ATOMIC FORCE MICROSCOPY LABORATORY EXCERCISE

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1. Introduction

Atomic force microscopy is a imaging modality that can generate maps of surface topology with nanometric precision. The purpose of this unit is to understand and learn how to use an atomic force microscope (AFM). In order to do this unit, you will need to understand how an AFM works, and how it is operated. An introduction to the AFM, from the AFMWorkshop TT-AFM manual, can be found here. A set of tutorial animations that explain the principles of the AFM can be found on here

Please note: the TT-AFM manual is provided to us by AFM Workshop for use by students in the Advanced Bio-engineering Laboratory (83-411). This link should not be published or shared outside of this course.

In this set of lab exercises, you will:

(1) learn how to use the AFM.

(2) measure a set of calibration samples to check the calibration of the instrument.

(3) use your calibration data to correct the XYZ measurements of the AFM

(4) measure a set of nanometric scale objects (gold nanoparticles, DNA plasmids)

(5) process your results and present them in a report

2. Materials and Methods

2.1. AFM. The laboratory is equipped with two AFMs. One is a TT-AFM and the second is a TT2-AFM, both designed by AFM Workshop (Hilton Head Island, SC, USA). They are very similar, and both will be referred to as TT-AFM in this document. The manual for these instruments can be found here. The manual decribes Version 3 of the software. We are now using Version 4 of the software, whose manual can be found here. Also, the appendix on measurements is very useful.

2.1.1. *Modes of operation.* The TT-AFM can scan images using either vibrating or non-vibrating modes. In the non-vibrating mode (often called contact mode), the AFM tip contacts the surface, and the image is generated by either following the displacement of the optical cantilever, or (more commonly) by using feed back to maintain a constant cantilever displacement and measuring to vertical movement of the Z-piezo that is required to do that.

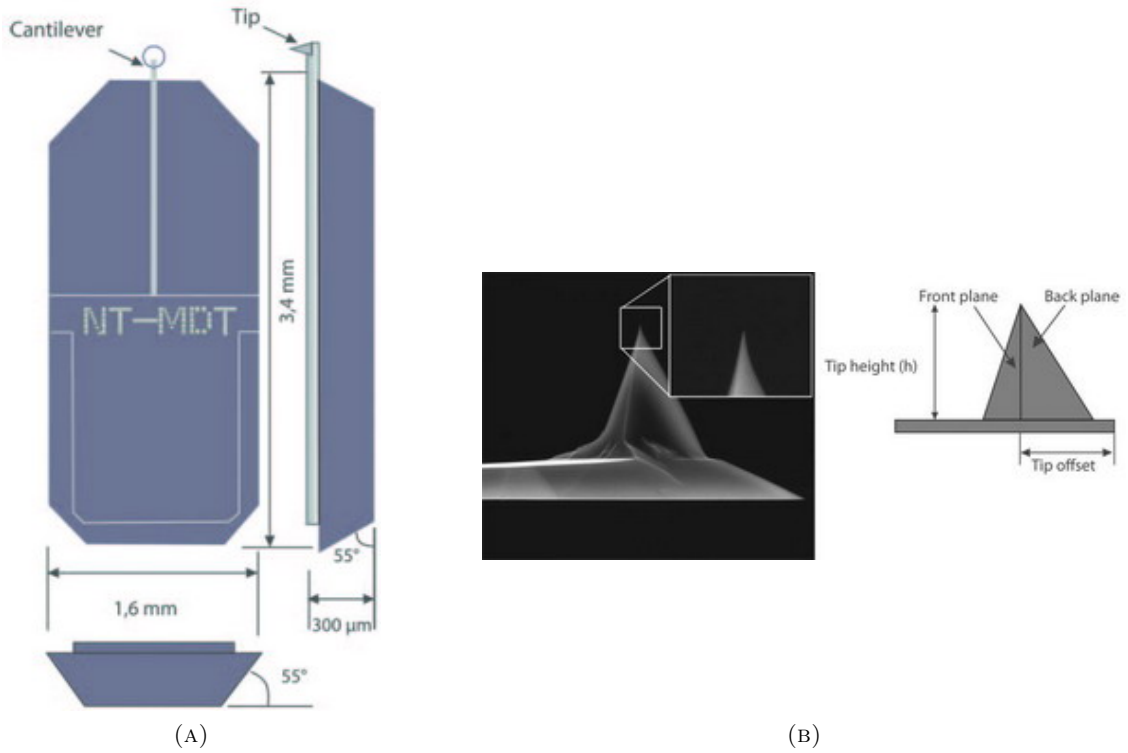
In the vibrating mode, the cantilever is excited by a piezo-electric "tickler" at its mechanical resonant frequency. As the tip approaches the surface, the amplitude of the mechanical vibrations decreases due to mechanical damping caused by interaction with the surface. Typically the instrument will be set to hold the tip at a distance where the amplitude has decreased to 60% or 70% of the free space amplitude. As the sample is scanned, the Z-piezo uses feedback to maintain the damped vibration amplitude, and the feedback signal is used ot construct the image.

