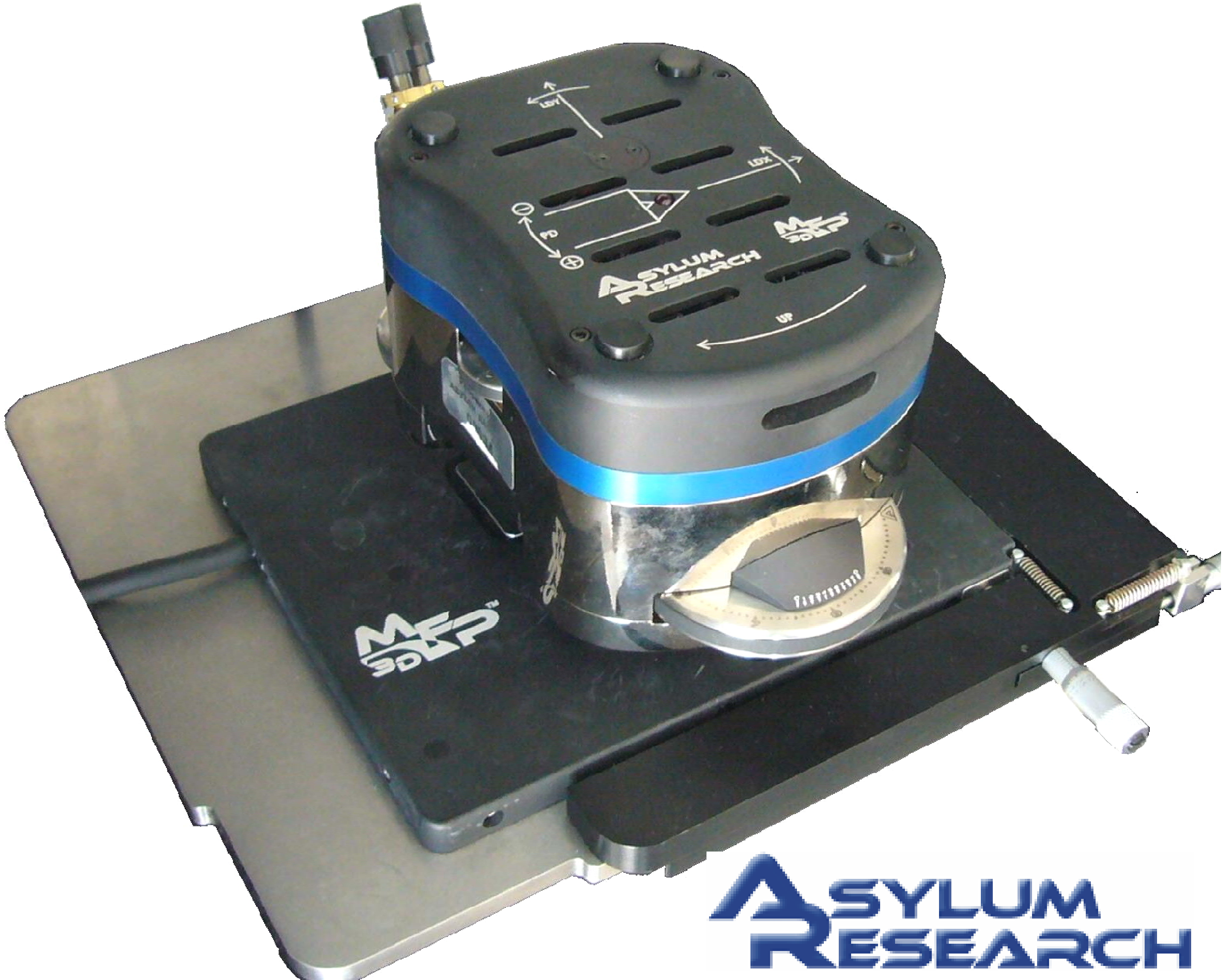
Atomic Force Microscopes Santa Barbara, CA 93117 (805) 696 6466

Procedural Operation 'Manualette'

Version 10.5 (Ocotber 2009)

Ryan Fuierer, Ph.D.

Based on software version 080501



Science First

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Acknowledgement-

The author would like to acknowledge all the inmates at Asylum Research who have answered the many questions between my post doctoral days when first starting to use the MFP-3D until today, allowing this document to be prepared and presented as accurately as possible. More than ¾'s of the company has been involved in the technology transfer to the author.

▪ The author is also especially grateful to Scott Maclaren (UIUC material research lab) for taking the time to carefully edit this document, convey some interesting software techniques, provide valuable discussion and detailed descriptions of document typos and format issues that became transparent over time from excessive staring. Scott Rocks! http://cmm.mrl.uiuc.edu/staff/ScottM.htm

▪Mike Falvo, who got me started in AFM, and still let's me ask dumb questions about it today.

▪Mark Van Landingham/ Krysten Van Vliet- thanks for letting me arbitrarily bounce nanoindentation questions off you.

Disclaimer:

The purpose of this 'Manualette' is to offer new and infrequent MDP-3D™users some supplemental documentation on how to *procedurally* operate an Asylum Research MFP-3D™ AFM. It's like a manual, but more brief and to the point regarding basic 'how-to' operational procedures. It started off as a small document, and was termed the manualette. Its inception was to alleviate time spent training lab members during the author's post doc days, but has evolved to this document in efforts to reduce the operational learning curve to achieve your efficiency.

Also note that this instrument's standard software is highly versatile, and describing even the most straightforward techniques in our user base is quite an undertaking. Adding the openness of the software, MFP-3D accessories (heaters, ORCA, VFM, etc.) and signal routing with the crosspoint switch, makes the instrument mind-numbingly versatile, and there may be a good chance it's not described.

It is assumed that the official MFP-3D™ manual has been perused. The MFP-3D™ is an expensive, high precision instrument, and should be treated with great care and common sense. AFM does take patience since it is an instrument that must be tuned and have proper sample preparation to be successful.

A great effort has been made to ensure this manualette is as accurate as possible, but its accuracy and completeness cannot be guaranteed. Despite the best efforts by the author, this manualette may contain mistakes and the reader should use this only as a general guide for operation of the MFP-3D atomic force microscope, and is not the ultimate source of information about this instrument. The author assumes no responsibility for any damage whatsoever by persons or property that may result from readers undertaking operation of the MFP-3D.

This Manualette:

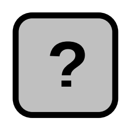
▪ Is broken down into sections that are in a (seemingly) logical progression for obtaining AFM images or simple force spectroscopy experiments, and the subsequent (basic) data analysis.

▪ Only discusses operation of the MFP-3D™ itself, and does not include any optical microscopy techniques (if your MFP-3D happens to be sitting on an inverted optical microscope), or operation of electrical techniques or accessory modules at this time.

▪ Is in continual development, so more insightful, updated versions will occur in the future. Version 10 by far is the largest and most comprehensive to date. Expect Version 11 by late 2009. Meanwhile, much of this content will be converted into an official AR MFP3D operational manual that will have multiple editors, will be linked and downright fancy.

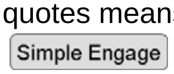
If you are not familiar with Igor Pro, you should read through the 'Getting Started' manual that comes with the software documentation. It's a worthwhile use of a couple hours.

The author also suggests when updating new software from Asylum, to read the 'what's new read me' files; they are often very descriptive.



The 'MFP3D Help Files' under the Igor Help menu are also very useful, especially for image analysis and display. The programmers write these for a reason, and are generally very insightful, yet sometimes not procedural- hence, the manualette.

Some features of this manualette-

-Any word in gray bold text in single quotes means it's an execution button in the software; example '**Simple Engage**', represents this button: 

The ▪ bullet points denote a new concept or comment.

✓ Checkmarks denote part of a procedure, typically these are set up in a series of instructions and are included to help guide the eye through specific operations.

Blue text (accompanied by one of the little note icons in the margin) represents a Note or empirical trick- something the author has picked up along the way, and is included in efforts to reduce time to arrive at epiphany.

These icons represent caution should be used to proceed.

Advanced questions should be directed towards the excellent, friendly staff at Asylum Research via emails to: support@asylumresearch.com, which are forwarded to all of our technical people. It is company policy to respond to email within a 24 hour period- quite often that actually occurs!

Have fun imaging! - Ryan

1. Introduction

An AFM image is typically comprised of a signal representing some Z distance of cantilever motion per X,Y point on the scan raster . These signals can be from various measurement points in the instrument: the voltage to move the Z piezo actuator in response to the feed back loop; the closed loop sensor monitoring the movement of the top plate of the optical lever detector; the amplitude, phase or deflection signal from the position sensitive detector; or some kind of error in the feedback loop. All signals are typically read as a voltage in the MFP3D system.

