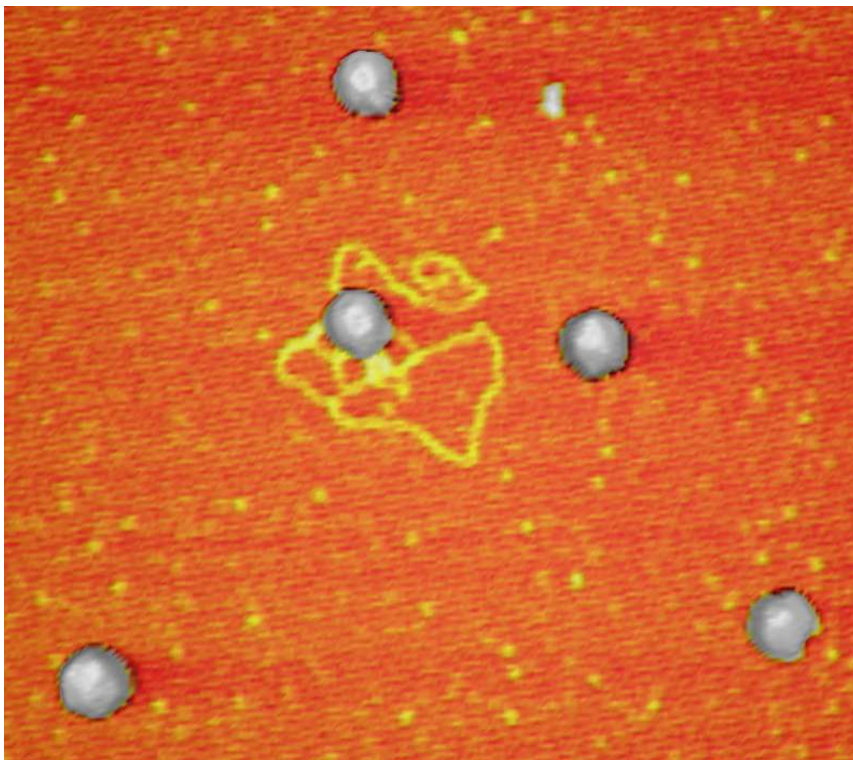




AFM imaging and Force measurements using Asylum MFP-3D

I. Introduction

In this lab we will perform AC mode AFM imaging of virus particles in liquid. The virus particles are adenovirus that were provided by Prof. Aravind Asokan from the UNC Gene Therapy Center. The main purpose of this lab is to introduce workshop participants to the general issues involved in AC imaging in fluid. If time permits and samples cooperate, we will also perform mechanical evaluations of the virus capsids using force vs. distance tools available to the AFM instrument.




Adenovirus particles imaged by Atsuko Negishi (UNC-CH).

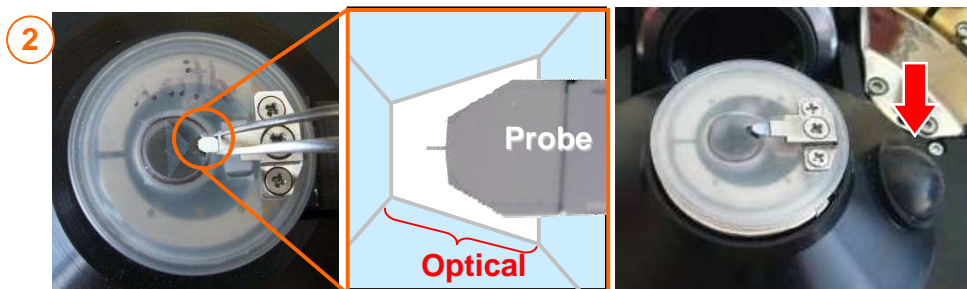


Most of the following lab protocol describes how to operate the Asylum MFP-3D. This write-up was made using edited excerpts from materials produced and generously provided to us by Ryan Fuierer of Asylum Research.

II. AFM setup and AC mode Imaging in Air.

10 steps to acquiring an AC mode image on the MFP-3D

- 1) **Open MFP-3D™ software:** click on icon on computer desktop 
- 2) **Loading Probe:** A) gently slide probe under tongue such that cantilever is in middle of clear trapezoidal window; tighten screw finger tight with provided screwdriver. **Do not over-tighten screw!**
B) Depress kinematic lever in head (red arrow); place cantilever holder in head



- 3) **Place head over sample; Using two hands, gently place head legs through holes in scanner; be sure to have legs up high enough to avoid crashing tip**



- 4) **Turn on CCD camera, and Fiberlite:** click on CCD icon in software (bottom left hand side) to bring up CCD window in software; Turn Fiberlite on with remote knob on desktop. Adjust camera position and focus located on back of head.

