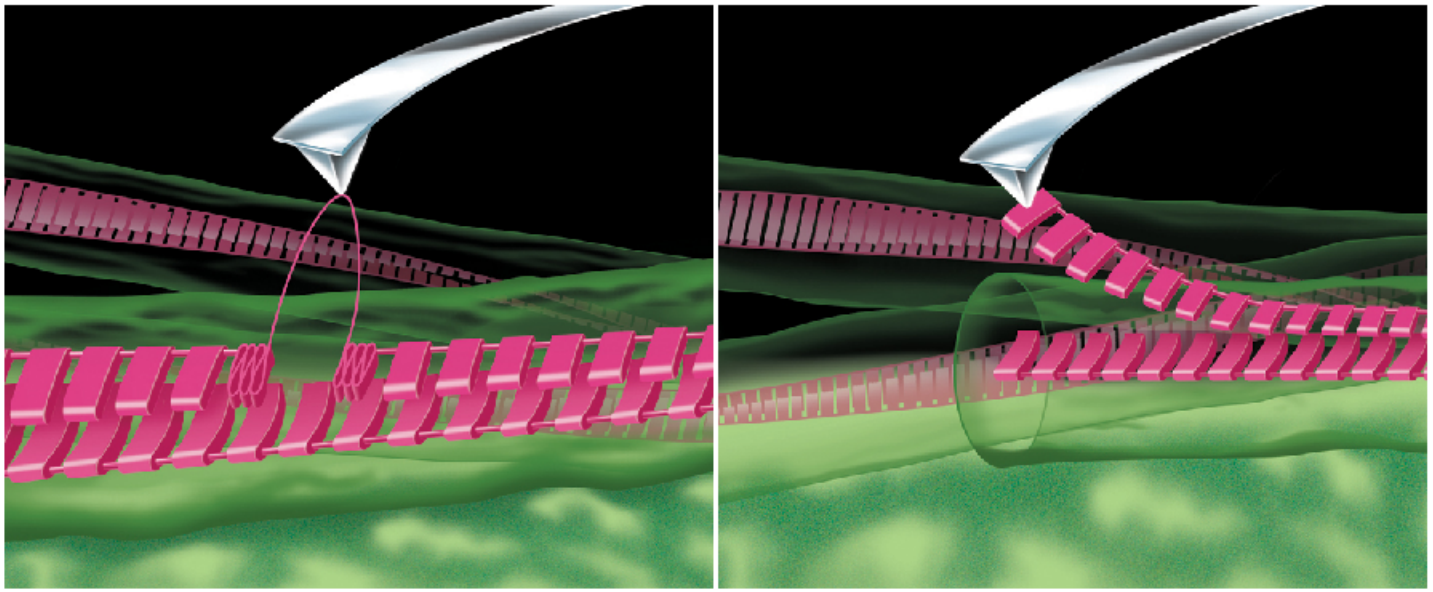
Unfolding individual molecule from the fiber

Peeling a beta sheet from the underlying layer of the fiber

Fukuma et. al. (2006) Tribology Letters 22(3): 233-7

Pulling Amyloid Fibers

Dr. Suzi Jarvis' Research, Trinity College Dublin, Ireland

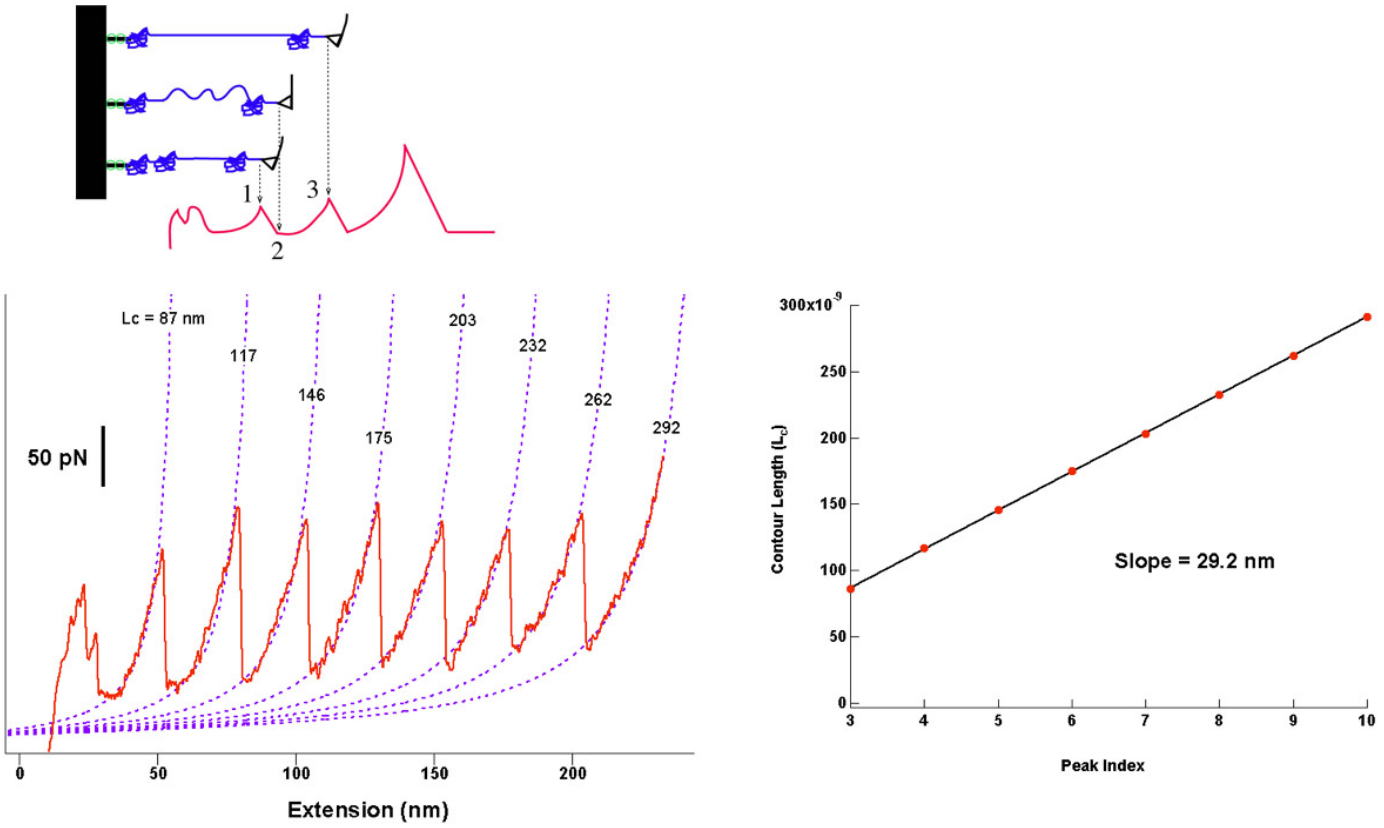


Unfolding individual molecule from the fiber

Peeling a beta sheet from the underlying layer of the fiber

From Dr. S. Jarvis' website: http://www.nanofunction.org/

Worm-like Chain Model



Courtesy of J. Clarke, Cambridge University.

Lipids

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Atomic Force Microscopes

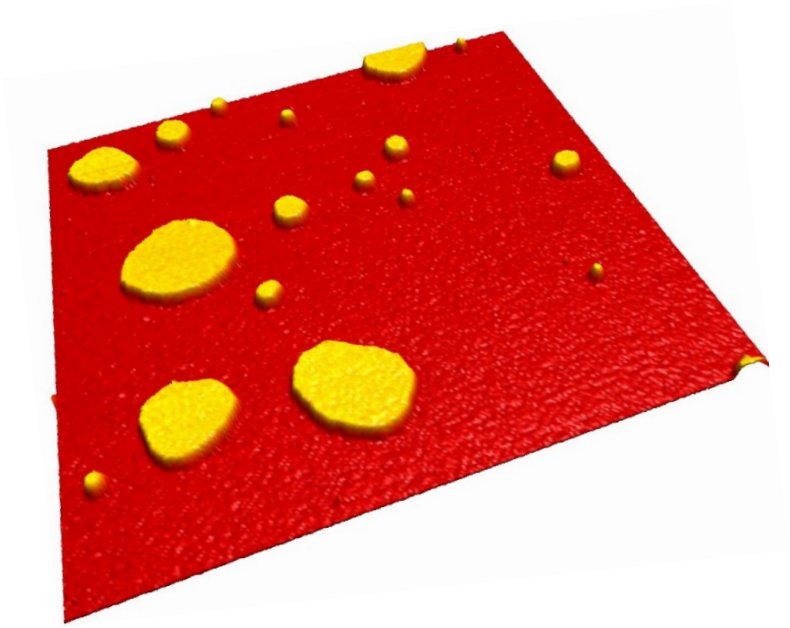
Sample Prep: Lipids (Expt. #2)

Imaging Conditions:

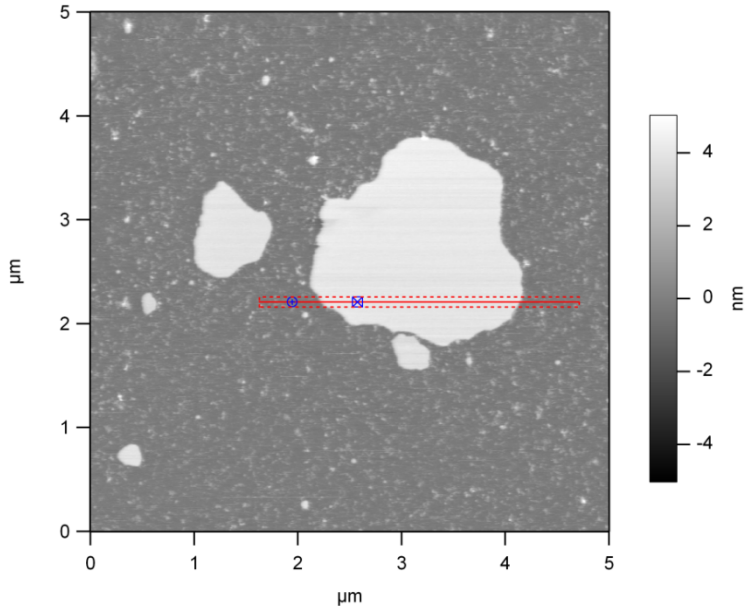
Substrate: mica (negatively-charged)