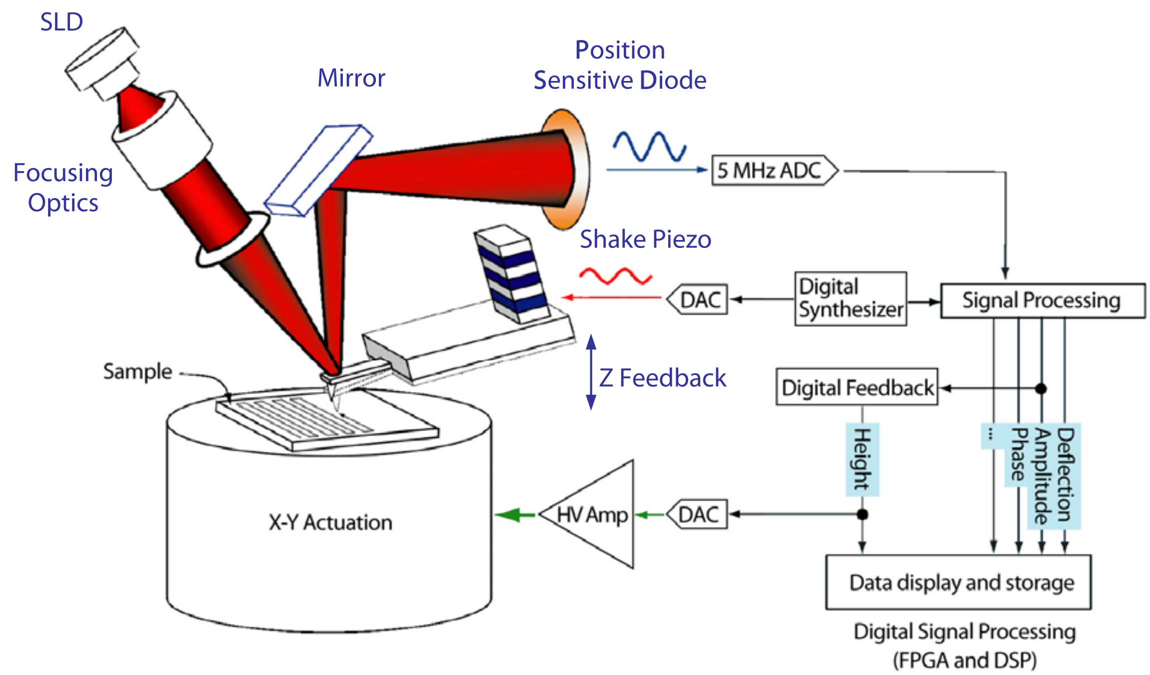


Figure 1.1: Simplified schematic of MFP-3D system.

If you are new to the MFP3D, a simplified schematic is seen in Figure 1.1 to allowing a better understand of the instrument. A superluminescent diode (SLD) provides columnized light that is reflected off the back of the cantilever to a position sensitive diode (PSD), which sends a voltage value to a feed back loop that is trying to maintain some user defined setpoint- this is typically a deflection or amplitude (voltage) signal from the PSD, depending on the imaging mode employed (AC or Contact mode).

Optical Lever Detector: this is the design that monitors how the SLD light source measures the deflection of the cantilever- Figure 1.2 shows more detail important to the MFP3D design: The lower part of the figure shows the optical lever design- what's novel about it is that this entire optical detection assembly (the SLD, cantilever, mirrors and PSD) moves together- the importance of this design is it maintains the laser spot hits the back of the cantilever in the same spot across the XYZ scan regions- hence, the same optical lever sensitivity (i.e., nm/ V on the PSD) is maintained. *Why is this important?* Anytime the SLD spot moves on the back of a cantilever, the optical lever sensitivity changes, hence the forces measured aren't directly comparable to each other. Additionally, the SLD directs light on a 22° angle to the cantilever which helps eliminate optical interference from stray reflections off the sample that could convolute the PSD signal.

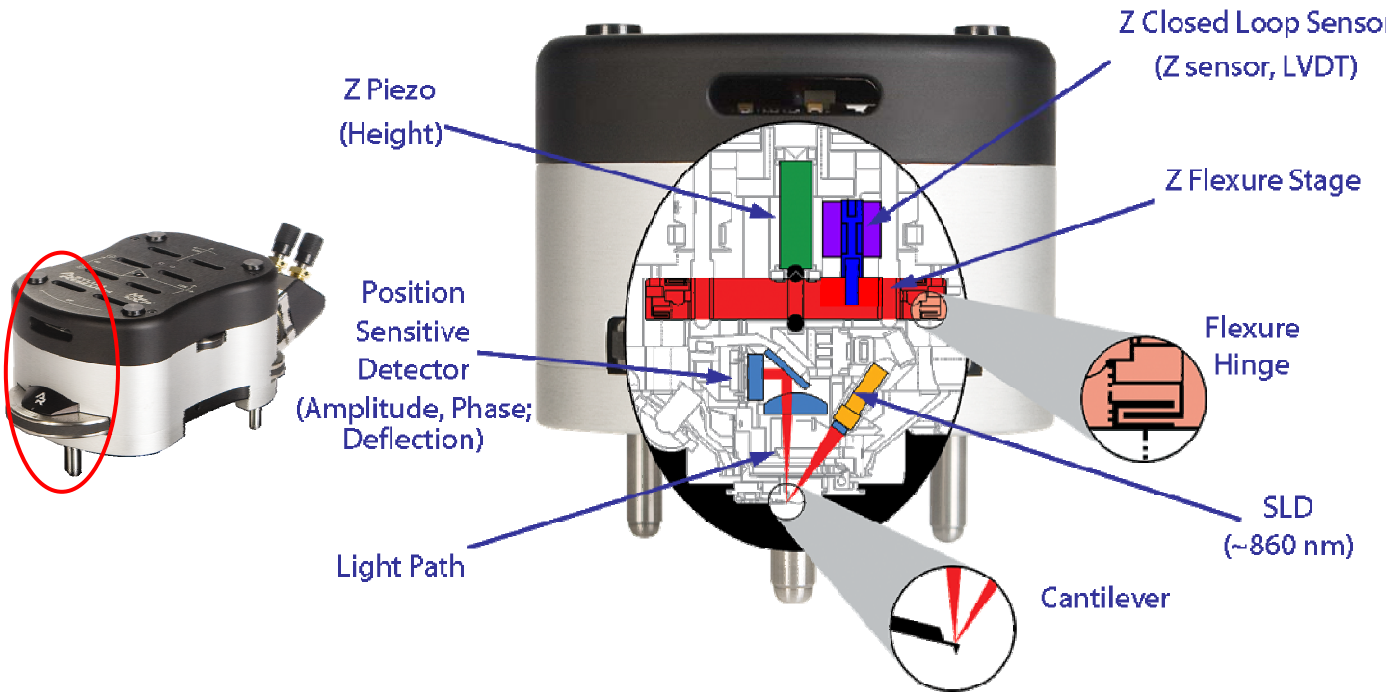


Figure 1.2: The optical lever detection schematic for the MFP3D. Parenthetical comments are where various data channels originate

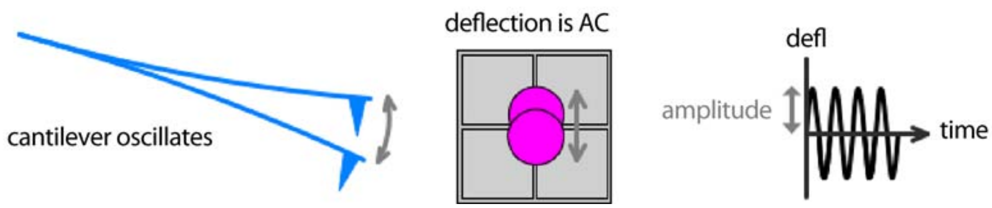
Scanning:

As the sample is rastered in an X,Y pattern under the tip, the feed back loop moves the Z piezo up or down to track the surface via what ever user defined set point it has been asked to maintain- usually some voltage signal from the SLD spot moving on the photodiode (peak to peak amplitude in AC mode or deflection in Contact mode). These signals are immediately digitized in an ADC and ultimately sent to the feed back loop. Extension of Z Piezo towards the surface will compress the flexure based springs of the plate holding the optical lever assembly; retraction of the Z piezo pulls the flexure away from the surface. These movements produce a three dimensional image from the Z piezo voltage signal (Height) per X,Y raster point, or from the closed loop sensor that monitors how much the upper flexure plate moves in response to the Z Piezo moving (Z Sensor) per XY raster point. The Z sensor signal is particularly useful in accurately measuring Z distances because of its linearity, relative to the Piezo (Height) voltage signal due to the creep, hysteresis and non linearity issues of the actuators. All MFP-3D scan axis are sensored with a proprietary linear variable differential transformer (LVDT) which is an extremely quiet closed loop sensor design- with an XY factory spec of <0.6nm, there is no need to turn the closed loop sensors off when scanning at very small scan ranges. Note that LVDT or Z sensor means the same thing in the MFP-3D software.