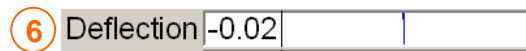


- 5) **Aligning the SLD:** turn on SLD on by turning key on front of controller 90° clockwise; using the LDX, and LDY thumbwheels on head, position SLD spot at end of cantilever with maximum Sum voltage in Sum & Deflection Meter.





6) **Zero PhotoDetector:** using 'PD' thumbwheel on head (left side), zero the deflection.

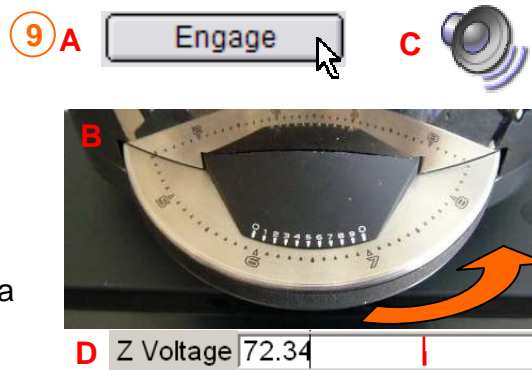


7) **Drive Frequency Auto Tune in AC mode:** In the *Tune Tab*, Click 'Auto Tune'; Software selects Drive Frequency and Drive Amplitude automatically.

8) **Parameter Selection:** in the *Main Tab*, choose a Set Point voltage ~20% of the 'free air' amplitude; Integral Gain of 10; Scan Rate= 1Hz; Scan Angle 0°. Choose scan line & point resolution to preference.

9) **Engage tip:**

- A) click the 'Engage' button on the Sum & Deflection meter;
- B) turn large thumbwheel on front of head counter clockwise until
- C) a chime is heard. At this point the 'free air' amplitude and Set Point voltage values match;
- D) continue to move thumbwheel to a value of ~70V on Sum & Deflection meter



- 10) **Start Imaging:** Click 'Do Scan' on the *Main Tab*. To fine tune imaging parameters, monitor the image quality, Trace and Retrace line scans to achieve good tracking between tip and substrate.
- Adjust Set Point Voltage (lower voltage value to increase force); - -
 - increase Drive Amplitude (typically),
 - Integral gain: low enough to still get good tracking
 - Scan Rate.



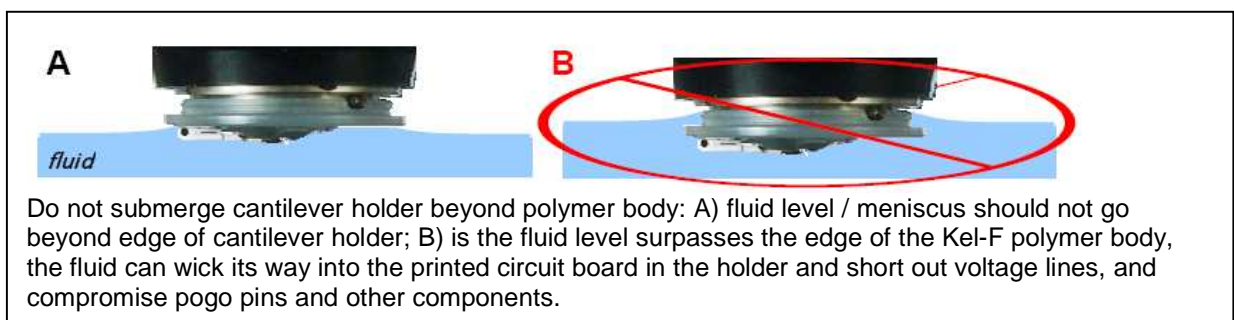


III. AC mode *IN FLUID*:

The MFP-3D™ was designed to operate in-fluid very well, and with great ease for the user. The procedure for tuning the cantilever is almost as easy as in air, but differs slightly....

Preparing for *IN FLUID* Imaging:

- Load an appropriate cantilever for your application into the cantilever holder. Typically, Silicon Nitride cantilevers are used for in-fluid imaging because they are more compliant/ flexible and can tolerate the buoyancy of the fluid dynamics a little better. Silicon cantilevers tend to chatter too much in fluid, but they are fine for force work.
- With the head on its back, gently wet the cantilever with some of the imaging fluid – this is done to help support the cantilever at the fluid/ air interface as it is plunged into the fluid around the sample. The figure to the right shows a few 10s of μL s is all that is needed- gently dispense fluid at the side of probe chip such that the fluid goes ('snakes') between the cantilever and the quartz window to ensure proper support.
- Using two hands, rotate the head, and place on sample stage. Make sure the legs are adjusted so the tip doesn't crash into the surface!
- Open CCD camera; turn on fiber light illumination so you can see the cantilever(s).