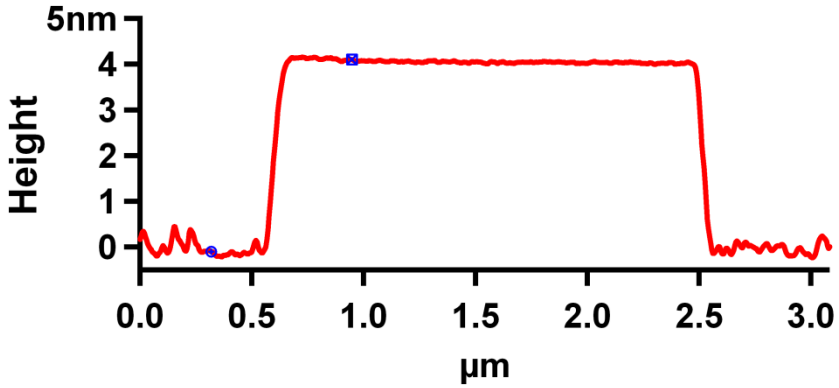
Protocol:

- Lipid vesicles will adsorb and flatten onto mica. Easy!
- Two Methods For Bilayer Preparation:
  - Langmuir-Blodgett technique
    - Excellent, uniform coverage
    - Requires non-standard equipment
  - Vesicle Fusion Method
    - Simple procedure with standard lab equipment
    - Difficult to obtain uniform coverage
- Buffer: specific to the lipid



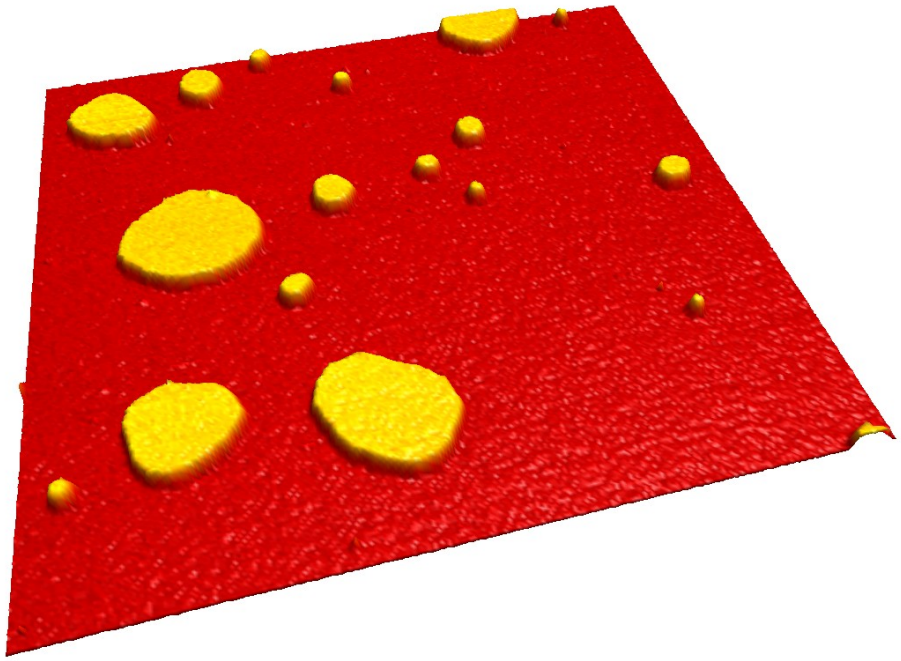
Supported Lipid Bilayers

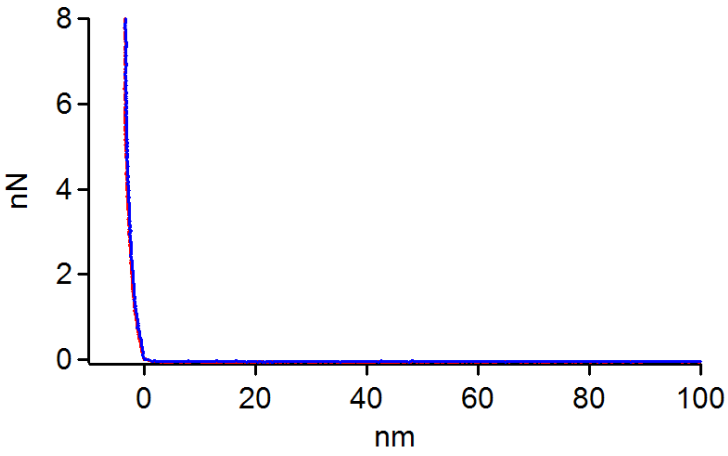


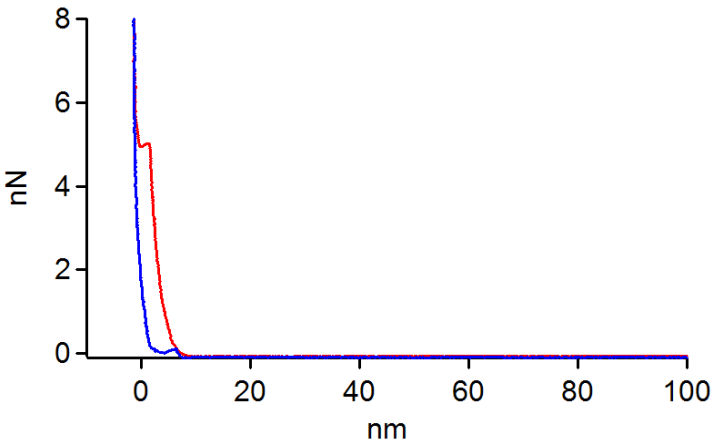


- Supported lipid bilayers are excellent systems for studying lipid-lipid and protein-lipid interactions.
- Because they form a very thin film on the support substrate, AFM is an excellent tool to study these model systems.
- These interactions can be studied *in situ* with changing environmental conditions and temperature without need for fixation or staining.

Lipid Mechanics



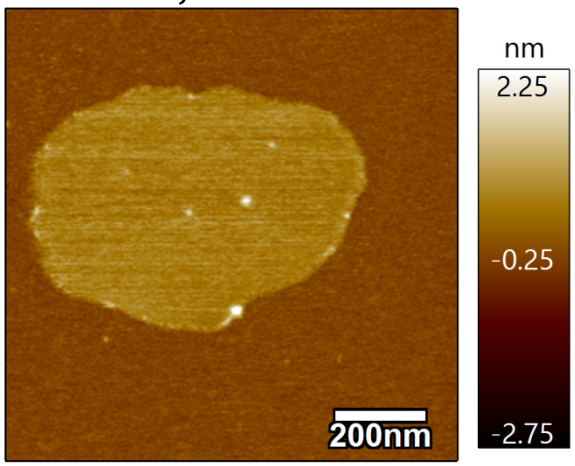




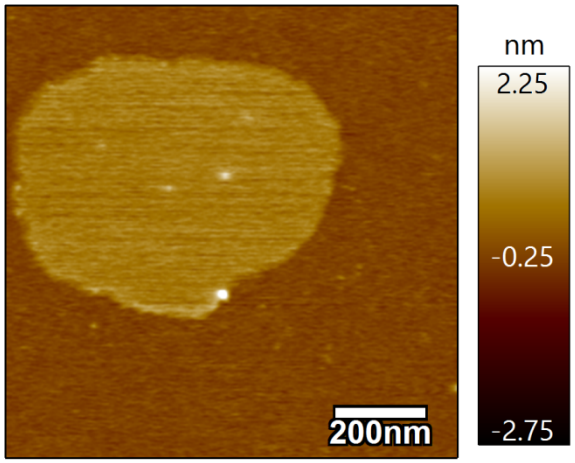
- The closed-loop XY scanner can position the tip to within ~450 pm of a feature of interest, and mechanical testing can be performed via force curves.