Imaging modes:

This chapter describes the introductory operation of the two most common imaging modes: AC mode and Contact. The former is the most common method used in AFM imaging, and therefore described first.

AC mode (intermittent contact mode; called AC because of AC bias driving a shake piezo in the probe holder) This technique drives the cantilever just off its fundamental resonant frequency at some user defined amplitude set point, typically some dampened value of the 'Free Air' amplitude of the cantilever (i.e., not being dampened by the sample). This set point is read as a voltage off the PSD. The feedback loop maintains this setpoint amplitude (voltage) as the tip is scanning across the surface, retracting the Z piezo when the amplitude gets dampened (bumps); or extending the Z piezo when/if it oscillates at a larger amplitude than the amplitude set point (over holes). The latter is why the set point must be a dampened amplitude ratio of the 'Free Air' ratio.



A qualitative relationship between the force applied relative to the set point amplitude voltage can be seen in Figure 1.3.

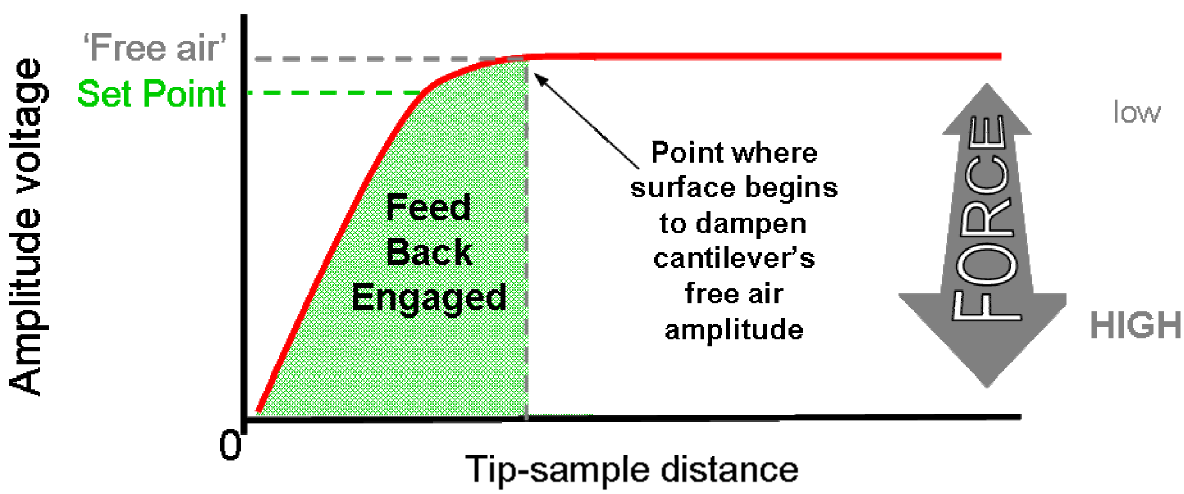
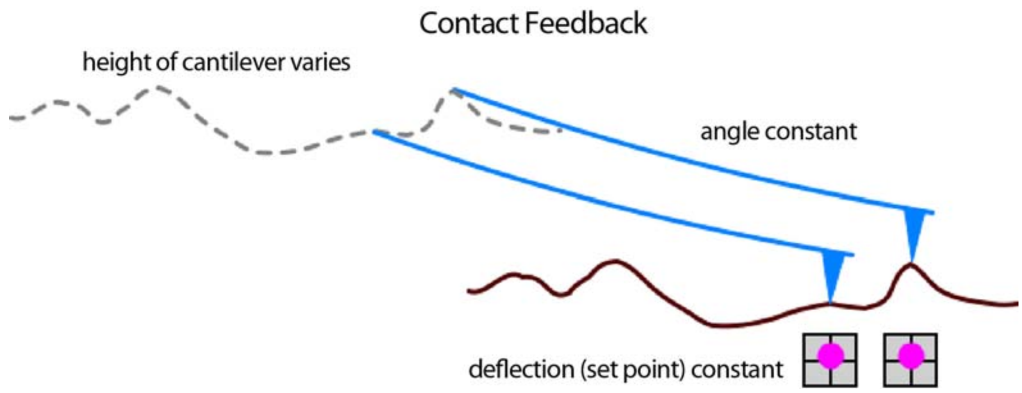


Figure 1.3: Qualitative relationship between tip substrate distance vs. amplitude setpoint (i.e. force).

The standard the images channels to have open are: (found in 'Master Channel Panel'; both trace and Retrace scans can be displayed & saved).
Height: the voltage applied to the Z piezo to maintain defined set point amplitude (voltage), per X,Y scan point.
Amplitude: the error signal of the feedback loop to maintain that user setpoint, per X,Y scan point.
Phase: As the oscillating tip interacts with the sample, the tip can experience NET repulsive or NET attractive forces, which in turn generates a lead or lag in the Phase signal in the photodiode.
Z sensor: The closed loop sensor (LVDT) that monitors the movement of the optical lever detection assembly (more specifically, the top flexure plate of that assembly). This channel is good for measuring accurate distances, especially >200nm, but not so much for sub 40 nm stuff.

Contact Mode: In this mode, the tip is in full contact with the surface as the sample is rastered in a XY pattern while maintaining a user defined deflection voltage (i.e., force) to keep a positive deflection on the cantilever (bows towards surface). Depending on how soft the sample is, and what forces are being applied, the technique can be more destructive than AC mode. However, this technique is great for hard surfaces, or imaging cells in fluid at very low set point forces.



A qualitative relationship between the force applied relative to the cantilever deflection voltage can be seen in Figure 1.5. Setpoint voltages slightly more positive than the free air deflection results in low applied force, while setpoint voltages much more positive than the free air deflection results in larger applied forces.

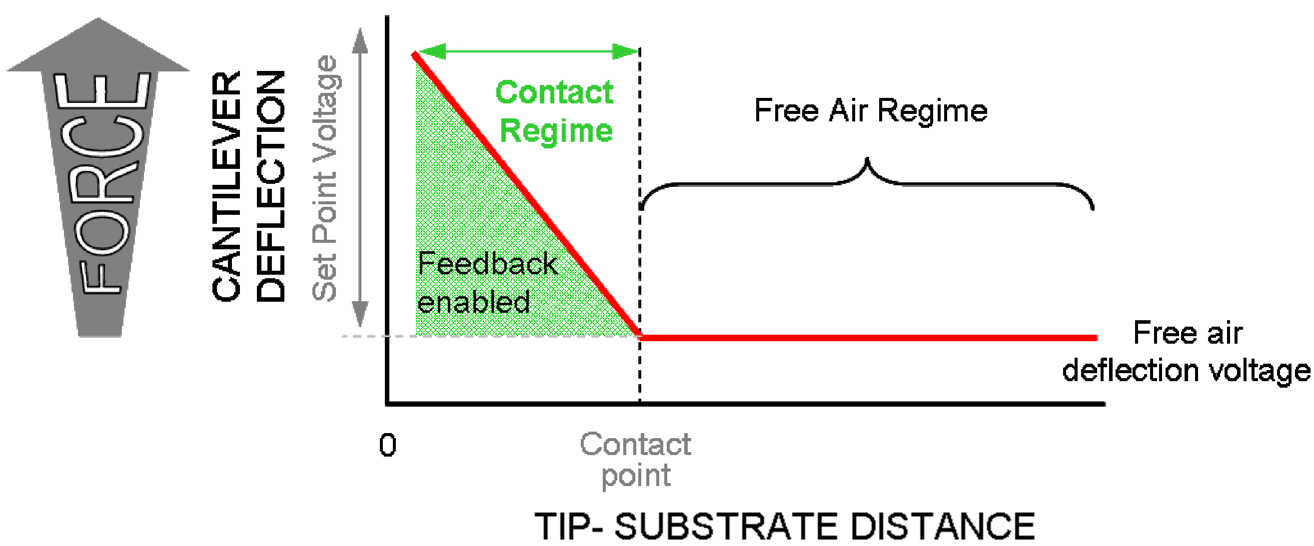


Figure 1.5: Qualitative relationship between tip substrate distance vs. cantilever deflection.