Aligning the SLD:

Once the head is on the stage, the best way to align the SLD spot on the cantilever when imaging in fluid is to use the top view optics CCD camera.

That being said, if the SLD spot can be aligned on the cantilever 'dry' (i.e., it isn't functionalized with some protein or chemistry that must stay wet), you can initially align it with the IR card in air; then plunge the tip into fluid (remember to wet the tip). After that, just adjust the LDX back towards the cantilever probe chip via monitoring the Sum Voltage in the S&D meter. The fluid's index of refraction (i.e., aqueous) causes the SLD spot to move off the cantilever once in-fluid. Generally, there is very little noticeable LDY shift when plunging the tip into fluid.

Determining Drive Frequency in Fluid

There are two ways to select a proper drive frequency-the first is the AR instructed (proper) way to do select it, and how to engage the tip; the second description is the hack way to do it, blindly developed by the author some years ago (this should be reserved for moderately desperate situations).

Traditionally, determining the drive frequency from a standard drive frequency sweep is difficult because the shake piezo oscillates the entire cantilever holder- this in turn sloshes the fluid between the probe and sample, termed a resonant cavity, and presents a 'forest of peaks' in the drive frequency sweep, often distinguishing the fundamental resonant peak from a resonant cavity peak very difficult. Picking the wrong peak can compromise the tip apex.

1) AR has worked a very nice protocol into their software in which the Thermal power spectrum can be overlaid over the Drive Frequency sweep, which allows a precise drive frequency to be determined (see Figure 6.2.5) because the cantilever's natural resonant frequency is known, relative to the peaks from the resonant cavity.

- Determine a range to do the frequency sweep from the thermal power spectrum; (i.e., perform a thermal tune); a window of 5 to 10kHz is a good place to start.
- In the Tune Tab, click the 'Append Thermal' checkbox.
- Using the Igor (Ctrl+i) cursors; enter fundamental cantilever frequency in the Drive Frequency parameter window in Tune Tab.
- Click the 'One Tune' button (with the proper frequency range selected); After the sweep, you'll see a frequency plot (black trace) over top the thermal power spectrum (red scatter points) as seen in Figure 6.2.5A. ▪ If the signal is weak, or





very noisy, you can increase the Drive Amplitude, and Click 'One Tune' again. This can give you something like Figure 6.2.5B- it cleans up the noise, or increases peak amplitudes- sometimes this is good to do to distinguish the proper resonant frequency of the cantilever from the resonant cavity peaks associated with driving the cantilever in the fluid.

- Using the cross cursor, right mouse click at the most pronounced peak that corresponds to the center or just left side to maximum of the resonant peak on the thermal tune scatter plot (red). A dialogue comes up (after you right click); choose 'Set Drive Frequency As'; this updates the drive frequency (Figure 6.2.5C).
- A plot like the one in Figure 6.2.5 D will result.
- Click the 'Center Phase' button once a drive frequency has been determined. This will center the phase at 90° , which can be seen in the S & D meter. This allows you to monitor whether the tip is in the attractive or repulsive regime, however, this doesn't hold very true in-fluid (using shake piezo drive) as it does in air.
- Select imaging parameters (i.e., set point to free air voltage ratios) and you'll be ready to Engage. Recall, choose a Set Point voltage that is lower than the free air amplitude voltage (i.e., some voltage percentage); for gentle engage, assign the Set Point $\sim 95\%$ of the 'free air amplitude voltage'.