Faster Scanning – DMPC + chol

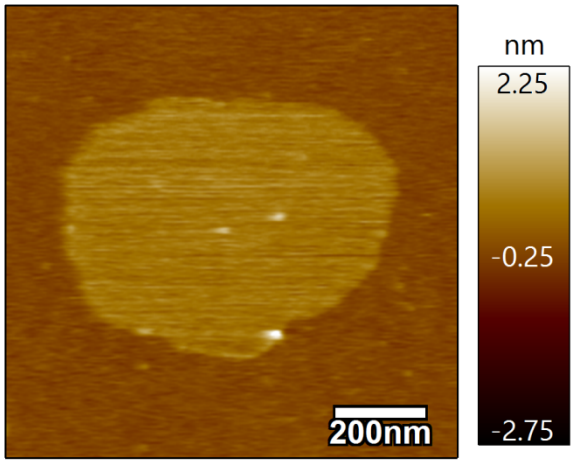
1.95 Hz, 2:11 minutes



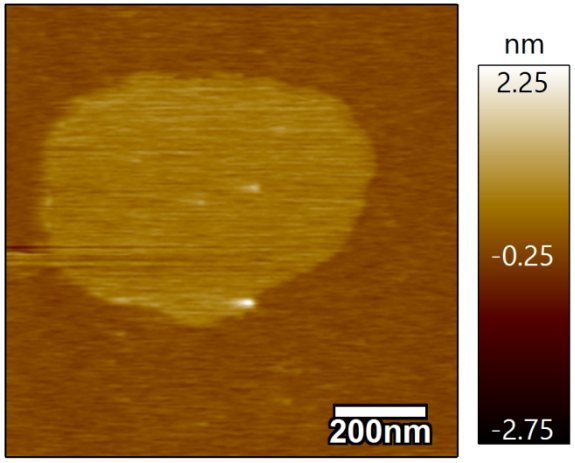
4.88 Hz, 0:52 minutes



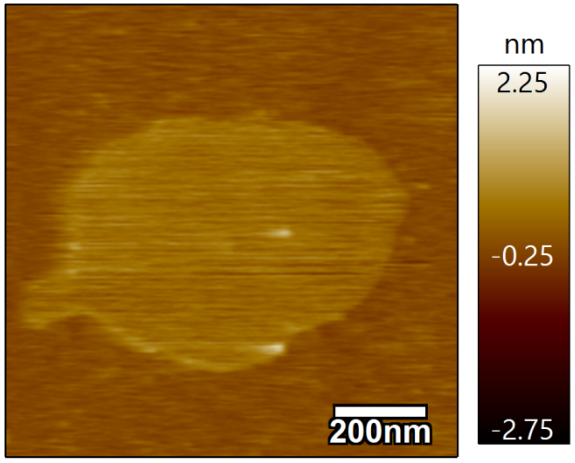
9.77 Hz, 0:26 minutes



13.02 Hz, 0:19 minutes



19.53 Hz, 0:13 minutes



Height
1μm
512 x 256

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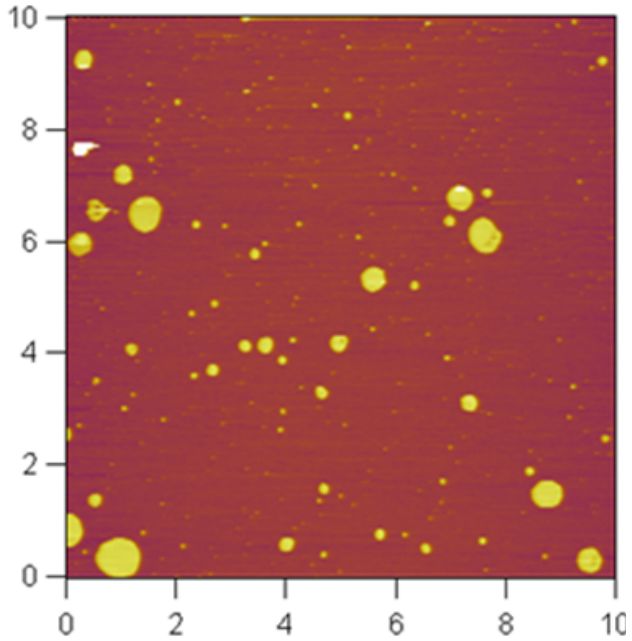
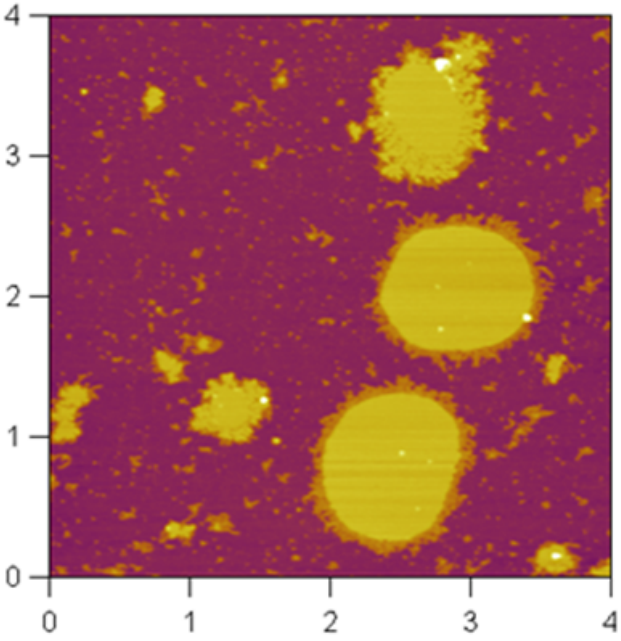
Degradation of Lipids with Antimicrobial Peptide

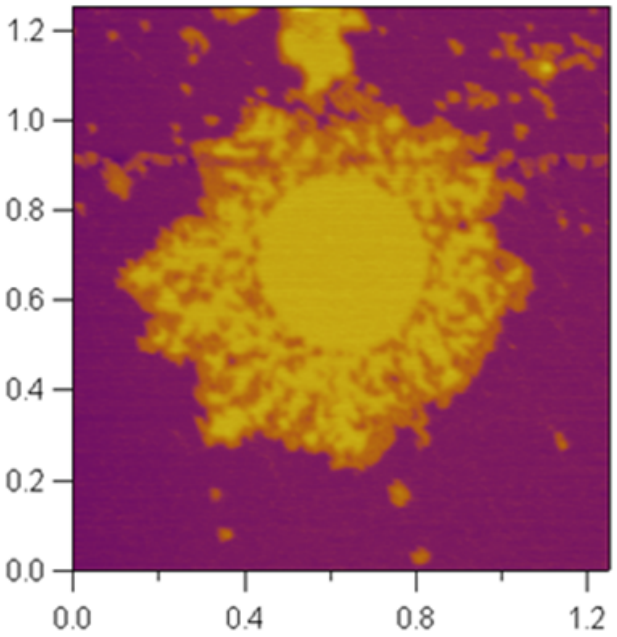
Time: 0



Time: 4 hours



Time: 6 hours



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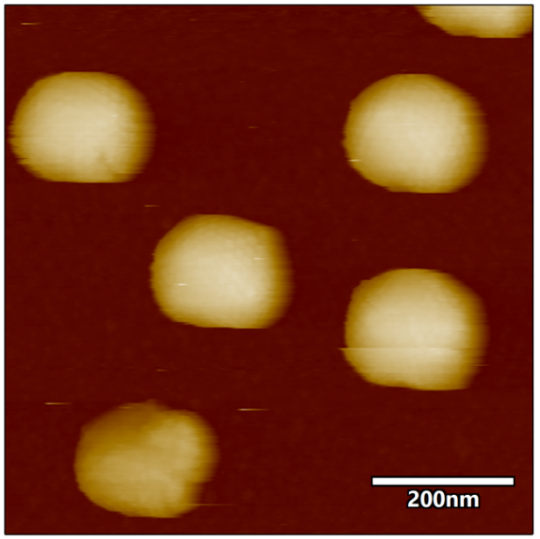
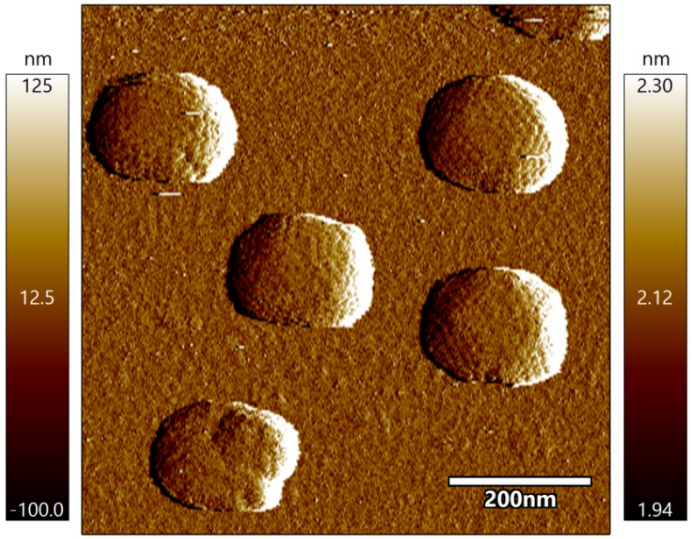
Viruses

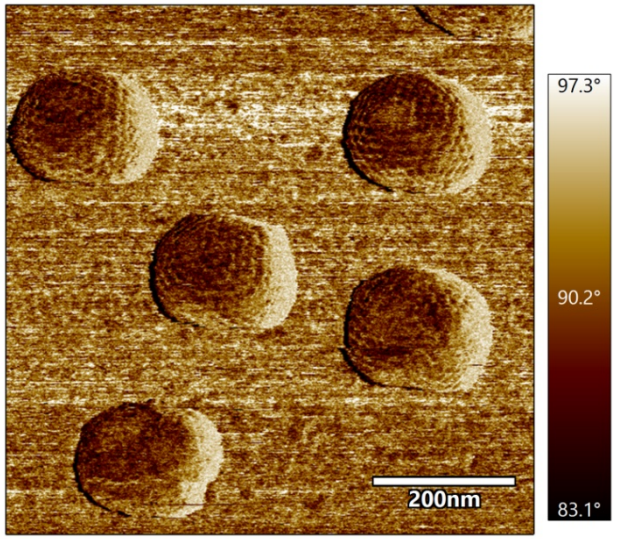


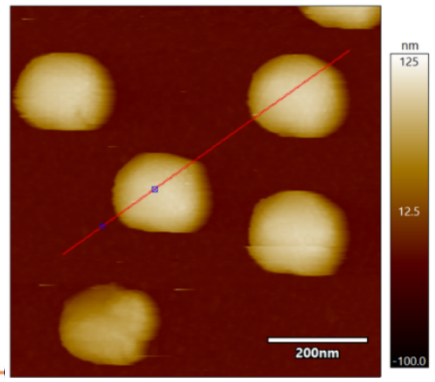
Herpes Virus: HSV-1

- High resolution scans of Herpes Simplex 1 viral capsids: resolve.
  - Parameters: survey scan of 750nm scan, 512 x 256, 1Hz, AC mode with Biolever mini cantilever
  - Capsids appear uniform in height. Applying our Particle Analysis routine to the 5 complete viral capsids in the image below, the average Maximum Z is 118.96 +/- 15.25 nm (expected = 120nm).

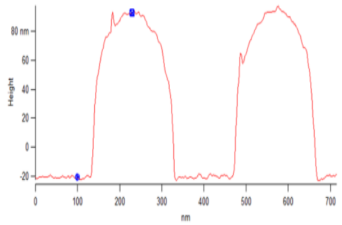


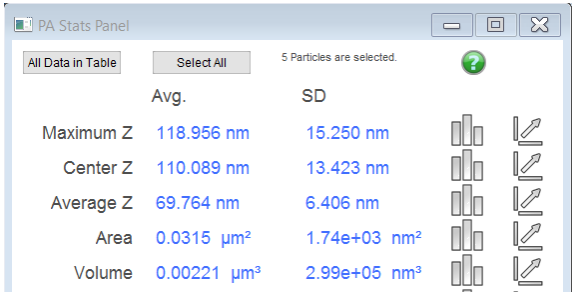






dZ of selected capsid = 113.65 nm

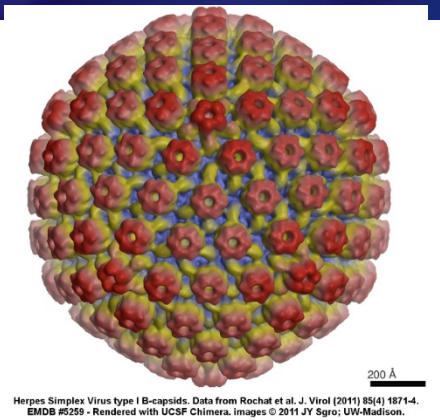




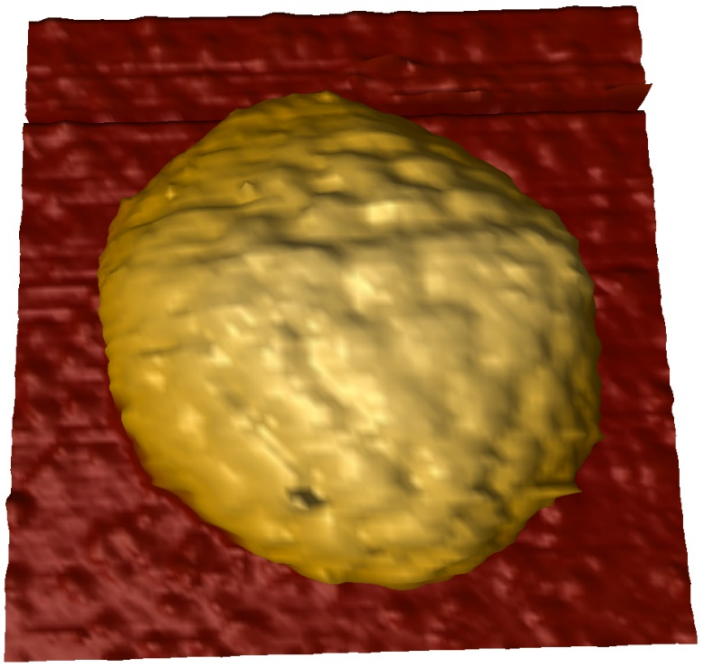
Sample courtesy of Alex Evilevitch, UIUC