The standard the images channels to have open are: (found in 'Master Channel Panel')
Height: the voltage applied to the Z piezo to maintain defined positive deflection, per X,Y scan point.
Deflection: the error signal of the feedback loop to maintain that user set point deflection (voltage), per X,Y scan point.
Z sensor: Closed loop sensor that monitors the movement of the optical lever detection assembly (essentially it's a plate). Better for measuring accurate distances, typically >200nm.
Lateral: used to monitor cantilever torsion (twisting) when scanning at 90° by comparing the amount of signal in the left and right halves of the 4 quadrant photodiode.

1.2: Introduction to the MFP-3D™ components:

Head: this is what contains the optical detection system and controls the Z piezo actuation of the cantilever; it contains thumbwheel adjustments to position the laser and zero the deflection on the position sensitive diode/detector (PSD); the top view optics position knobs and focus wheel to view the tip from above; has three independent legs that adjust height of head relative to sample. *Use two hands to lift the head due to its weight.*

Figure 1.2.1: MFP-3D™ head; 'Extended' Z range head shown

Probe: the probe is what the *entire* tip/cantilever/substrate chip is called. The MFP-3D™ cantilever holder accommodates all major brands of commercially available probes (Figure 1.2.2).

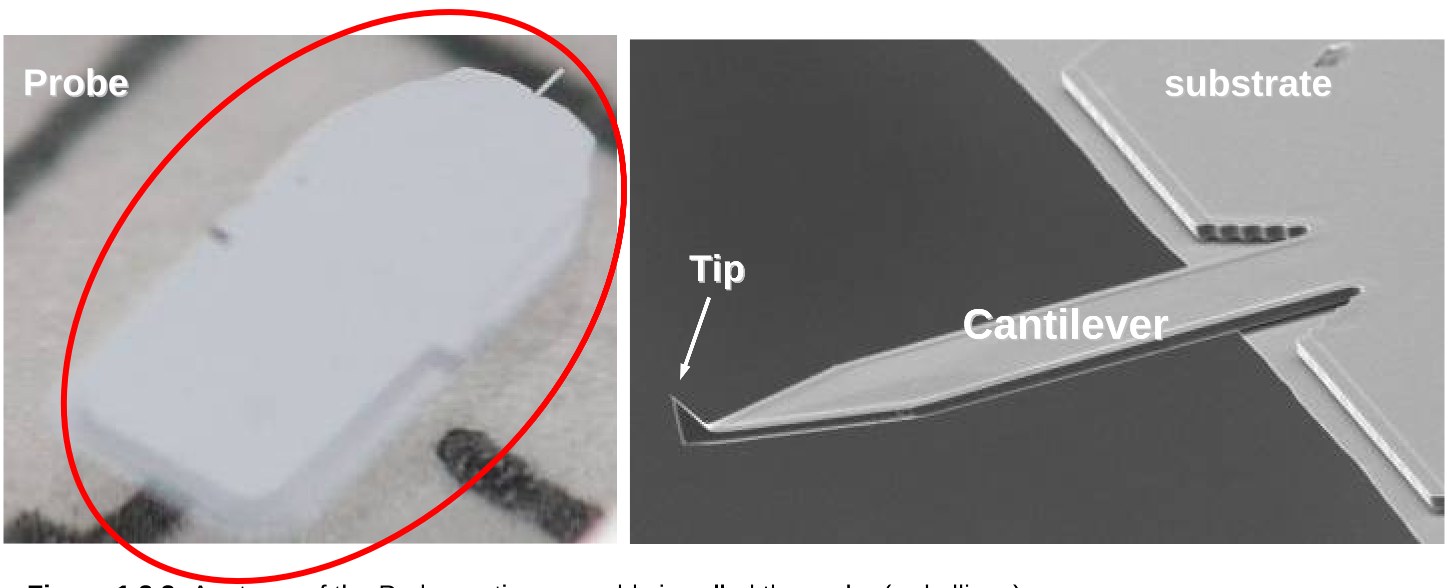


Figure 1.2.2: Anatomy of the Probe: entire assembly is called the probe (red ellipse).

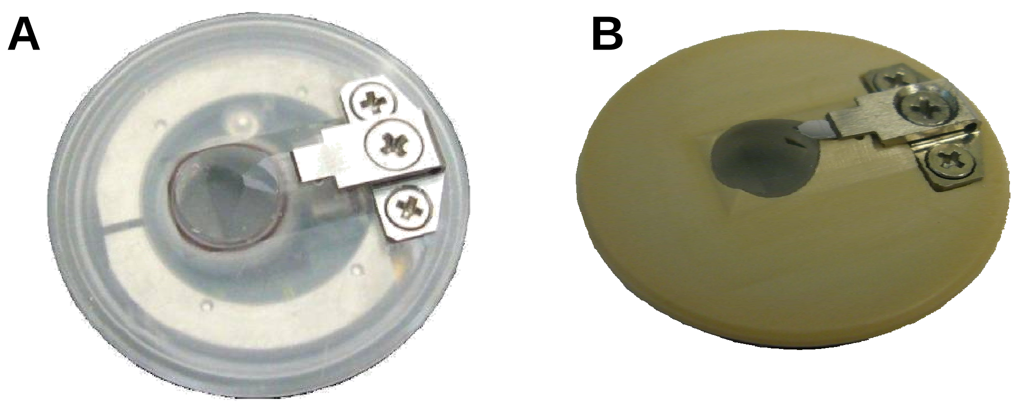


Figure 1.2.3: MFP-3D™ cantilever holder: A) older Kel-F design; B) newer PEEK filled glass design.

Cantilever holder: this is what secures the probe chip; where the 'shake' piezo is housed. It snaps into the head via kinematic machine mount.
It consists of:
-a Kel-F body (Figure 1.2.3A) or PEEK filled glass (tan colored; (Figure 1.2.3A)).
-stainless steel tongue clamp to secure probe
-quartz window for optical path where the laser bounces off the back of the cantilever & top view optics; pogo pins for shake piezo, applying bias to tip.

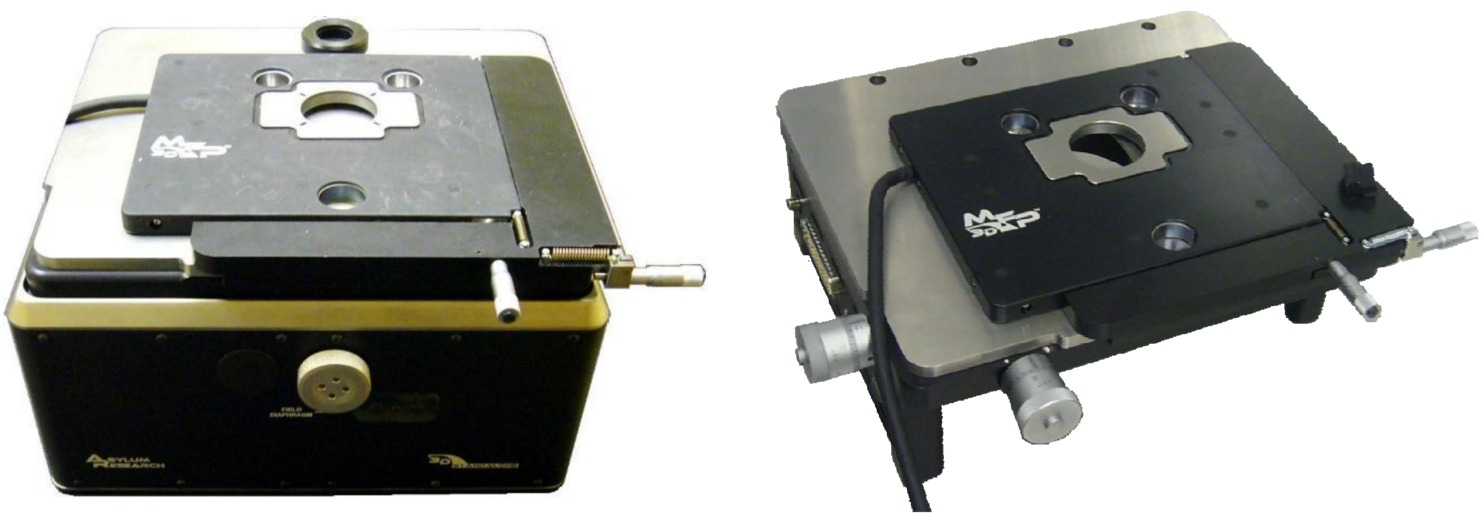


Figure 1.2.4: MFP-3D base with XY scanner: A) Stand alone base; B) Bio baseplate (not attached to inverted stage and without top view optics pillar).

X,Y Scanner and Base: The flexure based 90 µm x 90 µm X,Y scanner sites on the base. The smaller holes in the scanner are where the 3 legs of the head go through to sit on the baseplate stage. The scanner can be manually moved relative to tip using the coarse translation micrometers (front right corner). Figure 1.2.4 shows a stand alone base, however, the MFP-3D™ can also be mounted onto a baseplate that sits on an inverted optical microscope frame.

