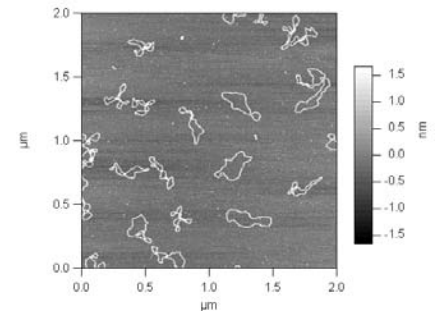
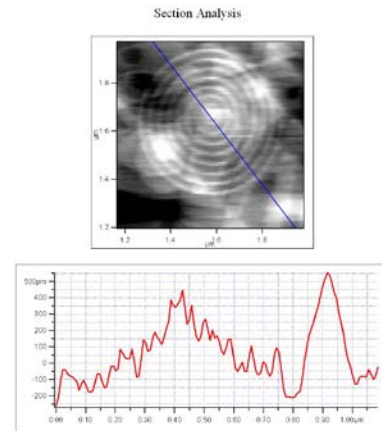
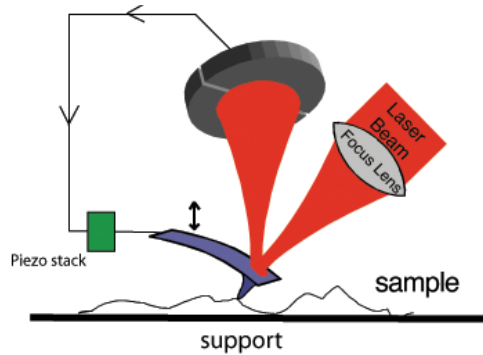
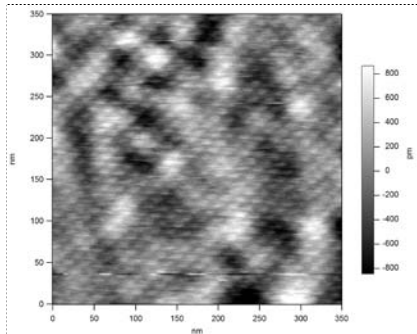


AFM Sample Preparation in Biology



Outline

1. **Substrates: flat and smooth**

- Mica/AP-mica
- Glass
- HOPG
- Other substrates

2. **Sample Preparation**

- DNA: See Experiment #2
- Proteins
- Lipids
- Cells

3. **Cantilevers**

- AC mode
- Contact mode

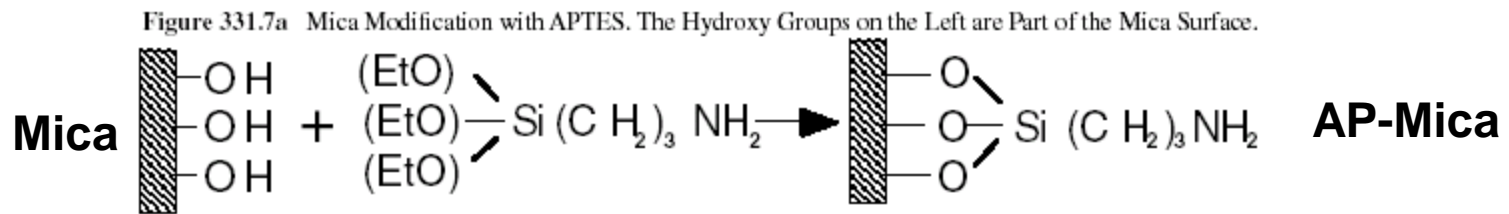
Mica

- Cheap, atomically flat
- Muscovite or Ruby
- Step of an individual layer = 1 nm
- Hexagonal lattice = 0.52 nm, RMS = 0.06 ± 0.01 nm
- Grade: typically V1 or V2 which means it is atomically smooth and has minimal steps, ideal for AFM imaging:
 - http://www.2spi.com/catalog/submat/mic_shet.shtml
- Clean surface once cleaved: use a punch set, do not cut with scissors!
- Known chemistry: negatively charged in liquid
- Prepare by gluing mica disks to glass slides using 5-minute epoxy (Devcon)

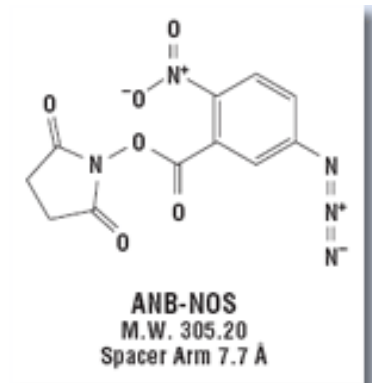


Modified Mica Surfaces

- Cross-linking groups to the surface
- Uses silane groups that protonates at neutral pH
- Example 1: 3-Aminopropyl-triethoxysilane (APTES) (Lyubchenko)