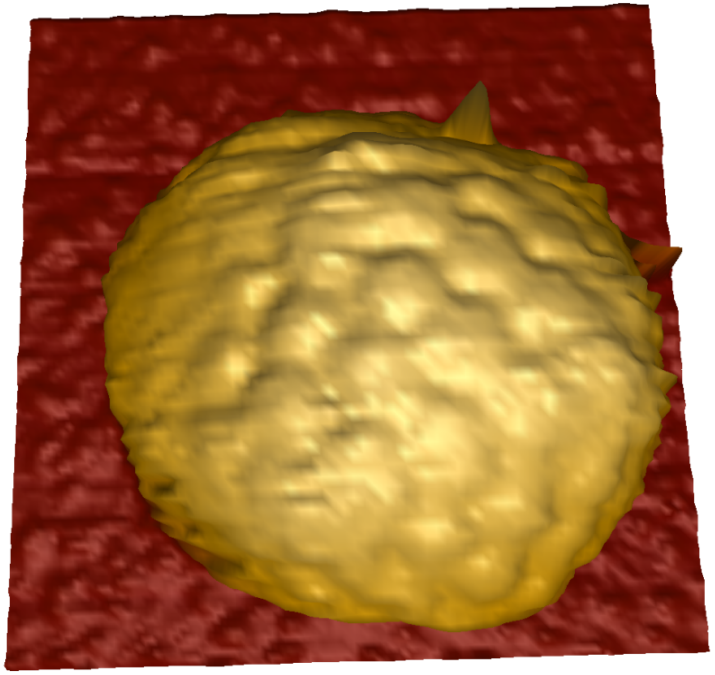
Herpes Virus: HSV-1

- High resolution scans of Herpes Simplex 1 viral capsids.
  - Zooming into a couple of capsids we can see individual capsomers.
  - As a comparison, a 3D diagram rendering of the capsid is included.



Herpes Simplex Virus type I B-capsids. Data from Rochat et al. J. Virol (2011) 85(4) 1871-4.
EMDB #5259 - Rendered with UCSF Chimera. Images © 2011 JY Sgro; UW-Madison.





Sample courtesy of Alex Evilevitch, UIUC

Bacteria and Cells

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Atomic Force Microscopes

Measurements on Cells

- Quantitative topography (i.e. cell height, volume)
- Cytoskeletal details
- Monitor dynamic processes
- Accurate force measurements
  - Adhesion forces
  - Ligand-receptor interactions
  - Unfolding membrane proteins
  - Mechanical properties (Young's modulus)
  - Cor
- Can be combined with optical techniques to locate target cells
  - Correlate fluorescence markers with AFM topography
- **Live** cells can be probed at near physiological conditions

AFM Studies of Cells

- Provides quantitative topographical measurements (i.e. cell height, volume)
- Accurate force measurements (ligand-receptor interactions, unfolding membrane proteins, elasticity)
- **Live** cells can be probed at near physiological conditions
- Can be combined with optical techniques to locate target cell(s), correlate fluorescent markers with AFM topography

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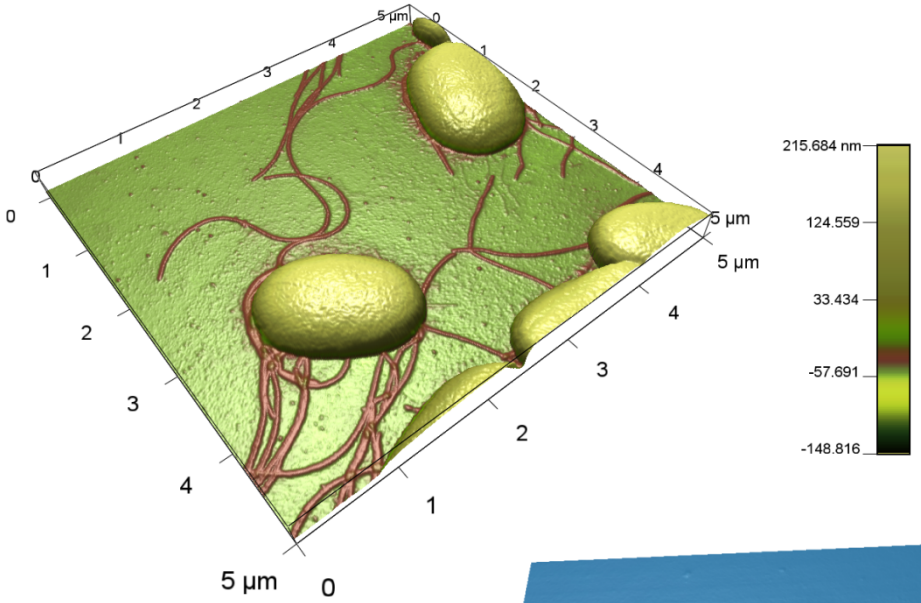
Sample Prep: Bacteria, Yeast

Helpful Hints:

- Bacteria and yeast cells are particularly hard to attach, unlike adherent mammalian cells.
- Successful immobilization methods include: “trapping” them in cellulose membrane pores or use coated glass slides (poly-L-lysine, gelatin).



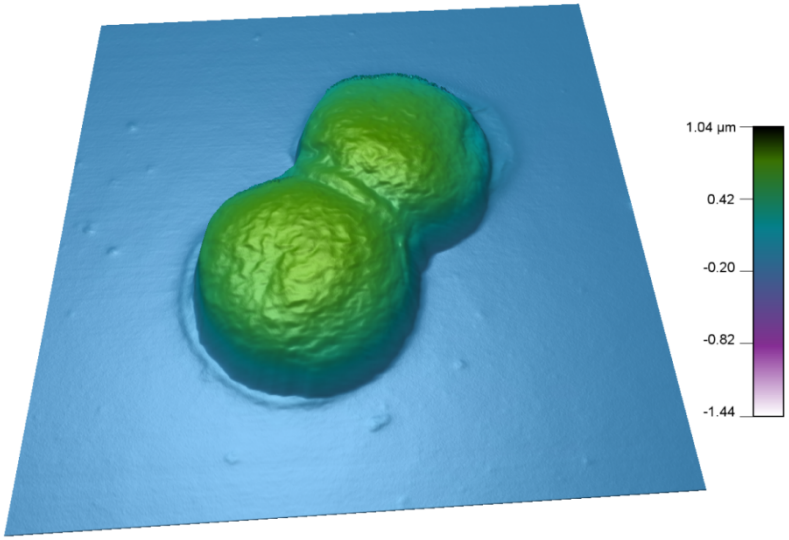
Various Bacteria Types



4μm scan



10μm scan



m scan

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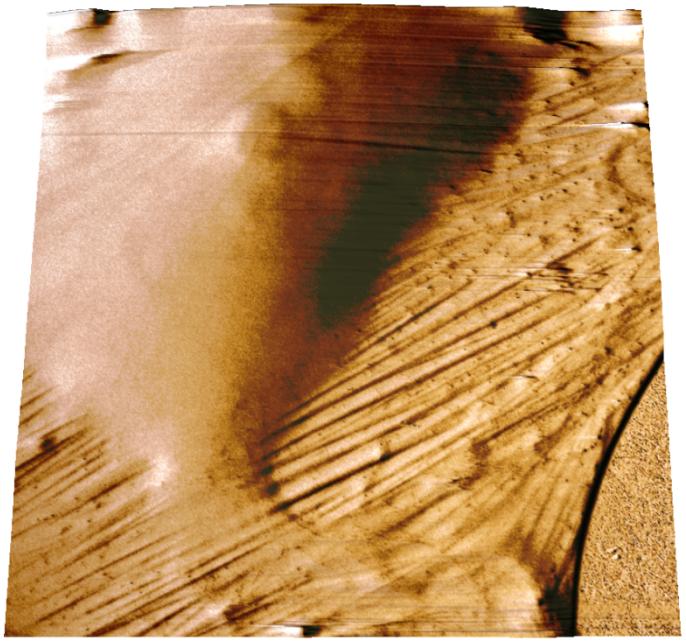
Sample Prep: Cells (Expt. #3)



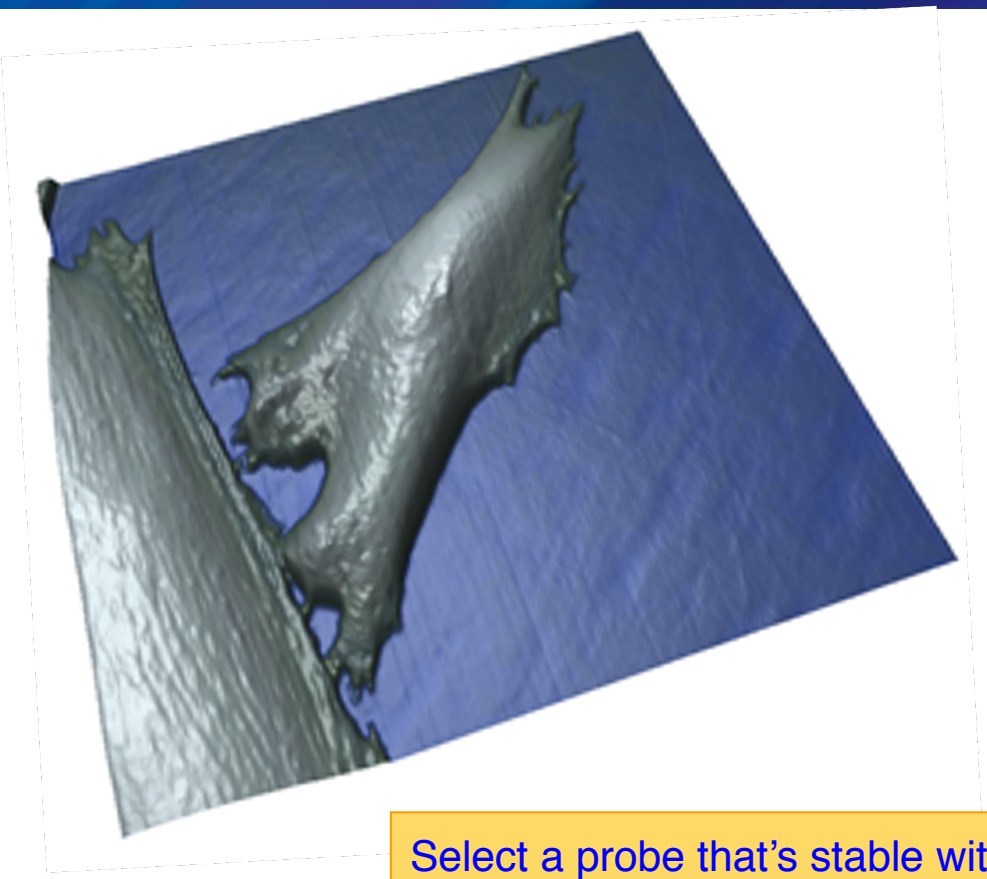
Imaging Conditions:

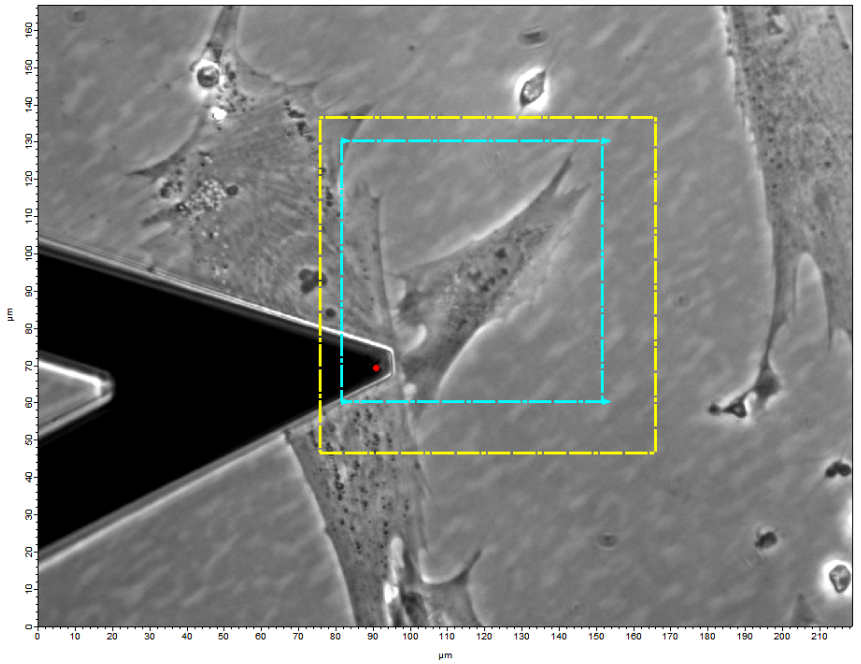
- Cell Concentration: 50-80% confluent
- Substrate:
  - Glass coverslips
  - Glass-bottom/polystyrene petri dishes
  - What do you need? fluorescence? TIRF? Confocal? High/low NA?
- Buffer: Culture medium, PBS
- Contact mode or AC mode
- Recommended parameters:
  - Scan rate (0.25-0.5 Hz)
  - AC Mode: Target free amplitude of 0.6V and Setpoint of 0.5V
  - Contact Mode: ΔV ~0.5V

Protocol:

- Grow the cells as usual. If the cells don't adhere, may need to use adhesives: poly-D-lysine, collagen, laminin, fibronectin, Cell-Tak, PEG-derivatives. You can also serum starve (i.e. use 2% serum) to promote flattening.

Cell Imaging: Fibroblasts

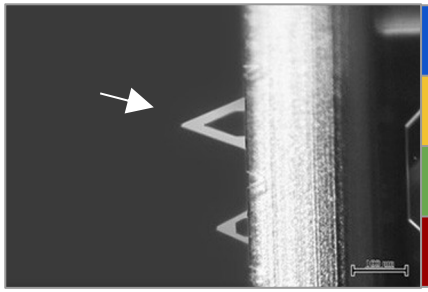




MFP-3D-BIO, TR400PB (long)

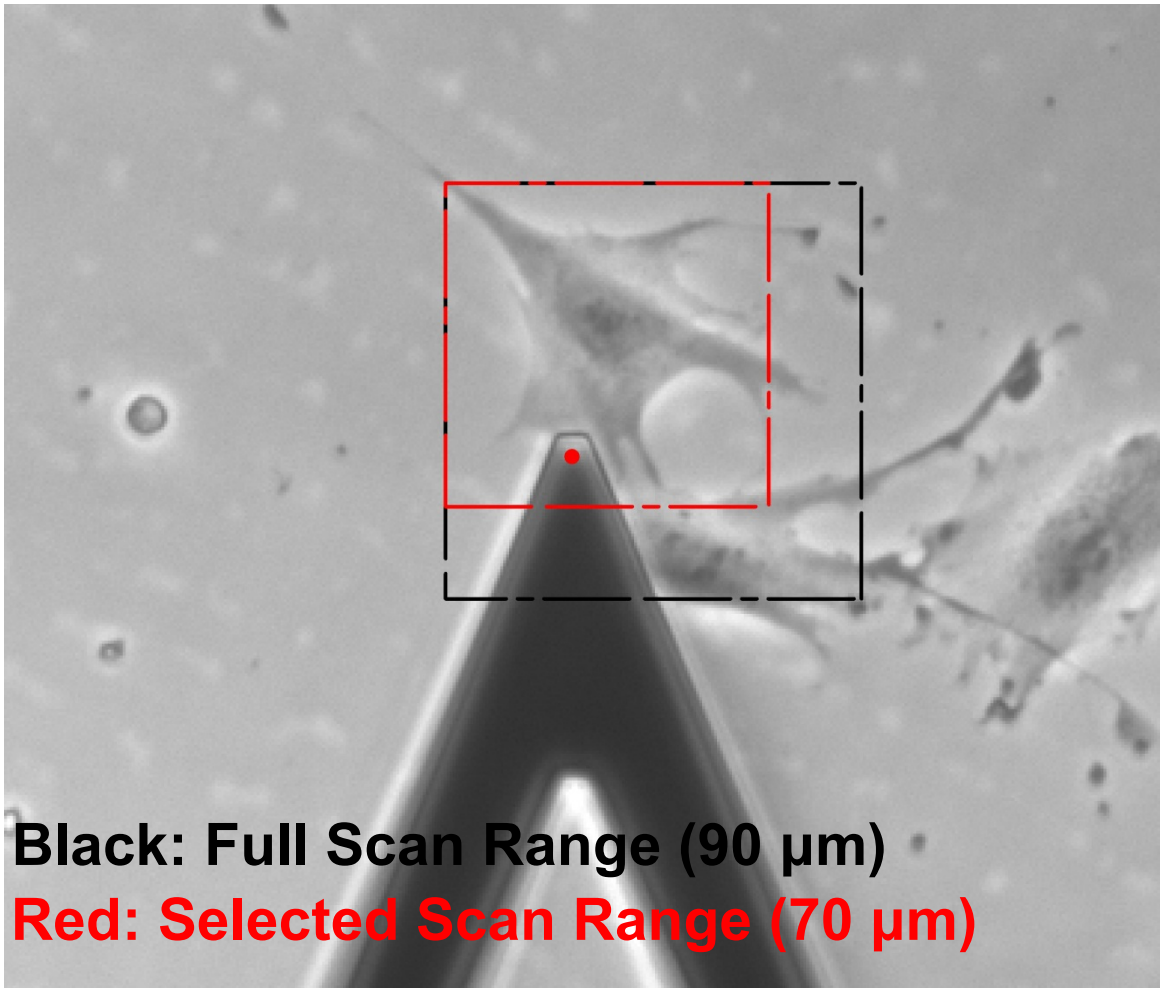
Select a probe that's stable with very low spring constant for **contact mode** imaging in fluid

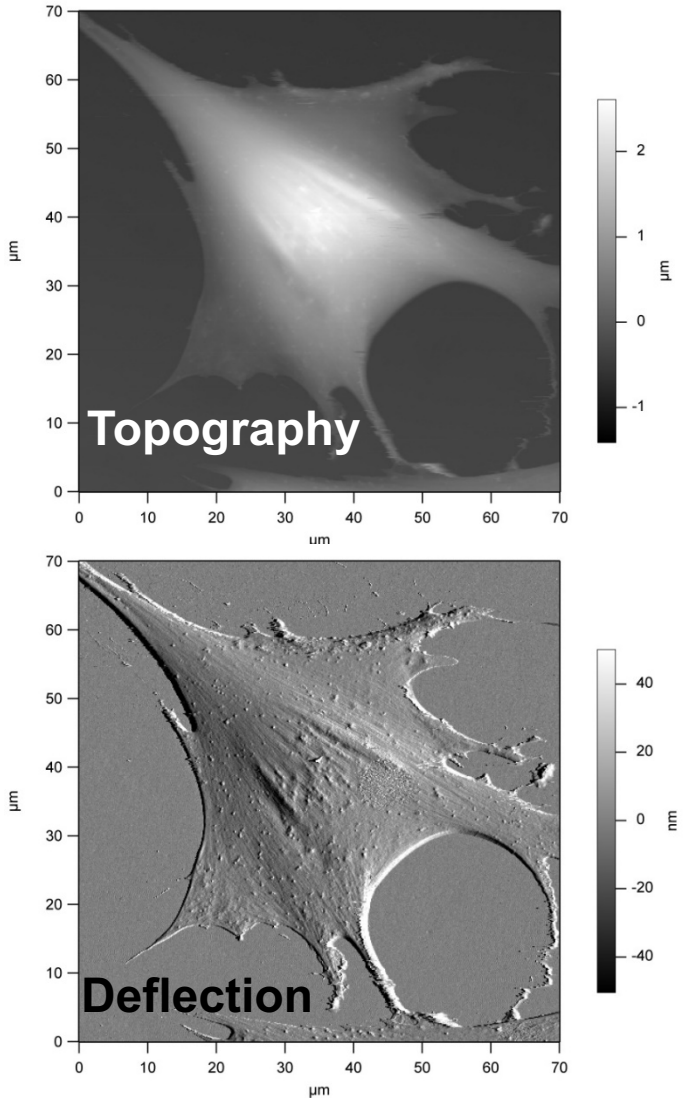
Note: for taller cells (non-adherent) you may need a **taller tip height**