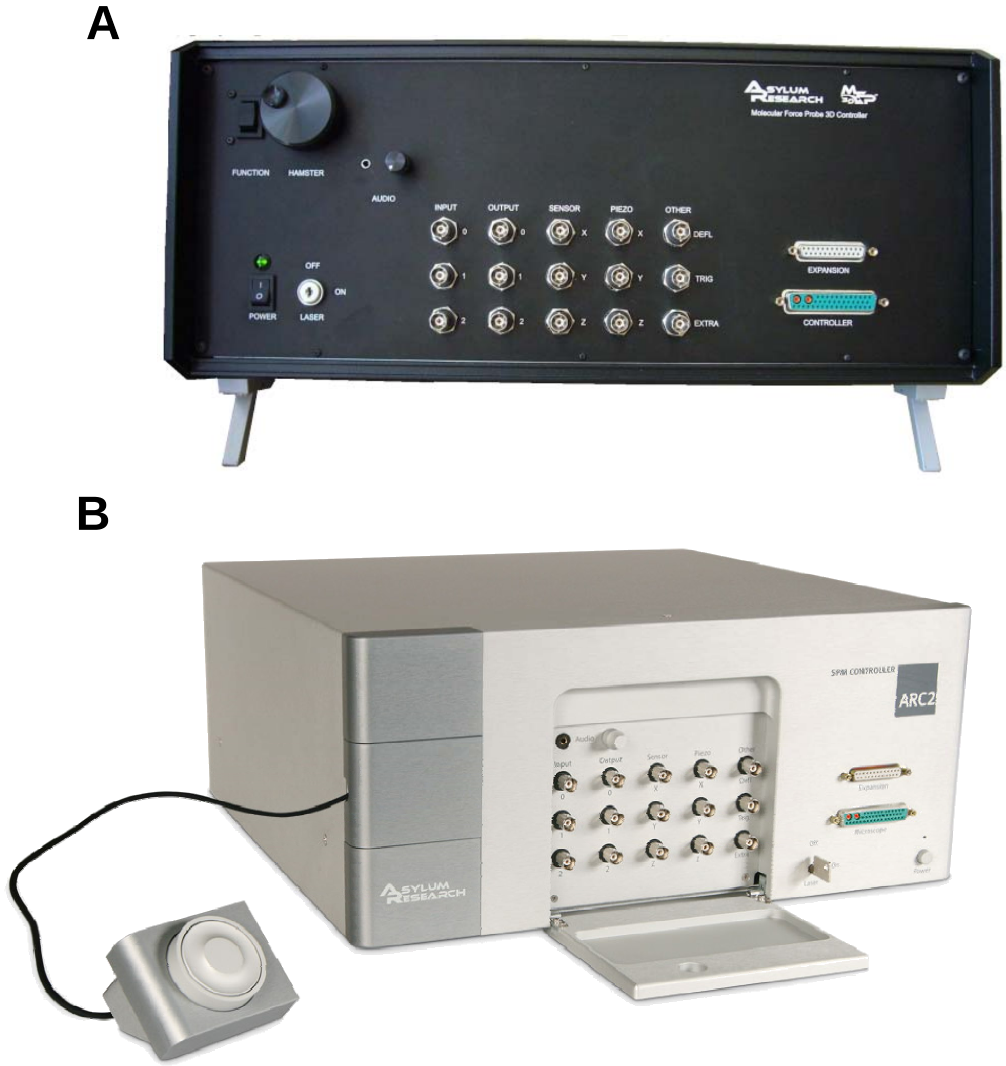


Figure 1.2.5: MFP-3D™ all digital controller: A) Original design; B) new ARC2 design (spring 2008).

Controller: The digital controller contains a DSP and FPGA, and software controlled analogue 'cross point switch' for rerouting internal and external signals for custom experiments. The front panel of the controller is where: the power switch is; the key to turn the 'laser' on/ off; the 'Hamster' wheel is located to fine tune imaging/ control parameters; BNCs for advanced input/output signal access. The controller communicates with the PC via USB interface. There's a lot more going on under the hood that will not be discussed here.

2. Opening the MFP-3D™ software:

✓ Open the MFP-3D™ software; the launch icon is on desktop.

▪ The software is fully loaded when Igor says it's ready (lower left hand side of window). When Igor is busy, it doesn't want to be interrupted and will ignore any subsequent requests until finished; while this is occurring, typically the 'Abort' button in the lower left of the MFP-3D™ software tray is present. The 'Abort' button can be clicked to abort a software request or calculation. This isn't recommended during software boot up because it won't load fully.

▪ When the software is loaded, Igor will say 'Ready' instead of the 'Abort' button, and will look something like Figure 2.1, lower left.

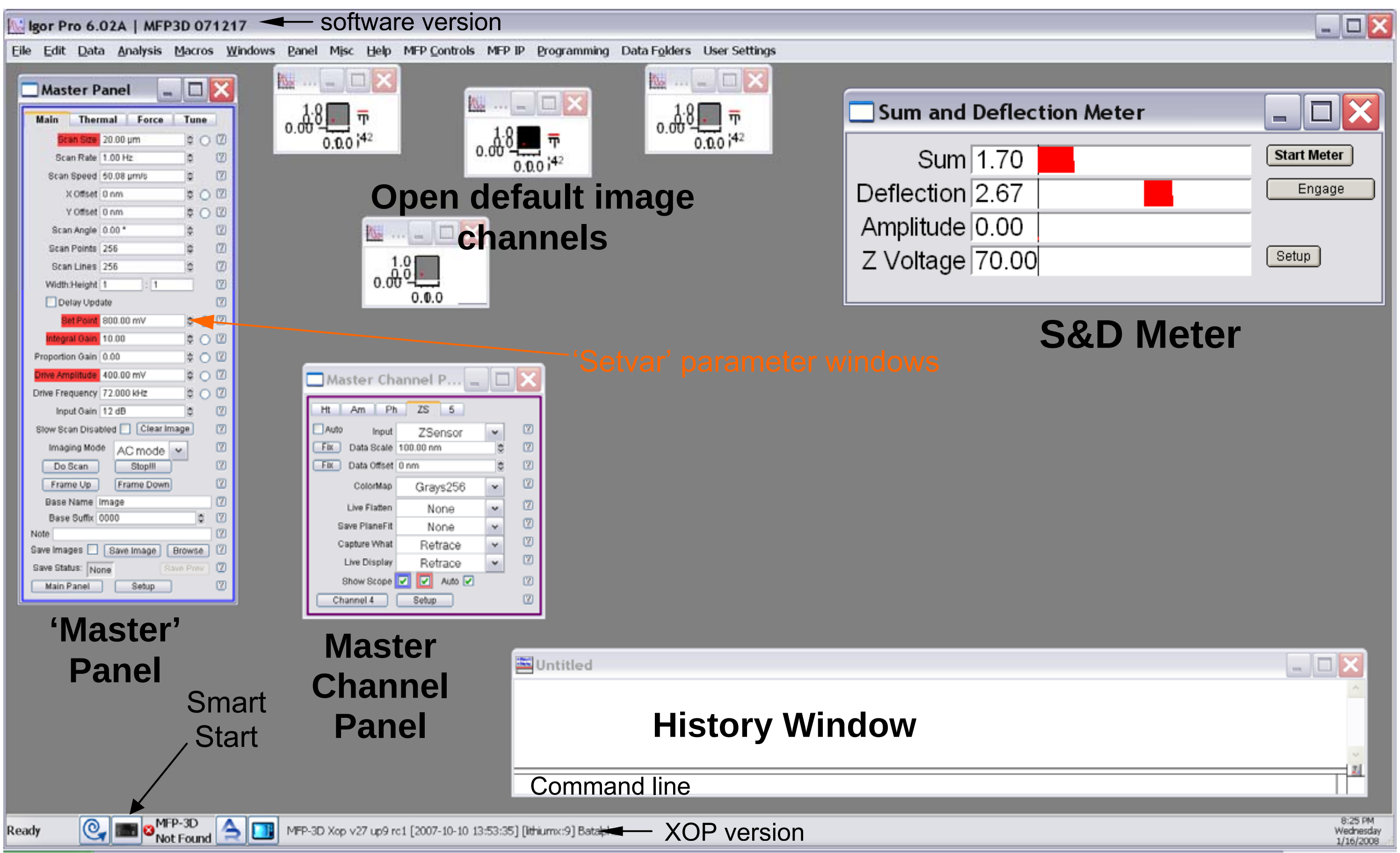


Figure 2.1: Default MFP-3D™ software upon launching.

Other features of the MFP-3D™ software tray (see Figure 2.2)

① Igor Pro status- if Igor is ready to accept a software request from you, it will say 'Ready'. If not, it's thinking and the Abort Button is present with a rotating quartered circle.