For more details on these modes, and for instructions on how to implement them on the TT-AFM, see the manual. We will mostly use the non-contact (vibrating) mode for the lab exercises.

2.1.2. *Tips.* The tip cannot be seen, as it is well below the resolution of optical systems that use visible light wavelength (for example, our eyes). The tip sits on an optical cantilever that is also quit small, but can be seen if one has good eyes. A description of the geometry of the chips, cantilevers and tips used in our lab can be found here. Figure 1 shows the (industry standard) chip dimensions for the chips used in our lab. The small cantilever can be see at the top of the chip.

For tapping mode the lab has standard long cantilever probes with a nominal mechanical resonance of 190kHz (note that they are actually specified for a range of 160-220kHz). The specifications can be found here. We also have one box of short cantilever probes with a nominal mechanical resonance of 300kHz (200-400kHz). The specification of these probes can be found here.

We also have contact mode probes, whose specifications can be found here.



(A) (B)

FIGURE 1. (A) Industry standard AFM chip that holds the cantilever and tip. (B) Expanded view of the tip. Images taken from NT-MDT website.

The tips that we use cost $20 each, and they break very easily, so you must be careful when inserting or removing samples, and in doing the tip approach that brings the tip to the surface. Being careful means:

(1) Be sure that the light lever is fully raised when inserting or removing samples.

(2) Always set the stepper mode speed to its minimum when lowering the light lever prior to automated tip-approach.

(3) Vibrating mode tips are not the same as contact-mode tips. Be sure that the mode matches the tip that is inserted into the light lever.

(4) Be sure that the resonant frequency is set when using vibrating mode. Also check that the free space amplitude is between 0.6V - 1V.

(5) Never operate the stepper motor when the tip is in contact with the surface. use the tip retract function to retract the tip before engaging the stepper motor.

2.1.3. *TT-AFM Software.* The TT-AFM is operated with a Labview based control program, which is documented in the software manual. The images that are acquired must be saved to the computer that controls the TT-AFM. We will the following conventions:

(1) Each pair of students will create a folder on the desktop for their data. Typically, the folder will be named in a way that identifies it with a particular pair.

(2) Each day's work will be stored in a subfolder named YYMMDD where YY is the year, MM is the month and DD is the day of the session. For example, 4 March 2020 will be 200304 (note that we zero-pad the single digits so that the names will align correctly).

(3) The images will be save using the standard name that the TT-AFM software assigns, except that the first words "AFM-scan" will be replaced by something descriptive of the sample (for example, SHS1A if region A of sample SHS1 was scanned.

At the start of the session, the directory in which the images are saved must be set to the folder that was created. Otherwise the images go to some default that we need to search for.

2.2. Samples. The lab is equipped with a collection of commercial test samples. These are samples with well defined geometries that can be used to calibrate the instruments. In addition we have some samples that are prepared in-house.

2.2.1. *Commercial standards.* Below is a list of standards that we have, together with links to their specifications. They include hyperlinks to their specifications, where these are available.

(1) HS20MG and HS100MG 20 nm and 100 nm height standards. The actual heights as indicated by the manufacturer are slightly different (for example, 113 nm for one of our HS100MG samples), are written on the box holding the sample. It is important to check this value, **and it is very important to return each sample to its box**.

(2) SHS-0.1 100 nm height standard. Our sample is specified to be 104 nm. We found that this sample is quite a bit under its specification on 104 nm, so it is not used for calibration any more.

(3) 628 AFM Tip and Resolution Test Specimen. Contains 1–5 nm cobalt particles.

(4) SiC/1.5 Silicon Carbide 1.5 nm height standard

(5) 677-AFM and 607-STM waffle patterns

(6) TGT-1 2D array of sharp tips.