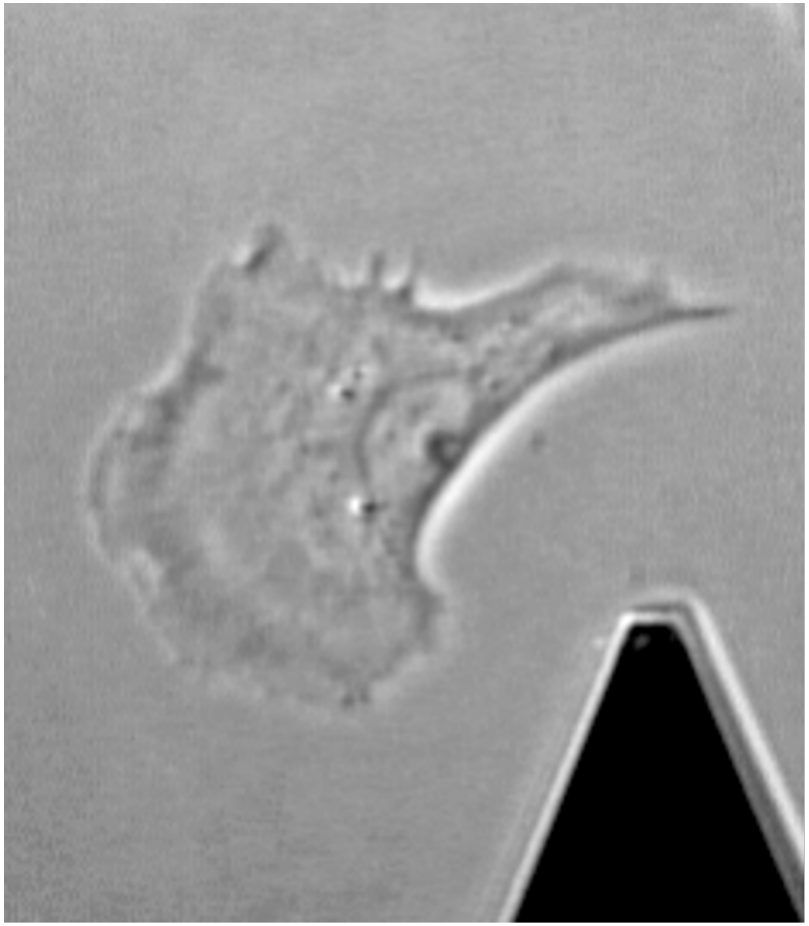
k	0.09 N/m
r	32 nm
f	32 kHz
Q	19

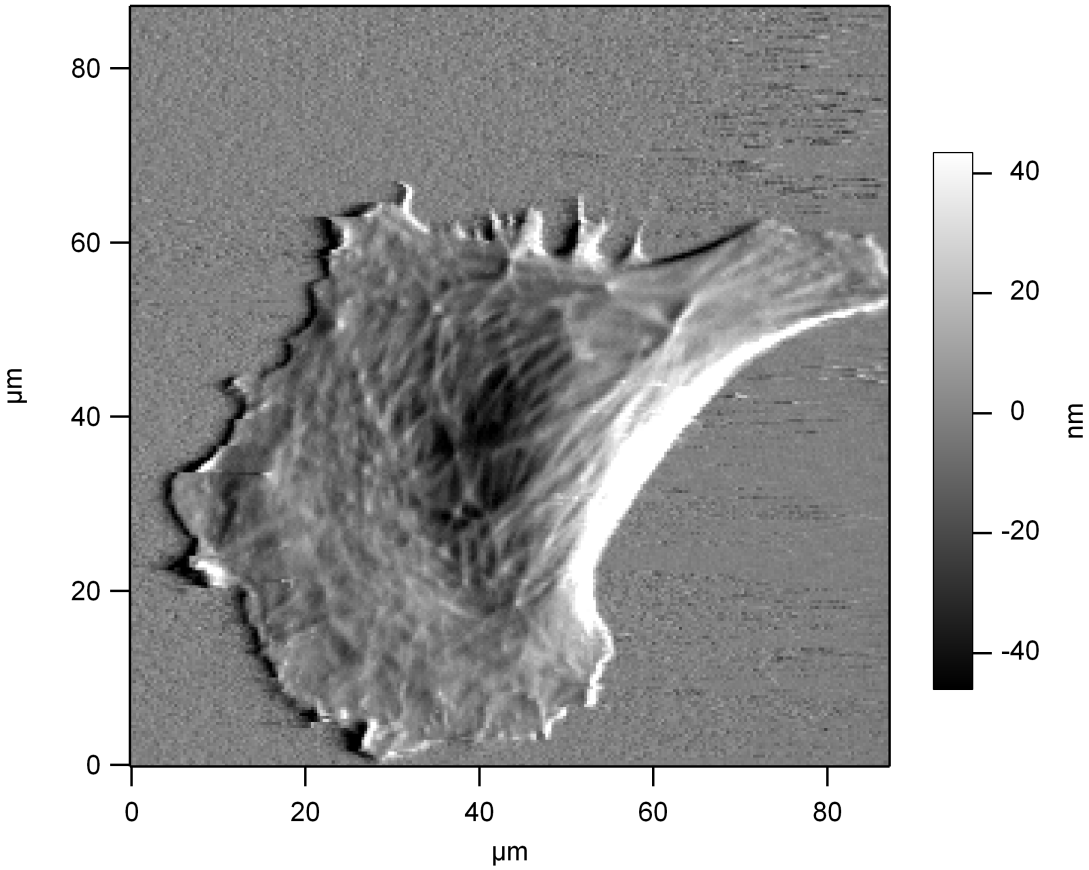
Select Scan Location with Optics





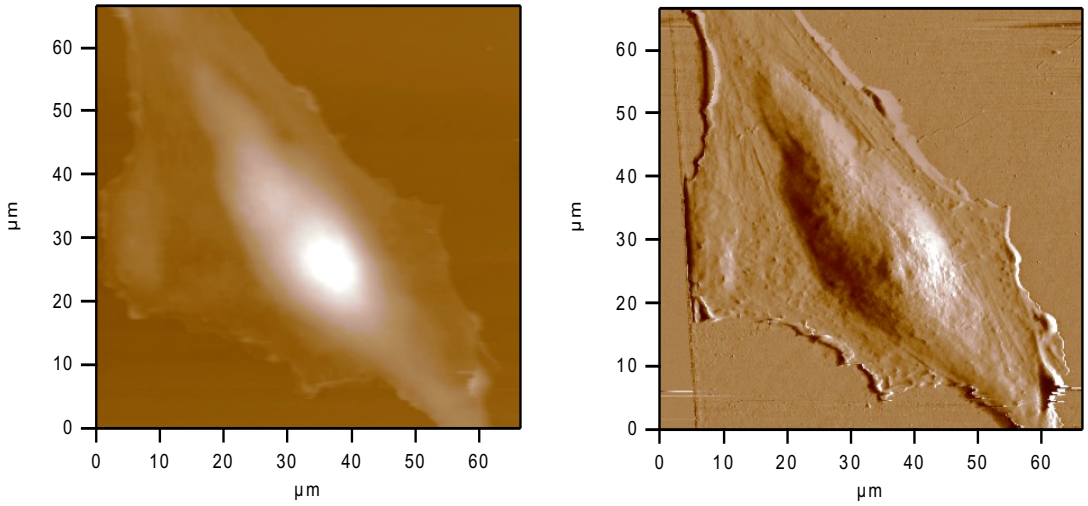
Complimentary Imaging – Cardiac Fibroblast



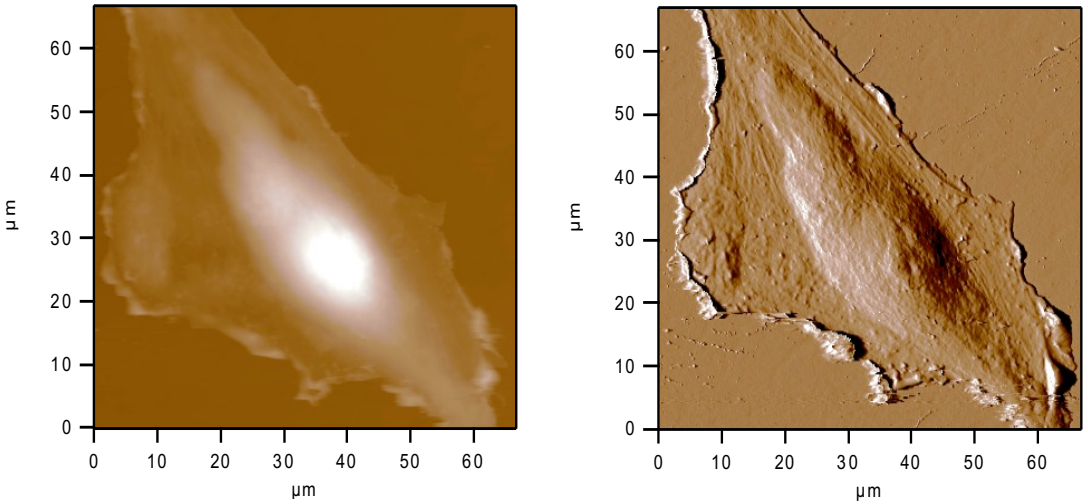


Live MRC-5 Fibroblasts

AC Mode:
Z sensor (left),
Amplitude (right)



Contact Mode:
Z sensor (left),
Deflection (right)



65μm scan

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Live MRC-5 Fibroblasts

- A series of images capturing cell movement.
- Images were acquired every 16 minutes.
- The sample was moved after Image 3 was acquired using the XY translational knobs in order to keep imaging the same cell which had moved out of the scan area.