- Example 2: N-5-Azido-2-nitrobenzoyloxysuccinimide (ANB-NOS) (Karrash)
 - Reactive NHS ester and nitrophenyl azide
 - Targets amino groups
 - Can be used as a crosslinker,
 - with AP-mica



Glass

- More rough than mica or gold
- For cells, large molecules and large samples in general
- Problem: Cleaning. Lots of recipes!

Highly Oriented Pyrolytic Graphite (HOPG)

- Bad reputation! Steps mimic DNA helix perfectly.
- But great for proteins. They seem to adsorb better than on mica.
- Cheap, easy to cleave, atomically flat.

Other Substrates

- Poly-L-Lysine coated glass: Positive surface
- Silanized glass: Glass is hydrophobic. Useful for air imaging. *(Not so useful for liquid imaging: Looks like silicon nitride tips interact strongly with hydrophobic surfaces and proteins tend to denature upon contact).*
- Gold: Vapor deposition onto glass or mica or epitaxially grown gold surface Au(111). Chemically inert, can bind thiols or bifunctional disulfides: binds proteins.
- AP-glass
- Silicon

Summary: Substrates + Samples

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

Sample Preparations

- Problem: Functionality of adsorbed molecules?
- Technique 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.

Sample Prep: DNA

Imaging Conditions:

- Concentration: 1-5 $\mu\text{g/ml}$
- Substrate:
 - mica (negatively-charged)
 - AP-Mica (positively-charged)
- Buffer: contains divalent cations
 - ie- 40mM HEPES-HCl, 10mM 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.

Sample Prep: Proteins

Imaging Conditions:

- Liquid is preferable. In air, salts crystallize.
- Concentration: 1 to 100 $\mu\text{g/ml}$
- Substrate: HOPG or mica
- Buffer: pH < pI to promote positive charges
- AC mode

Protocol:

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

Balancing Electrostatic Forces

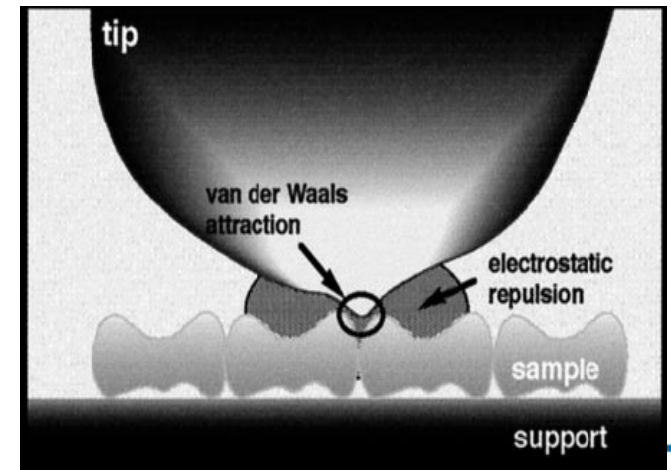
Main Forces in Liquid:

- 1) Van der Waals, globally **attractive**, short range.
- 2) Double layer forces: **Repulsive**, long range, dependent on pH and ionic strength. Due to ionic atmosphere over the surfaces of the tip and the surface. The 2 layers create the repulsive force.

Try to balance the attractive Van der Waals force with the repulsive double layer force, using high ionic strength. In general, play with the pH and the concentration of salts

Example: Müller et al. (1999), Biophysical J. 76: 1101-1111.

100 to 200mM of monovalent cations and 50mM of divalent cations at neutral pH.



Sample Prep: Cells

Imaging Conditions:

- Cell Concentration: 70-80% confluent
- Substrate: glass coverslips, glass-bottom/polystyrene petri dishes
- 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: $\Delta V \sim 0.5V$

Protocol:

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

Sample Prep: Bacteria, Yeast

Helpful Hints:

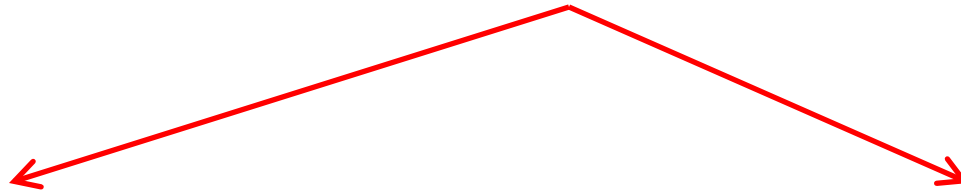
- Bacteria and yeast cells are particularly hard to attach, unlike adherent mammalian cells.
- Successful immobilization methods include: “trapping” them in cellulose membrane pores or use poly-L-lysine coated glass slides.
- AC Mode in Fluid only

Sample Prep: Lipids

Protocol:

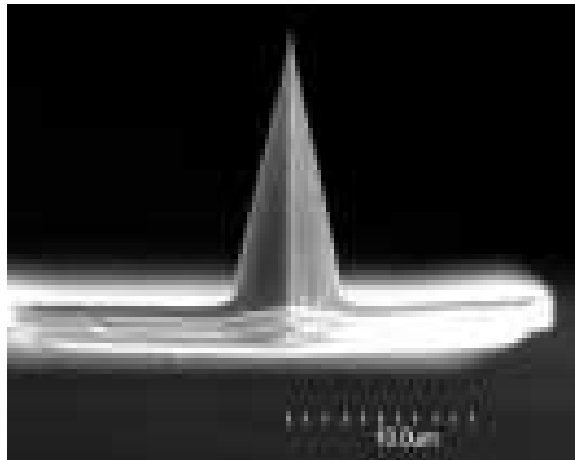
- Lipids will adsorb to mica. Easy!
- Two Methods:
 - Langmuir-Blodgett technique
 - Vesicle Fusion Method
- Buffer: specific to the lipid

Cantilevers



Silicon

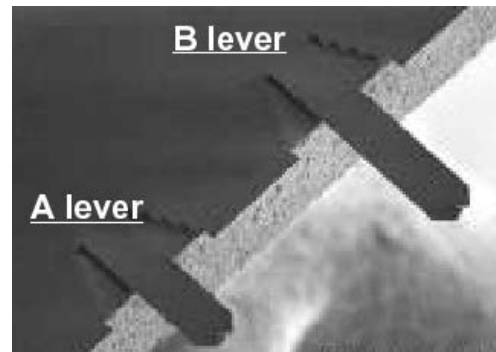
- For: AC mode air
- Typically are not problematic



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

Cantilever Specifications

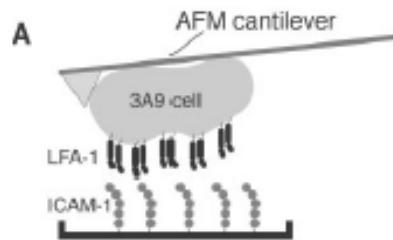
CANTILEVER	LENGTH (μm)	RESONANT FREQUENCY: AIR (kHz)	SPRING CONSTANT (N/m)	THICKNESS (μm)	TIP HEIGHT (μm)	TIP RADIUS (nm)
AIR IMAGING						
Olympus AC160TS	160	300	42	4.6	11	10
Olympus AC240TS	240	70	2	2.7	14	10
FLUID IMAGING						
Olympus Biolevers, RC150VB (coated on both sides)	60 100	37 13	0.027 0.006	0.18	5.0-10	25 (max 40)
Olympus TR400PSA	100 200	34 11	0.08 0.02	0.4	2.5-3.5	15 (max 20)
Olympus TR800PSA	100 200	73 24	0.57 0.15	0.8	2.5-3.5	15 (max 20)
Olympus RC800PSA (rectangular)	100 200	69, 71 18, 19	0.39, 0.76 0.05, 0.10	0.8	2.5-3.5	15 (max 20)
Veeco DNP-S	100 thin 100 thick 200 thin 200 thick	56 57 18 12	0.32 0.58 0.06 0.12	0.6 (0.4-0.7)	2.5-3.5	10 (max 40)
Veeco DNP (specs same as for DNP-S except for tip radius)	100 thin 100 thick 200 thin 200 thick	56 57 18 12	0.32 0.58 0.06 0.12	0.6 (0.4-0.7)	2.5-3.5	20 (max 60)

Special Cantilevers

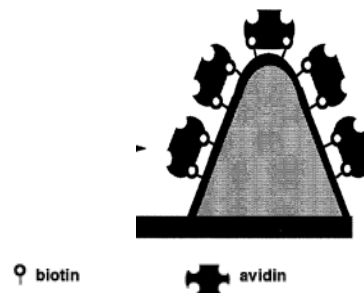
- High aspect ratio tips from IBM
 - EBD tips (electron beam deposited)
 - Nanotubes: multi walls, single walls, for air or liquid?
- Less and less hope!