(7) Tip Checker for AFM probes.

(8) BS01 circular plasmid DNA pUC19 and a filamentous bacteriophage M13.

(9) DNA01 Plasmid pGem7zf+ (Promega) which is linearized with the SmaI endonuclease. Linear DNA molecules (3000 b. p.) are deposited onto freshly cleaved mica.

We also have the AFM Workshop test sample kit, which includes:

(1) Highly oriented pyrolytic graphite.

(2) Graphite

(3) A piece of DVD with protective covering removed.

(4) Waffle grid

(5) Aluminum foil (shiny side and dull side)

(6) A blob of polymer.

2.2.2. *Samples prepared on-site.* We have some samples that are prepared in-house. These include:

(1) 16200 Gold Nano-Particle (GNP) calibration kit. This kit includes 5, 15 and 30 nm GNP.

(2) 30 nm GNP produced in Prof. Popovtzer's lab.

(3) Dried, fixed cells

We are open to trying samples such as graphene, or items produce in other laboratories, is such are available.

2.3. Image analysis software. The AFM images produced in the lab are processed using the open source Gwyddion software package. Gwyddion runs on Linux, Macos, and Windows. You will need to download and install it on your personal computers, in order to analyze the results. You will find a number of useful examples in a file called tipAndTricks.pdf. Gwyddion can load the WSF files produces by the TT-AFM, and import the metadata from these files.

If you wish to process the images using ImageJ/Fiji, then you will need a script that we wrote to import the WSF files into ImageJ/Fiji. It can be found here.

Note that you will probably not be able to email the WSF files anywhere, because Google's protection software thinks that WSF files are evil. Our recommendation is to upload them to a Google drive or some other cloud service.

3. Experiments – general issues

The experiments will mostly focus on the standard targets, and the objective will be to calibrate the instrument. That is, you will determine a set of values that can be used to correct any bias in the instrument's calibration. For example, if the 104 nm standard consistently measures 110 nm in height, then a factor of $\frac{104}{110}$ should be used to correct height measurements with this AFM. The same is true for the lateral (XY) calibration.

When imaging with the AFM, it is necessary to choose an imaging area, resolution, and scanning speed that are appropriate for the sample of interest. For example, when scanning a 3 µm grid, a scan area of 10–20 µm is appropriate, since that includes a reasonable number of the grid period, but is not so large that the fine structure is lost. The resolution (number of pixels in the image) must be high enough to resolve teh features of interest, bt not so large that the scan takes too long. We would like to scan quickly, but if the scan speed is too high, the feedback cannot keep up, and image features will be distorted of blurred. A rule of thumb is to do an initial scan at 128×128 pixels, with a scan speed of 0.5 or 1 Hz. Then, when an acceptable image is obtained, the scan area and resolution can be adjusted to fit the features of interest, and a scan speed of 0.25 or 0.5 Hz is typically used. The scans can take between 2–20 min, so it is a good idea to have a laptop so work on previous data, learn Gwyddion, etc during the scans.

Other parameters of interest include:

(1) **Extension factor:** this parameter determines when the tip is considered to be in contact with the surface. It meaning in mode-dependent:

vibrating mode: This is the percent of free space amplitude at contact. For example, if this factor is 0.7, then contact will be defined as a vibrational amplitude that is 70% of the free space (fully retracted) amplitude. Typically, this is set at 0.6–0.7

contact mode: This is the amount that the TOP-BOTTOM signal changes as the cantilever is pushed up by the surface. For example, if the free space value of TOP-BOTTOM is 0.7, and the extension factor is 0.2, then contact will be detected when TOP-BOTTOM reaches 0.9.

(2) **HV:** HV means "high voltage", and it refers to voltage applied to the lateral (X,Y) and height (Z) piezoelectric actuators. It ranges from 0–15. When set to 15, the X and Y axes have a 50 µm range, and the Z-axis range is 17 µm. Reducing HV will proportionately reduce the range. For example, if the Z HV is set to 5, then the Z-axis range will be 17/5 µm.

The reason for doing this is that the total range is digitized to a fixed number of bits (16). Sixteen bits can represent $2^{16} = 65536$ values, which means that resolution of the piezo will be (full-range)/65536. By reducing the full range, but retaining the same number of bits in the digitization, we have better resolution. Therefore, when measuring a objects whose height is not over a few nm, and when we want sub-nm resolution, we may trade off range for resolution.