Image 1: 50μm

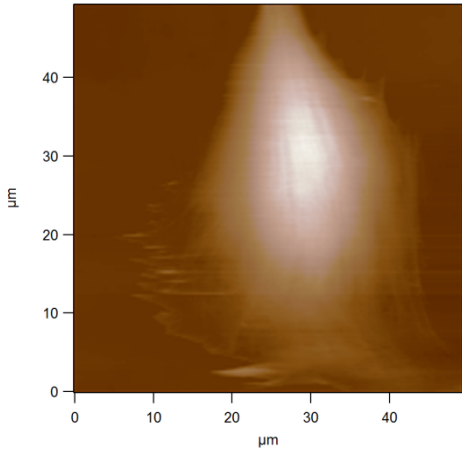


Image 2: 65μm

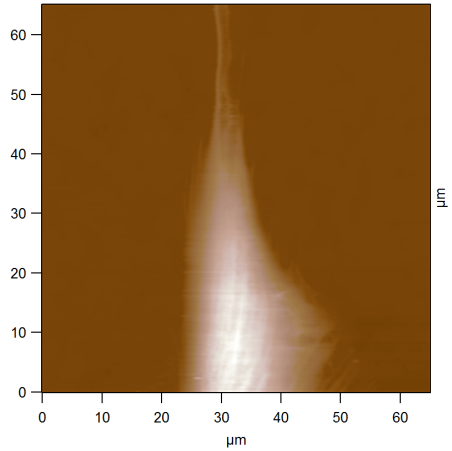


Image 3: 90μm

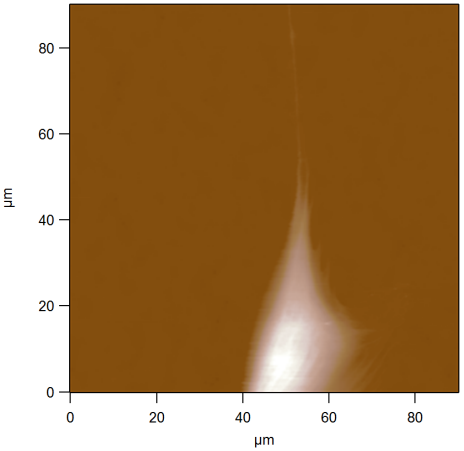


Image 4: 50μm

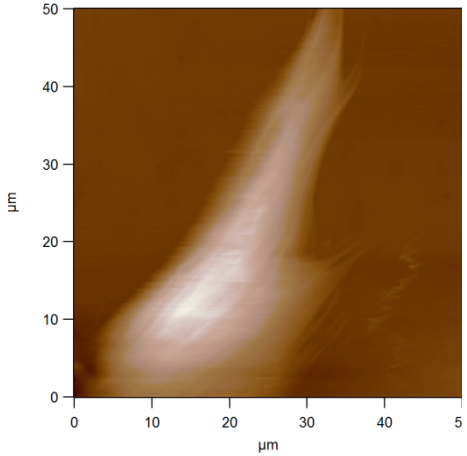


Image 5: 50μm

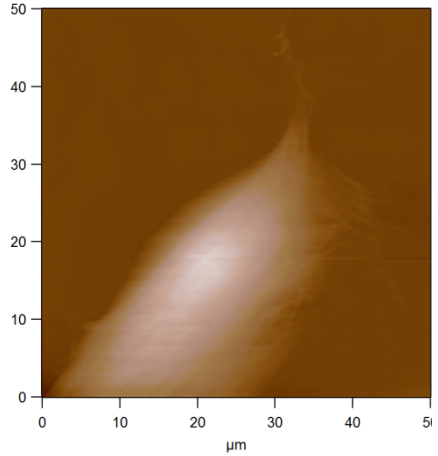
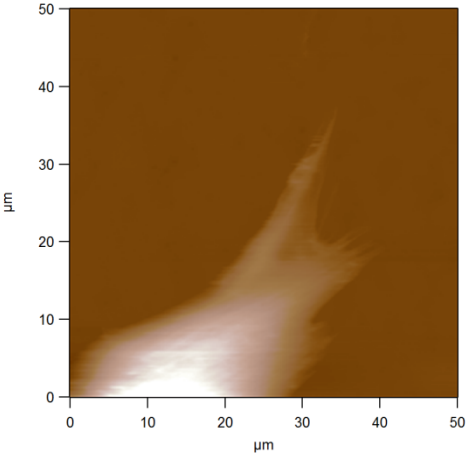


Image 6: 50μm



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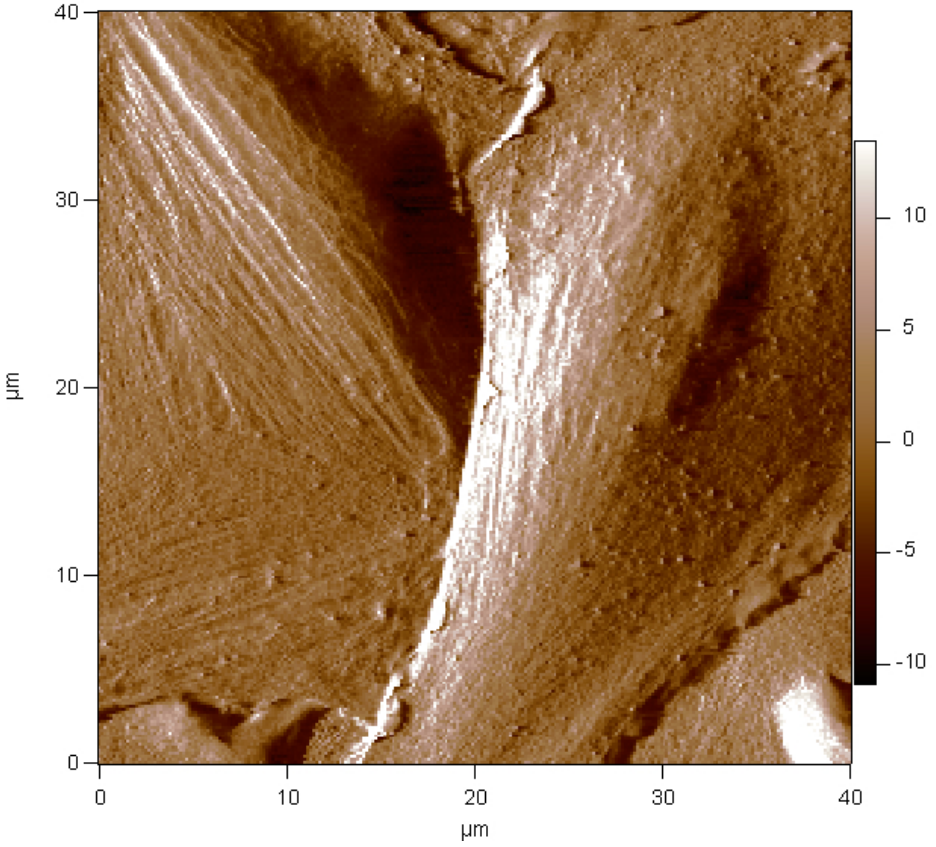
Live Endothelial Cells

Monitoring Dynamic Events: Treatment with Mannitol

Before



After

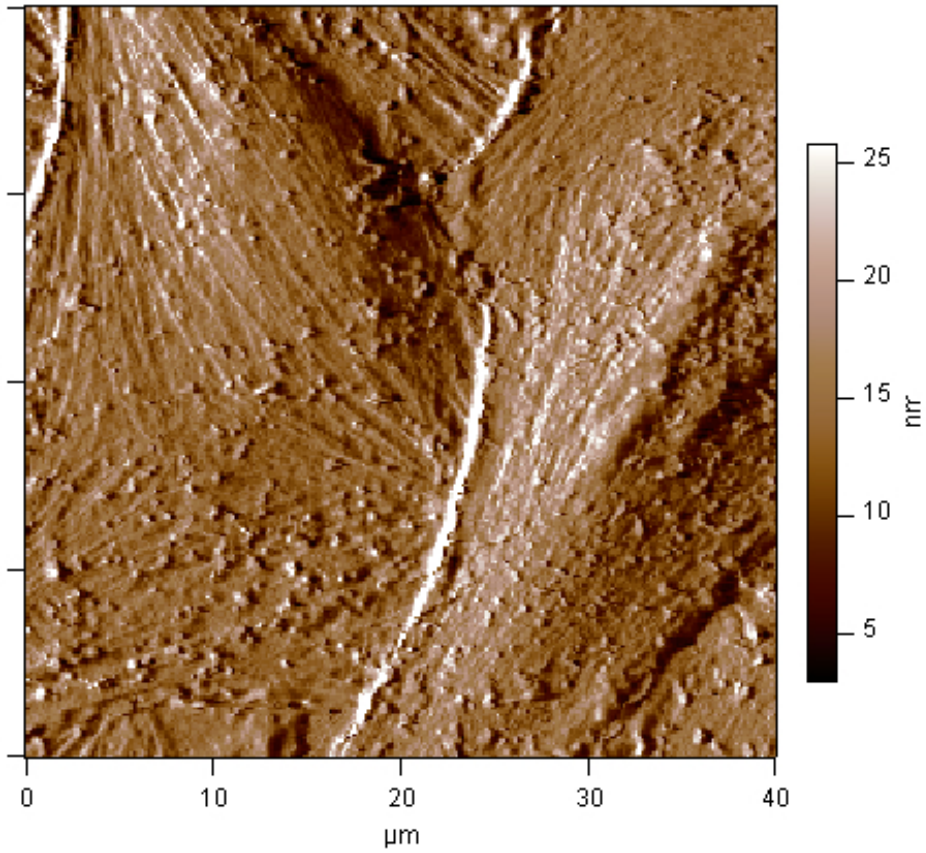


Image courtesy of Z. Bálint and Dr. G. Váró, Inst. of Biophys, Szeged, Hungary

Ghost Parental Osteocarcinoma

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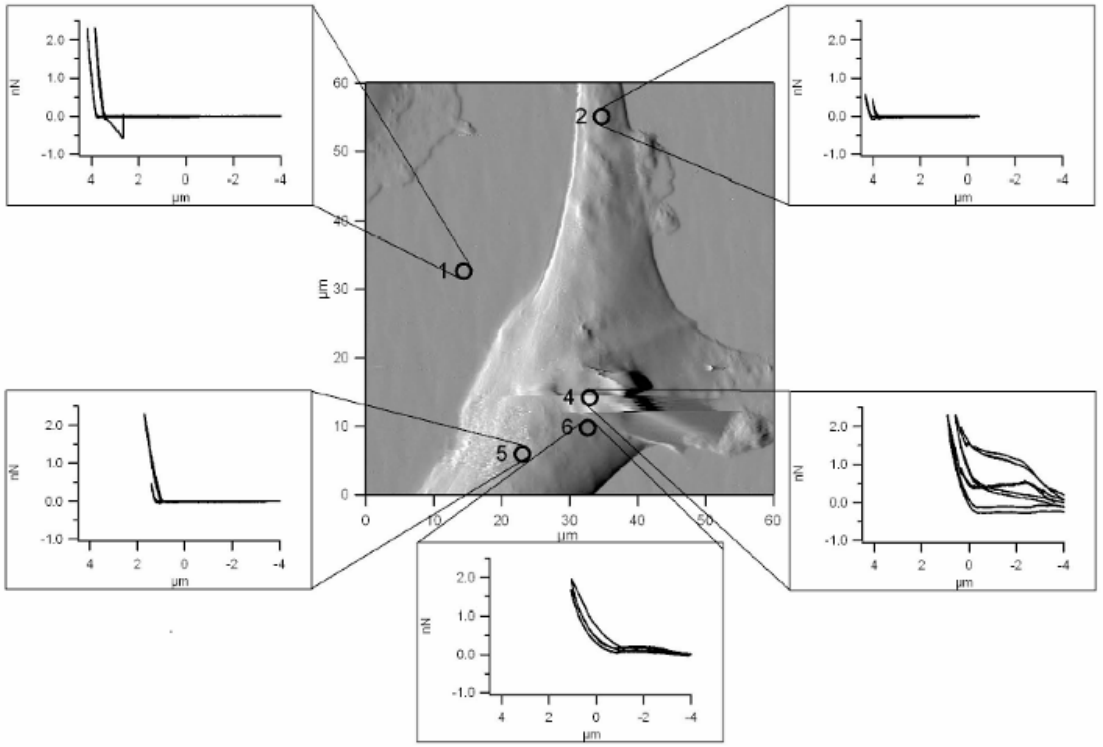
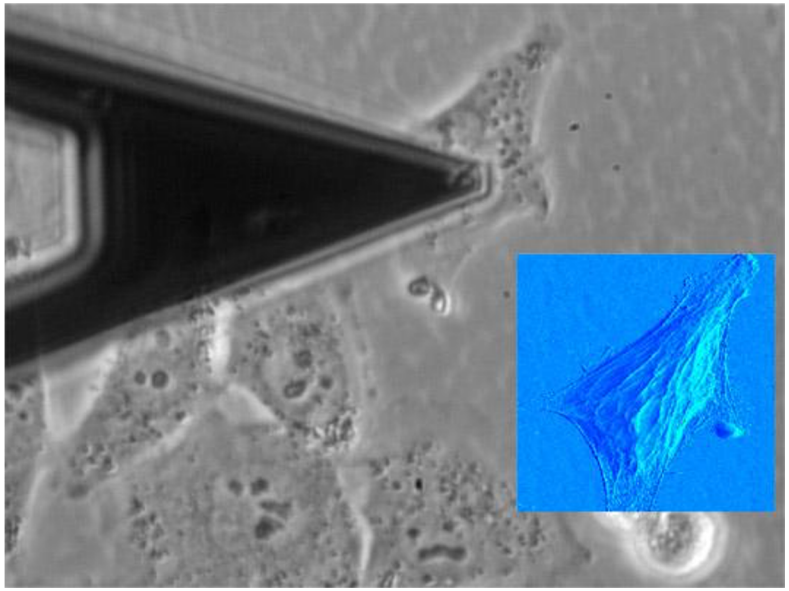
'Point and Click' Force Curves