Tip Functionalization

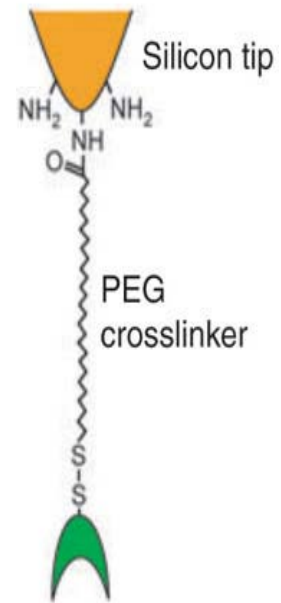
- Chemical coating: silanization
- Biological coating, for force measurements mainly:
 - non-specific binding of protein
 - specific binding of protein using a linker such as PEG
- Cells
- NOTE: The tip may no longer be sharp!



V.T. Moy Lab



Florin et. al. Science 1994.



AFM Tips & Cantilevers

Tips are critical for resolution but they are often dull, dirty, fragile and even broken! **They are completely inconsistent!**

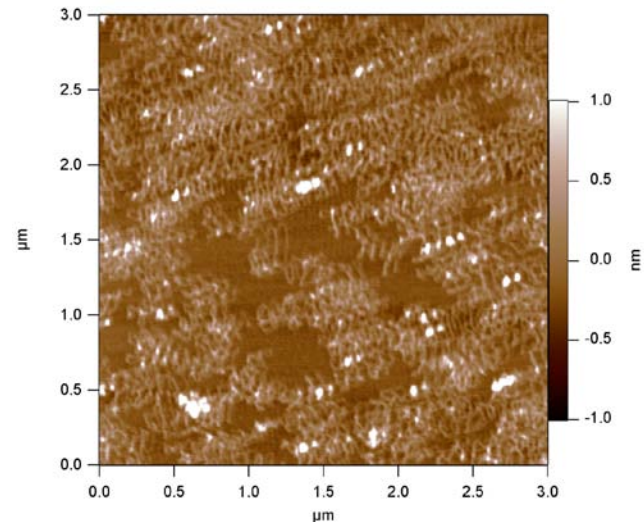
New users often don't recognize when they have a bad tip.

Tip Contamination

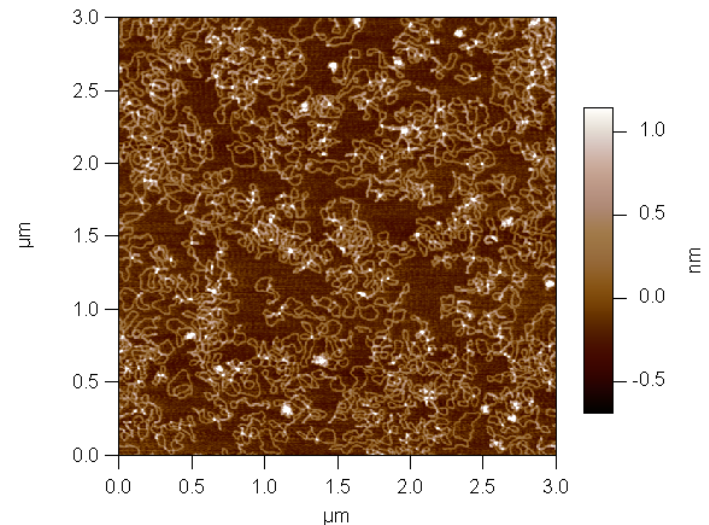
- Contamination is very common in biology, most biomolecules are sticky and will attach to the tip.

Symptoms:

- 1) features appear larger,
 - 2) multiple features appear in the same orientation – “double tip”
- Cleaning tips before use: UV light, piranha solution, plasma etching (argon plasma) but in most cases, using a new cantilever is the best solution.



DNA sample: “Triple Tip” Artifact



Same DNA sample: “good” cantilever

Tip Shape Evaluation

- EM: but resolution is too low.
- MFP-3D Software: Blind reconstruction of the tip shape using a surface with spiky features.

Conclusion

- Sample preparation is the most important step but can also be the most frustrating -- it is the first step and without a good prep you will never get to optimize your AFM imaging.
- When you cannot obtain an image, you don't always know if there is a problem with sample preparation or the cantilever (blunt/dirty tip, etc.). But if you change the cantilever (and use cantilever from different production batches) and continue to see the same thing, it is most likely due to sample preparation.