3.0.1. *Z_DRIVE vs Z_SENSE.* Usually, the TT-AFM is operated in feedback, so that height measurement is derived from the Z_DRIVE voltage to the Z-piezo that is required to maintain constant cantilever signal. However, for large displacements (even 10's of nm), there is significant hysteresis in the piezos that caused the height to vary as a function of scan direction.

The Z_SENSE signal is derived from the strain gauges that actually measure the piezo displacement, and can also be used to measure the height. This signal does not suffer from hysteresis, but it has higher noise than Z_DRIVE. In general, Z_SENSE will be used when the noise is acceptable, and Z_DRIVE will be used when measuring small displacements where the noise must be as small as possible.

This issue is discussed in a document that can be accessed here.

4. Experiments to do

Here is a set of experiments that are done in the AFM lab. However it is likely that in a given year, we may decide to focus more on certain issues (eg, spatial calibration, periodic noise, piezo-electric hysteresis, etc)

4.0.1. *Height standards.* Scan three regions of the HS100MG standards. You will image one of the 5 µm grids, one of the 10 µm grids, and one of the 5 µm gratings. With the HS100MG, we have a choice of features that are wells or pillars, so we will try to image at least one of each type. After these images are acquired, we may have the two parallel groups exchange samples, in order to see how the two instruments compare.

Use the Gwyddion softare to process and measure the lateral (XY) periods of grids or gratings, and the height of the features. First level the image, as then use Gwyddion's measuring tools to measure distance or height.

4.0.2. *DVD or Blu-Ray disk.* Image a piece of either a DVD or Blu-Ray disk. We will give one group Blu-Ray and the other group DVD. Start with a 5 µm × 5 µm area for the initial scan, and then choose an appropriate area to include 3–4 tracks in the scan.

You should measure:

(1) Intertrack distance

(2) Width of the features that are burned into the optical disk.

(3) Try to find the smallest length of the bits that are burned into the disk.

Find the DVD and Blu-Ray standards on line (whichever is appropriate to your measurement), and compare your results to the published standard. Discuss your results in view of the diffraction limit of visible light.

4.1. Waffle test pattern and TGT-1 test sample. Other test targets such as the waffle test pattern and the TGT-1 test target will be measured as time permits. For these patterns, the height is not tightly specified, but the lateral spacing is. You should scan these targets, and compare the instrument's calibration ot the known target geometry. In this case, you should use your results from the HS100MG or SHS-0,1 to correct the measurement, and then compare to the known geometry.

4.2. Silicon Carbide (SiC) test sample and 628 test target. The SiC test sample is a cleaved crystal of SiC that has atomic sized terraces of 1/5 nm height. Scan a 1 µm–2 µm square of this sample, to image 5 to 10 terraces. You will want to lower the Z-HV parameter to 5 in order to have finer height resolution, and use the Z_DRIVE signal. You will need to figure out how to flatten the image. Since the SiC target is by a set of steps, global flattening methods will not work, at least not by themselves.

The 628 test target is very challenging, becasue unlike the SiC test sample, there is not a large XY area over which to average. However, it is relatively simple to flatten. If there is time, you will scan this sample and try to detect the cobalt nanoparticles.

4.3. Gold nanoparticles. Scan samples of gold nanoparticles (GNP). Again, the Z-HV and other scanning parameters should be appropriate for high resolution scans. The exact setting will depend on the sample. Presently, we have 30 nm GNP samples prepared.

4.4. DNA and plasmids. As time permits, you will scan either the DNA01 or BS01 samples,and try to detect plasmid DNA.

5. The lab report

All material that is quoted or based on outside sources must be referenced. This is done by putting a number next to the relevant text and listing the citation in a reference list at the end of the report. All figures and tables must be numbered, have a caption, and be referred to in the body of the report.

The lab report should contain the following sections:

(1) A brief introduction that explains the goals of the experiments that are presented in the report. You do not need to give a detailed explanation of how the AFM works. You only need to state what is unique to the experiments that are presented.