② The **Rescan** button- push this when Igor gets confused- usually necessary if Igor freezes up, or when adding new components to the system (heaters, different cantilever holders which have identity resistors in them.

③ **Smart Start**- this shows you what MFP-3D™ components are communicating with each other. Further more, individual information on each component can be accessed by expanding using the triangular button (▶) to the right of each component icon

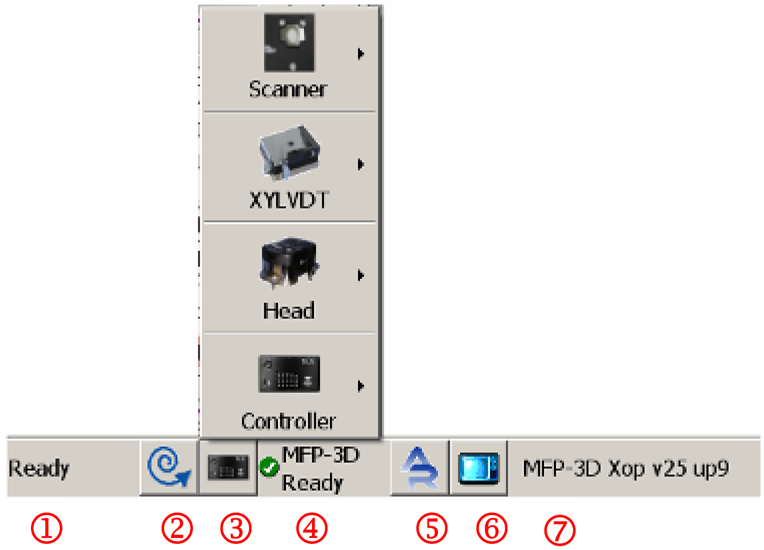


Figure 2.2; The MFP-3D software tray:

④ MFP-3D™ status- this will tell you if the instrument is ready, or if the controller or other hardware isn't found, etc. If new hardware is plugged in, it will notify you as it reads the calibration files off the new hardware.

⑤ Quick link to AR online support (AR engineers can control your MFP-3D™ over the web during online help sessions).

⑥ CCD top view camera icon- click this to get camera screen to come up.

⑦ MFP-3D™ XOP version

NOTE: For more about having multiple version of the MFP-3D software on your machine, see Section 14.16, or go to http://www.asylumresearch.com/Support/FAQ3D.shtml#IgorCopy.

Mode Master Panel-

In versions **after** March of 2008, an additional panel during the MFP-3D software boot will appear with the Mode Master Panel (Figure 2.3)- this was created in efforts to make the software more user friendly for new our infrequent users. What's great about the Mode Master is that it can be set up such that clicking on a technique reconfigures the panels to be parameter ready for the technique at hand. For example, clicking on the electrical then SKFM brings up the electrical tune panel and the NAP panel, along with the NAP channel panel.

✓ To use the Mode Master, just click on a function button- for example, say you want to imaging in Contact mode:

✓ Click 'Topography' button; this will bring up a panel with all the different topography acquisition modes.

✓ Click the 'Contact Mode' button; this will bring up all the parameters used for contact mode imaging (See Chapter 8).

▪ This seems to be experimental for the more complicated techniques because of how new it is to the software, but works well for the simpler straightforward imaging modes.

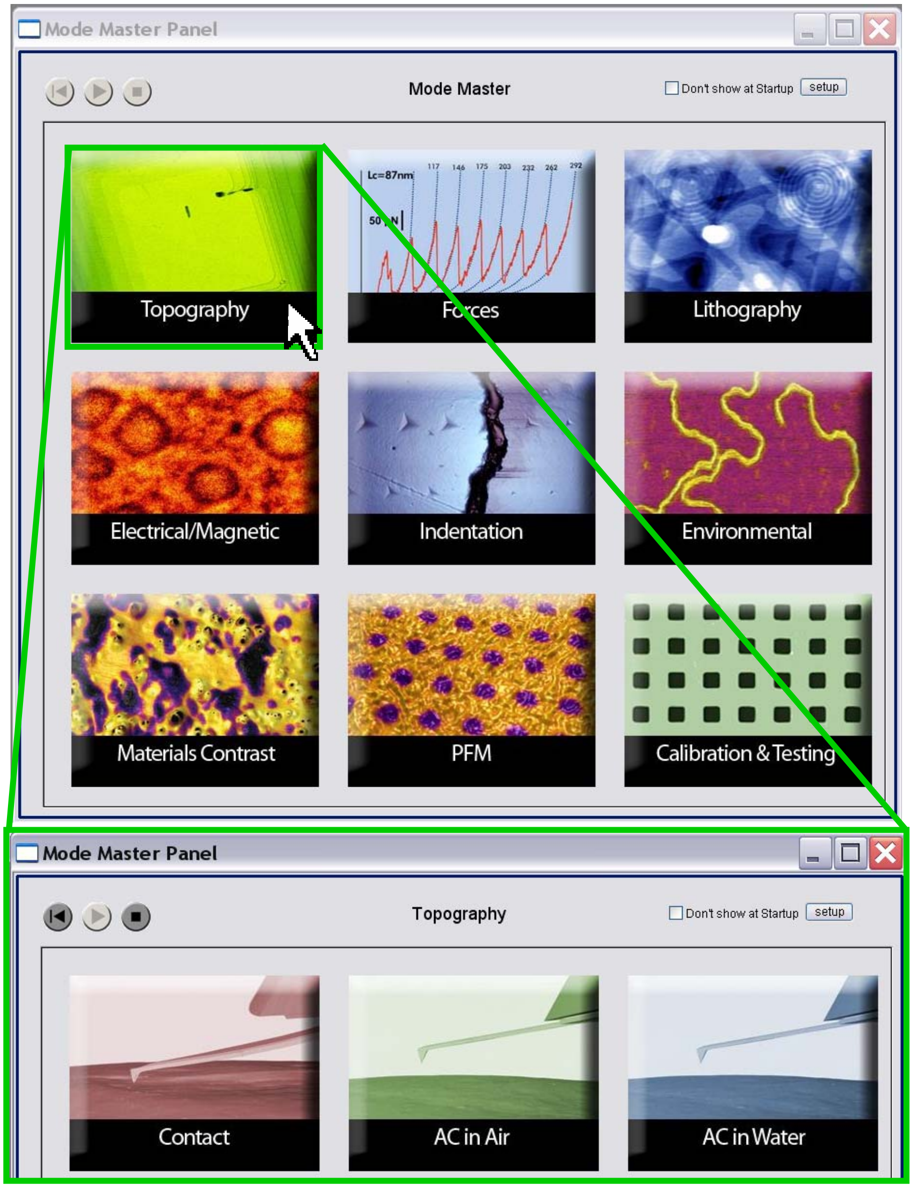
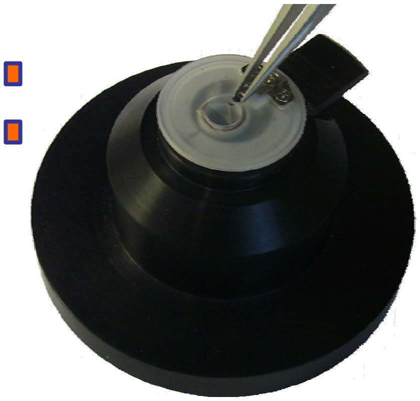


Figure 2.3: MFP-3D Mode Master Panel simplifies parameter selection with preset or user defined parameters established prior. B) clicking on topography brings up a sub-panel allowing the user to select between different topography imaging modes. Each of these modes will reconfigure software parameters to best suit the experiment.