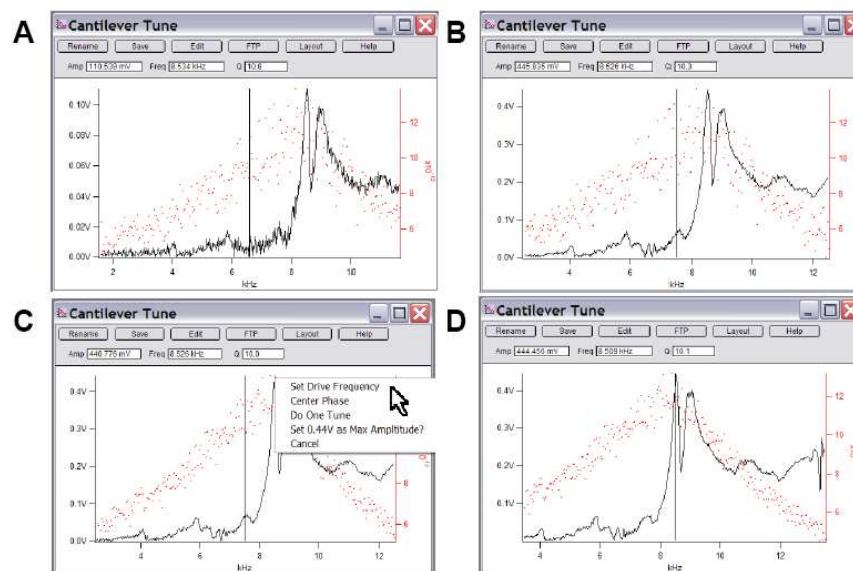


Figure 6.2.5: Performing a 'One Tune' to select Drive Frequency in AC mode: A) 'One Tune' Drive Frequency spectrum (black) with 'Append Thermal' spectrum (red scatter plot); B) Drive amplitude increased to increase S/N; C) Setting Drive Frequency with right mouse click on curve of interest; D) resulting 'One Tune' with cursor (black vertical line) at user defined Drive Frequency.





TIP ENGAGEMENT IN FLUID: Here is the way to do a soft/ gentle engage in fluid- SPECIFICALLY WITH PROTOCOL 1 for DETERMINING DRIVE FREQUENCY

- Click 'Engage' on the S&D meter.
- Take notice of the 'free air' amplitude value.
- Manually thumb the approach wheel until the feedback servo is activated.

Depending on the cantilever being used, sometimes using this 'gentle' engage approach (i.e. 95% Set Point trick) doesn't work because the piezo retracts fully upon clicking the 'Engage' button. In this case, decrease the Set Point voltage until the Piezo extends fully awaiting the amplitude dampening needed to achieve the Set Point. Then continue with the below protocol-

- As you thumbwheel down, you will notice that the Amplitude value in the S&D Meter increasing. This is (believed to occur) because of the liquid being compressed between the tip and sample from the oscillating shake piezo in the cantilever holder, effectively imparting a larger 'Free Air' amplitude onto the cantilever as it approaches the surface.
- Use the 'Hamster' wheel to occasionally decrease the amplitude to pre-approach free air amplitude voltage value to maintain the proper Set Point (i.e., ~95% of the 'free air' amplitude). If not, the Drive Amplitude will keep increasing (because the efficiency of the resonant cavity keeps increasing), until it gets very near the surface, then it will snap to contact ('hard' engage), likely damaging the tip or sample.
- If you use the 'Hamster' to continually adjust the Drive Amplitude a maintain the initial 'free air' amplitude voltage , you'll notice as the tip gets very close to the surface, the Amplitude will decrease, and will engage at the Set Point value you defined. (just as it does when soft engaging in air).
- Slowly decrease the Set Point voltage with the 'Hamster' Wheel such that the tip 'hard' engages on the surface .
- Move piezo into middle of Z range (~70 V).
- Start scanning.
- Tune parameters as described in the next section. With some samples, do not surprised if you cannot get the tip to track the surface as well as it does in fluid.





Tuning Imaging Parameters IN FLUID:

Tuning and arranging the proper imaging parameters in fluid can be a little trickier than it typically takes in air. The author suggests exercising patience, especially when imaging soft biological samples because this should be done at low scan rates (< 0.5Hz), increasing acquisition times.

NOTE: Depending on the sample, obtaining really good tracking is usually NOT a frequent occurrence when imaging in-fluid. When you get something that looks real, go with it-

It is also possible to image at very low Drive Amplitude and Set Point voltages- although usually after engagement because sometimes engagement cannot occur with these parameters ratios. The trick to this is once the tip is engaged and imaging, slowly step down the Set Point and Drive Amplitudes iteratively. It can take a little while, but the results can be very good- lower tip oscillations mean less sample perturbation (especially with cells and other bags of water).

Additionally, aside from adjusting normal tuning imaging parameters, the Drive Frequency can be adjusted with the 'Hamster' wheel until the 'sweet spot' is found. The figure below shows the results of changing the Drive Frequency on the fly to find the 'sweet spot'. During parameter tuning, monitor the image quality in the height and phase images, as well as the scan traces below the height image. The Phase image can be monitored for noise as seen in the left image. A slight adjustment reduces this noise for better image quality. The image is of a patterned protein (lighter color), and poly ethylene glycol terminated thiolate (dark region).