Above: Deflection image of cancer cell with force distance curves at user defined locations

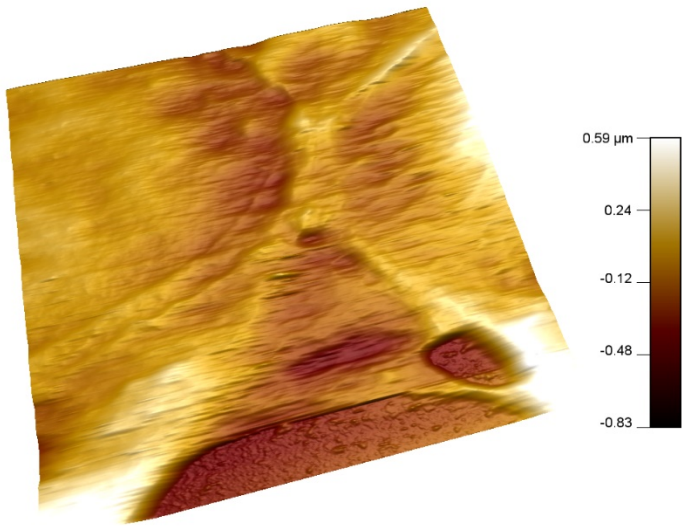
Left: CCD image of tip and cells

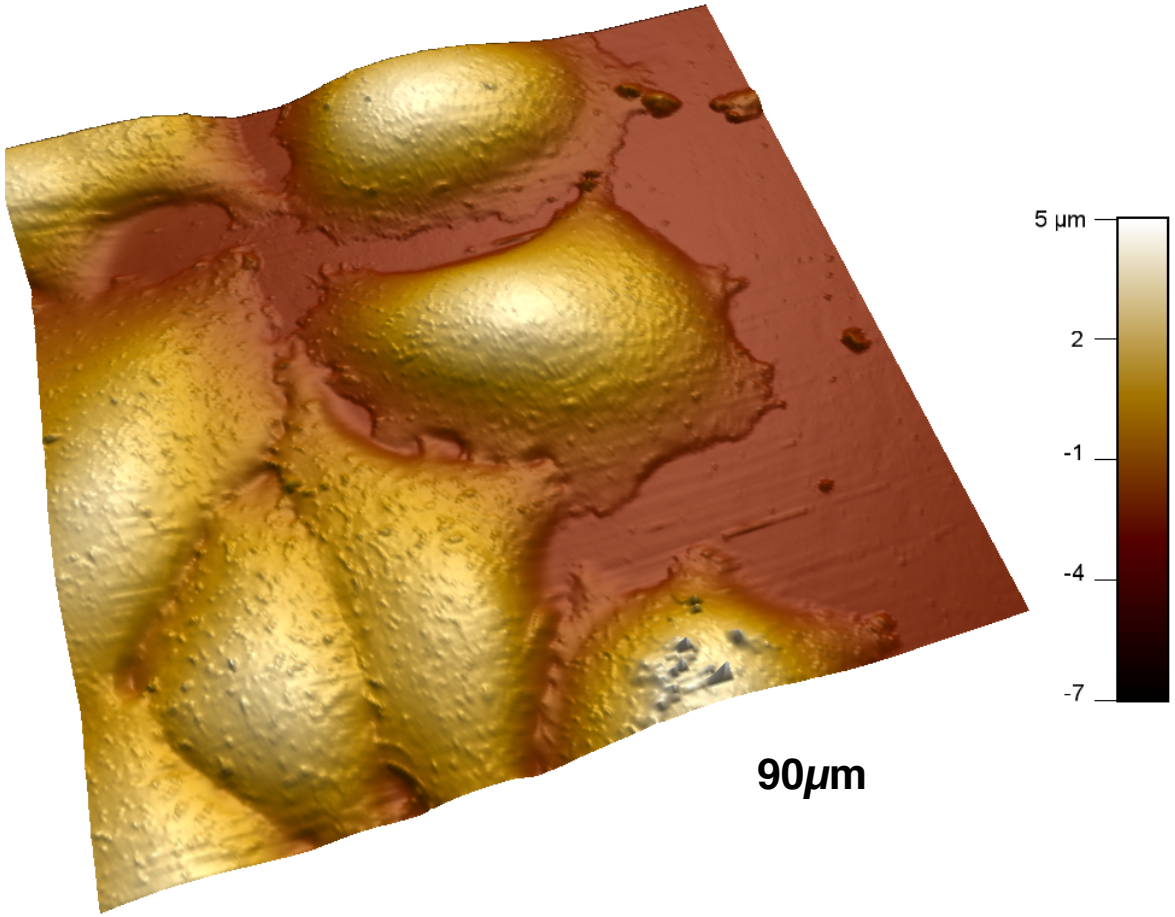
Sample courtesy of Denis Wirtz, Johns Hopkins University

Breast Cancer Cells

MCF7 Cell Line

Closeup: between cells 25*µ*m



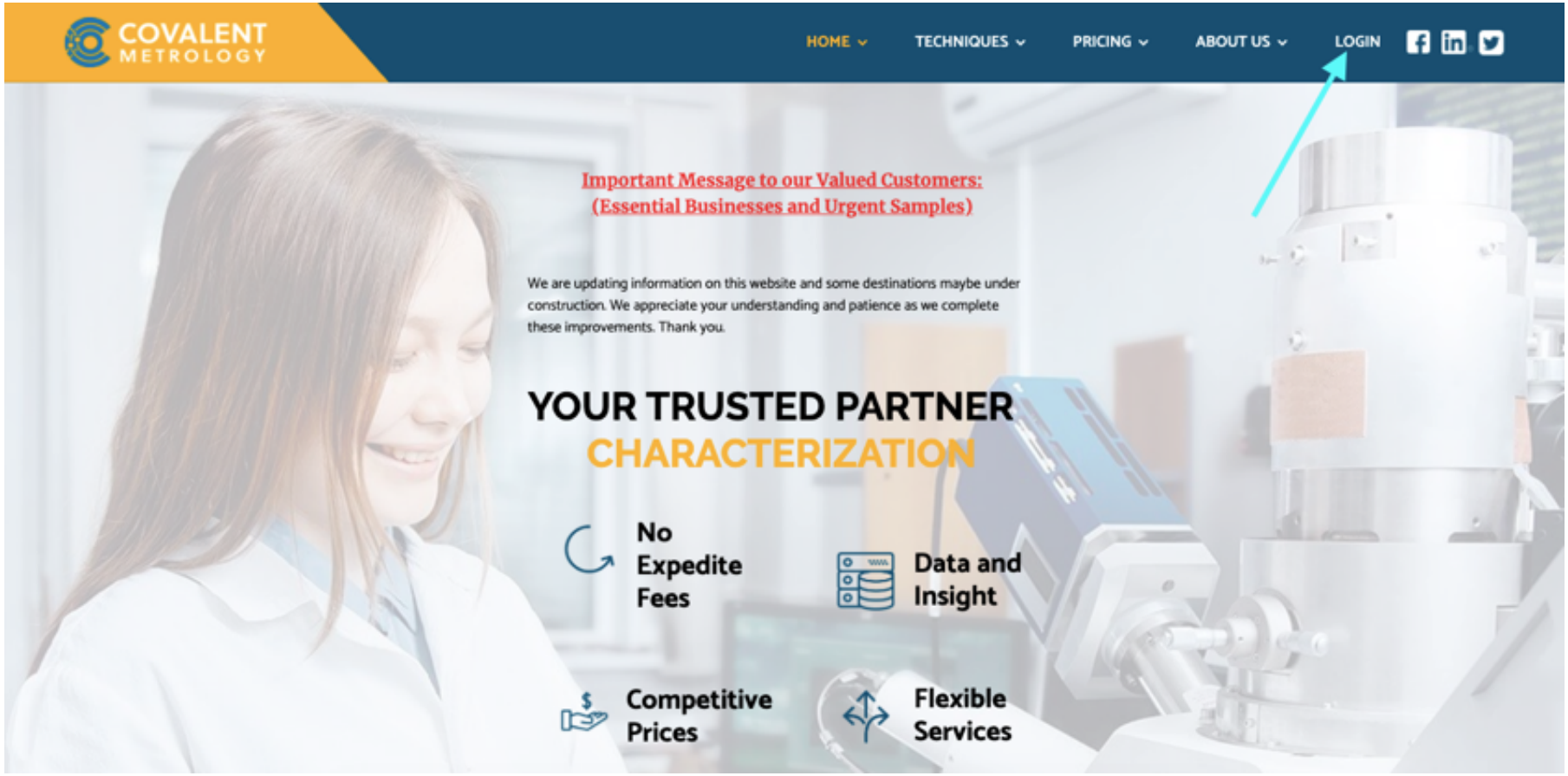


90*µ*m

The Next Episode



Covalent Community



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Covalent Community

All Other Users

The COVALENT COMMUNITY PORTAL requires password entry. It contains webinar and other metrology and characterization-related content that we believe would be useful and educational for the materials science innovation community. It does not provide access to any customer data and should only be used by individuals that are not Covalent customers or lab partners.

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