3. Loading the Probe:

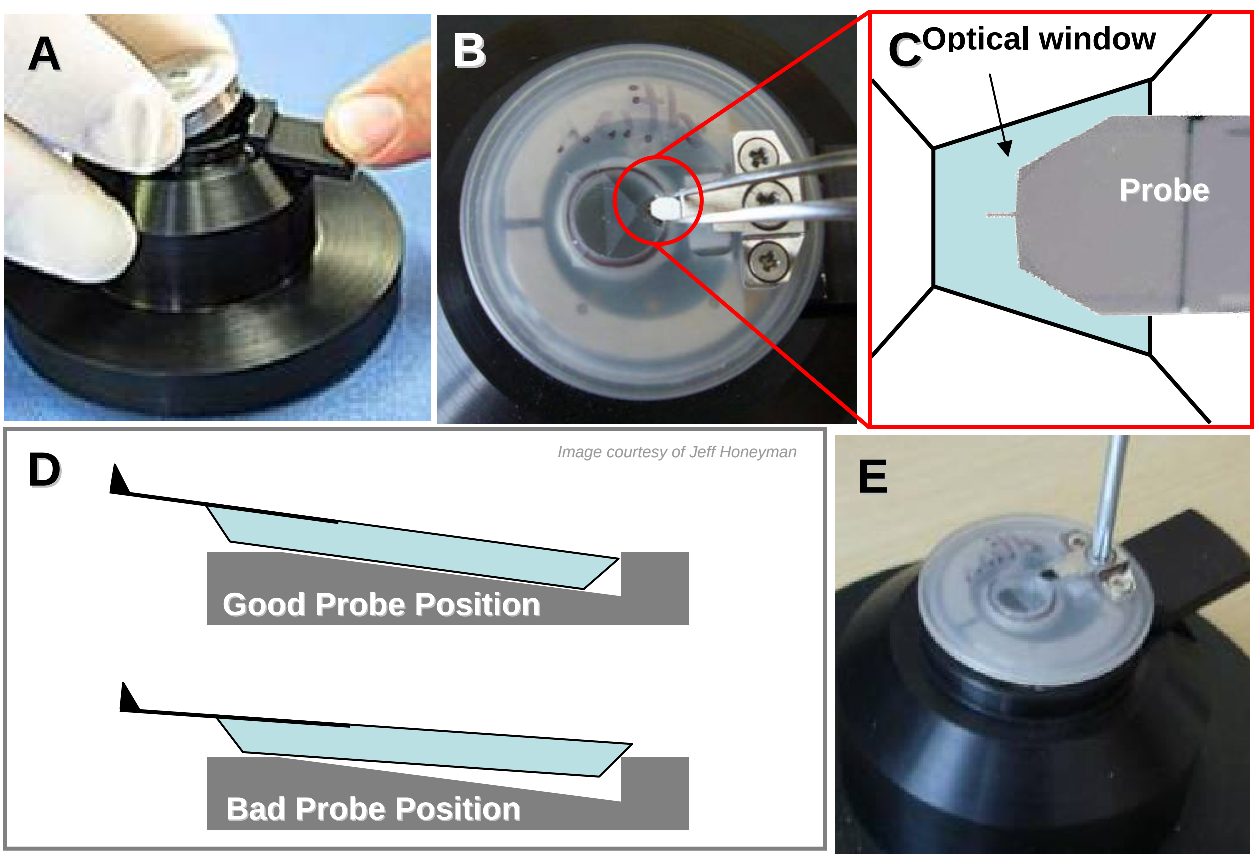


This section describes how to properly load a probe into the cantilever holder, and place the head onto the stage.

3.1: Loading the Probe:

The MFP-3D™ cantilever holder accepts most brands of commercially available probes. The quartz window is resilient from tweezer scratches. It can be cleaned a variety of ways- see official MFP-3D manual. It can be found on the AR forum found under manuals and documentation:
http://asylumforum.com/forum/showthread.php?t=372

✓ Load tip holder into loading pedestal apparatus (Figure 3.1A), which offers stability when loading tips. The cantilever holder has a kinematically machined ball bearing port that snaps into place using the lever on the apparatus and head port. If working in buffers or solvents that must be rinsed post experiment, the cantilever holder pedestal apparatus works great for rinsing the tip holder after imaging (i.e, so you don't drop it into the rinsing collection container).



✓ Loosen tongue clamp screw with provide Phillips head screwdriver (it's a right hand thread).

✓ Slide probe chip under tongue clamp. Position cantilevers so that they are centered (more or less) in the clear trapezoidal shaped quartz optical window (Figure 3.1B & C). The cantilever holder was designed to be resilient to scratching from tweezers, so don't be paranoid. For best results, DO NOT push probe chip substrate all the way back in the pocket: it can cause the probe chip to lift off the floor of pocket, compromising the deflection signal (i.e., no signal). Figure 3.1D illustrates this schematically.

✓ Tighten the screw in the center of the stainless steel tongue assembly (NO MORE THAN finger tight!). This generally allows for suitable chip coupling to the tip holder. (Figure 3.1E)

NOTE: DO NOT OVER TIGHTEN TONGUE CLAMP on cantilever holder- this can strip screw threads; or result in excessively bending the tongue, and cause some issues with obtaining suitable deflections between different Probe manufacturers or cantilever lines. If this does happen, the clamp can easily (*yet gently & patiently*) be bent back into place. (See Misc. Operations Section 14 to review this procedure.)

Accommodating Thicker Probe Substrates:

Often when switching between silicon probes to SiNx probes, the thickness difference offers some difficulty in gently sliding the probe chip under the tongue clamp near the hinge (not at end of tongue!- *see Figure 3.2*)- this is because there is a thickness difference between the two types, and from manufacturer to manufacturer. If some resistance is felt, and the probe just won't gently slide under the tongue clamp into the pocket, here's what to do:

Figure 3.2: **GENTLY** lifting tongue clamp to accommodate thicker probe chips.

✓ GENTLY take tweezers or flat head screwdriver to GENTLY pry up on tongue near base (see tweezer placement in Figure 3.2.). *Did I state GENTLY?* The author's experience is that it doesn't even have to feel like the probe tongue clamp was moved at all. This is usually enough to allow new probe to slide under without resistance.

Install cantilever holder into MFP-3D head:

✓ Put cantilever holder into the MFP-3D™ head- the author finds it easiest to put the ball bearing on release lever side (red arrow, Figure 3.3 below) of the kinematic system first; then ease the holder in from the back. Make sure the cantilever holder is *parallel* to the top of the head; other wise it is not properly seated (*see note below*).

Use extreme care when putting cantilever holder into the MFP-3D™ head. The 'pogo' pins used to get signals from the cantilever holder can be easily bent with excessive force (*Figure 3.4 below).*

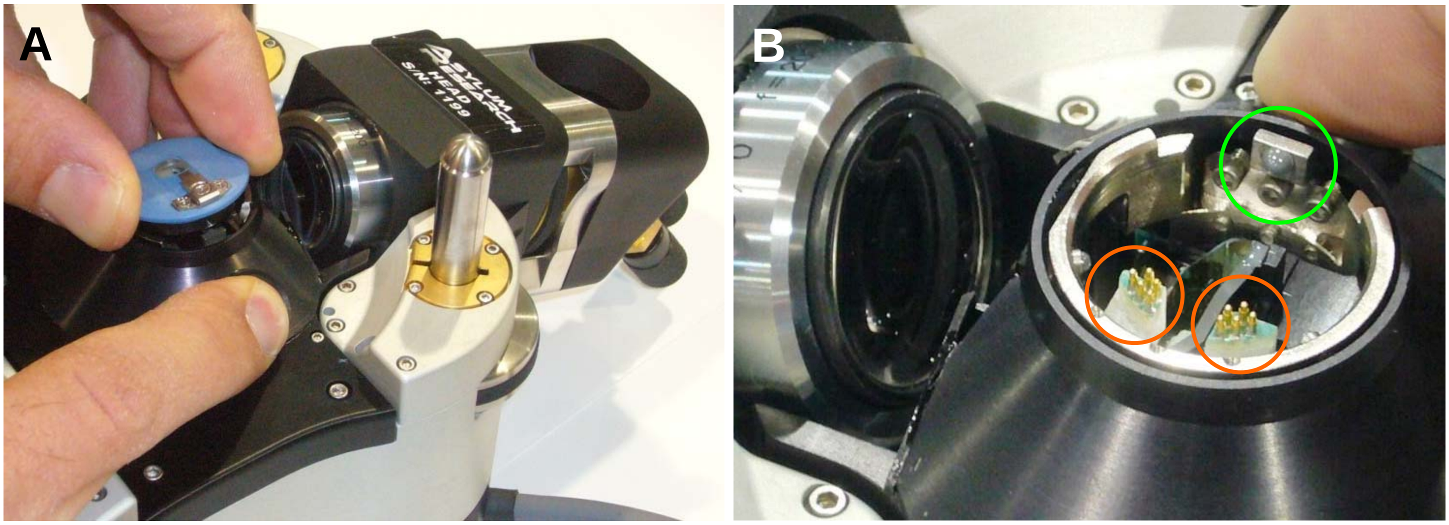


Figure 3.3: Push release button, line kinematic ball in socket initially, then gently press into place; B) location of kinematic ball on lever (green circle); don't damage pogo pins (orange circles).

3.2: Loading samples onto XY Scanner:

The MFP-3D comes in either the Stand Alone base (Figure 3.2.1A), or the Bio baseplate mounted onto an inverted optical microscope (Figure 3.2.1B). Either way, the sample is typically held down magnetically to the stainless steel scanner plate in the center of the XY scanner.