(2) A materials and methods section that describes the instrument, samples, and tools tha were used. Again, you do not need to repeat the TT-AFM use manual. This section must include:

- Name and model number of the instrument (TT-AFM or TT2-AFM, Hilton Head Island, SC, USA). Note that one of the instruments is a TT-AFM, while the other is a TT2-AFM, Be sure to specify which was used.
- A list of the sample that were used, and a brief description of the main features. A hyperlink to their specs is a good idea.
- A brief description of the analysis software (Gwyddion and maybe Fiji) with a reference to where it came from. You should also include a brief desciption of the methods that were used (eg, flattening methods, measurement tools, etc).

(3) Experimental results. These should be concise. Do not flood the report with images. Any image that is included must add information to the report. In particular, do not add 3D images everwher just because they look nice. In general, a representative image of each sample can be included, and then the results should be tabulated as appropriate. For example, if three images of some grid were acquired, just include one representative image in the report.

The conditions for which the image was acquired must be stated. These include:

- AFM mode (vibrating or contact)
- scan area (a scale may be sufficient)
- Important instrument conditions (Z-HV, scan speed, scan resolution, extension factor, other paramters if changed from default). Which channel (Z_SENSE, Z_DRIVE, Z_ERROR, etc) is the iamge from.

You must state which methods were used in the analysis. Methods that will be used probably include:

- Flattening algorithms. many are available, and you must choose waht works. You need to state what was chosen, and why.
- Distance and height measurements. If you measured using line profiles, you must have a figure that shows where the line was placed, and you need to explain how the measurement was done. If you defined a mask and measured (for example) the average height of the masked area, you need to show an image with the mask, so that he reader knows which area was measured.
- Noise reduction operations. If any filtering or averaging was done, this mus be stated.

If your results had some problem, document it in the report. There may be periodic noise, random noise, a worn tip, damaged sample. write was is, not what should have been.

(4) Conclusions. This section summarizes the report, and lists the major conclusions. Do not just write "The AFM is a cool intrument". Write about whether the calibration was correct, noise issues that you encountered, what worked, and what did not work.

Appendix **A.** Operation of the AFM

You will need to read the TT-AFM manual in order to operate the instrument. Here, we will outline what needs to be done, to help you look for what you need to know.

(1) Initial steps: turn on the AFM, the computer, and the coaxial LED that illuminates the sample.

(2) Set up the output folder as described above.

(3) Confirm that the light level is fully raised (should have been raised at the end of the previous session)

(4) Launch the TT-AFM software and the camera software. The camera software is independent of the AFM software, and enables you to see the sample and cantilever. Insert a sample and verify that the sample is in focus (the cantilever will be out of focus and not seen if the light lever is raised).

(5) Do a range check, verify that the reflected spot is centered (vibrating mode) and that T-B is in the range 0.7–0.8 V

(6) Do a resonance check (vibrating mode only)

(7) Manually lower the light lever until the tip can be seen approaching the sample. For samples that scatter the laser light back to the camera, you will see a red spot approaching the tip. Stop **before** the tip hits the sample. Samples that do not scatter light are more challenging, and you will need to develop a feel for when the cantilever is just a bit out of focus, at which point it is near but not touching the surface.

(8) Do an automated tip approach. When the tip in feedback, go to the scanning tab.

(9) Set up the scan parameters, scan the sample, and save the scan in the appropriate folder, after naming it as desired. **If you close the save windows without saving, you will not have another chance to save your image.**

(10) At the end of the session, upload your folder to a Google drive or whatever cloud service that you use. You will probably not be able to email the files, because they use wsf as their suffix.

Appendix B. Processing the data

The AFM scans will produce images that will need to be processed. You will need to learn how to use the Gwyddion software package. This package should be installed on your personal computer, and we recommend that you have a laptop in the lab, so that you can learn how to use Gwyddion while waiting for scans to finish.

As noted above, you will find a number of useful examples in a file called tipAndTricks.pdf.

Appendix C. Issues of Interest with the AFMs

Most of the AFM lab unit is devoted to characterizing and understanding our two AFMs. This is not just done as a standard teaching lab exercise whose result is already known. Instead, our results often raise questions that require further study. Below is a set of links to files that document some of these issues.

Scan distortion: The first few scan-lines of the TT2-AFM appear distorted. This phenomenon is documented here.

Height standards: Our SHS-0.1 standard appears to be much lower that specified (around 70 nm instead of 104 nm. In addition, Z_DRIVE measurements are affected by piezo-hysteresis. Z-SENSE, which follows the signal on the strain gauges, should be used wherever the SENSE signal is not too noisy.