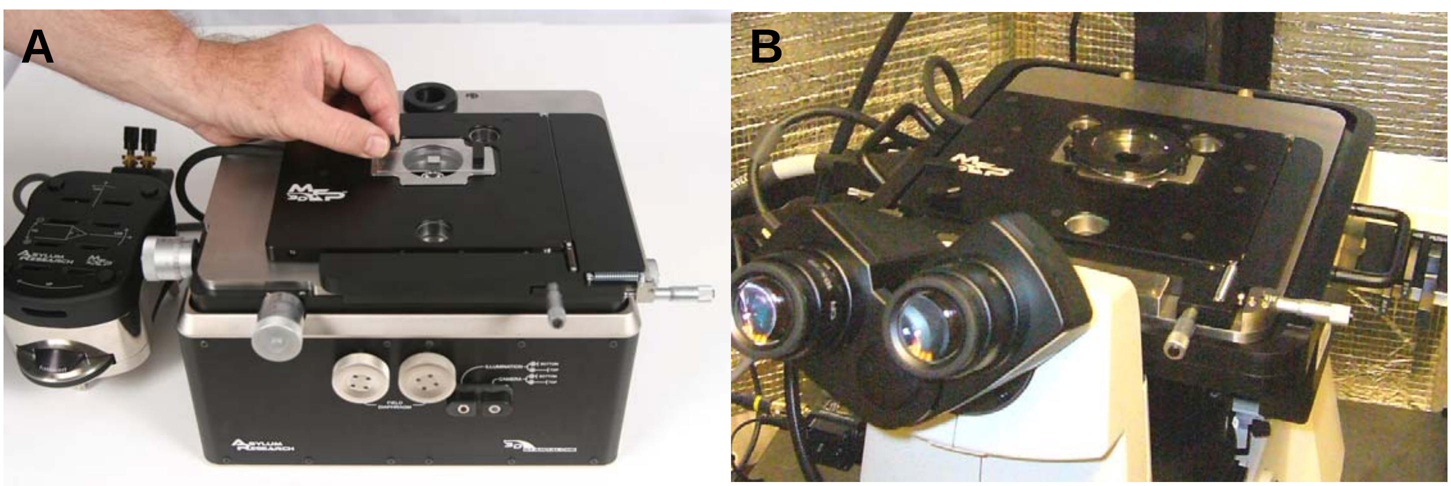


Figure 3.2.1: MFP-3D baseplates: A) the stand alone base; B) the Bio baseplate.

✓Place sample, with area of interest in center of ocular opening in XY scanner plate. (Figure 3.2.2A). Use the provided magnetic hold-downs (green blocks in Figure 3.2.2C). The ORCA sample holder has built in magnetic hold downs (Figure 3.2.2B), while the PEEK dishes for most AR fluid cells and heaters contain built in magnets (not shown)

Figure 3.2.2: A) Place sample area of interest towards center of scan plate; B,C) multiple sample holders can be magnetically held down on the MFP3D.

3.3: Placing Head on Scanner Stage:

Once a probe is properly installed in the cantilever holder, the superluminescent diode (SLD) can be aligned on the stand next to the base (with kinematic machined leg divots) *IF* using the IR card (Section 4.1A),
-OR-
over the sample *IF* using the CCD camera (Section 4.1B). To do the latter, the head must first be placed on the stage.

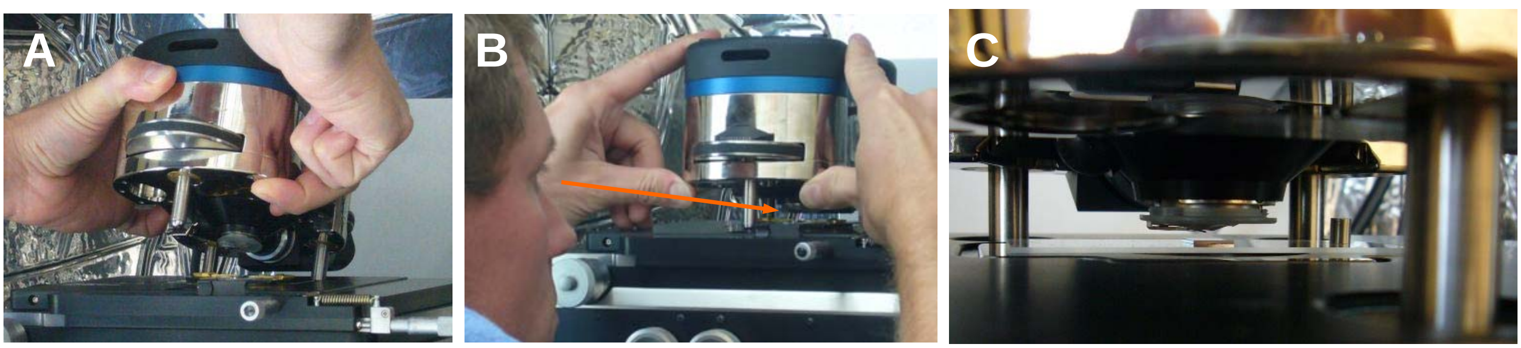


Figure 3.3.1: Placing head on sample stage: A)place back legs into kinematic ball joints ; B) look at tip sample distance as front of head is is lowered down, always ready to pull head up if tip appears to get too close (<1mm) ; C) space between tip and sample should be visible when all three legs are on stage baseplate.

✓Lift the head <u>with two hands</u> and place the back two legs in the kinematically machined divots on the MFP-3D baseplate (Figure 3.3.1A).

✓ Move hands so thumbs are under the front of head, and slowly lower head towards stage using back legs as pivot point; continually monitoring the tip – sample separation (Figure 3.3.1B) with your visual spectroscopy. If it looks as though the tip will crash, lift/ pivot head back up and adjust legs down to increase tip-sample separation, and repeat process.

▪ When complete, there should be a visible gap between tip and sample (Figure 3.3.1C).

▪ The top view CCD camera can also be monitored.

▪ Once all three legs of the head are on the stage, align the SLD on back of cantilever via camera, as described in Section 4.1B.

When working in a fluid droplet or fluid dish, it’s more difficult to monitor this tip sample separation- a safe approach is to have a great deal of distance (>200µm) between the two, and then use the CCD camera to monitor this approach. Figure 3.3.2 shows the meniscus created between tip and sample when plunging cantilever holder into fluid. See Section 6.2. for more on fluid imaging (AC mode).
MAKE SURE NOT TO SUBMERGE CANTILEVER HOLDER IN FLUID!- *See Figure 6.2.2B.*

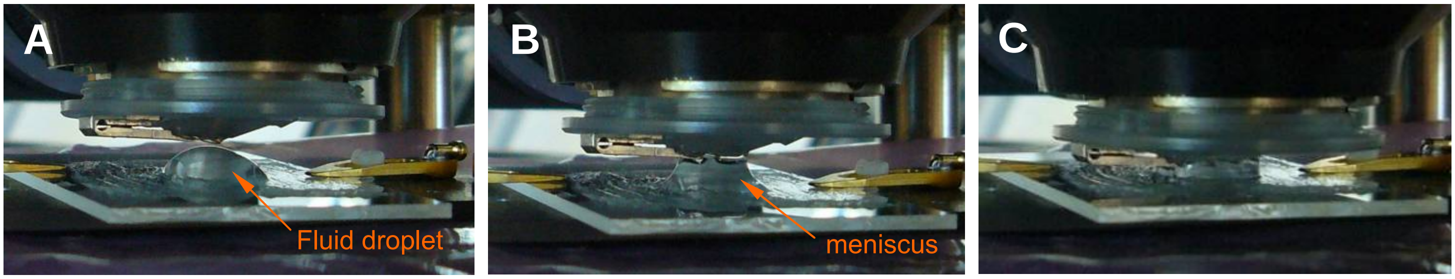


Figure 3.3.2: Installing head over sample with fluid droplet: A) tip approaching droplet; B) Surface tension/ capillary forces connect fluid meniscus to cantilever holder; C) head is lowered until front leg is on stage. Be sure fluid meniscus stays around tip and sample to avoid scanner damage.

4. Aligning the 'Laser':

This section describes multiple ways to align the 'laser' on the cantilever, and zero the Photodector prior to imaging. The 'laser' is actually a super luminescent diode (SLD, emits at ~860 nm), and will be referred to the *SLD*.

4.1: SLD Alignment:

There are two ways to initially (coarse) align the SLD on the cantilever: 1) use an IR card (*described in Section 4.1A*); 2) use top view optics CCD camera*(*described in Section 4.1B*). Fine alignment involves using the LDX & LDY alignment thumbwheels to maximize the sum voltage in the S&D meter, and subsequently 'zeroing' the SLD spot on the photodiode detector (PD)- *see Section 4.3*.

* *for use top down view optics CCD camera only; there are MFP-3D heads out there with no top view optics*

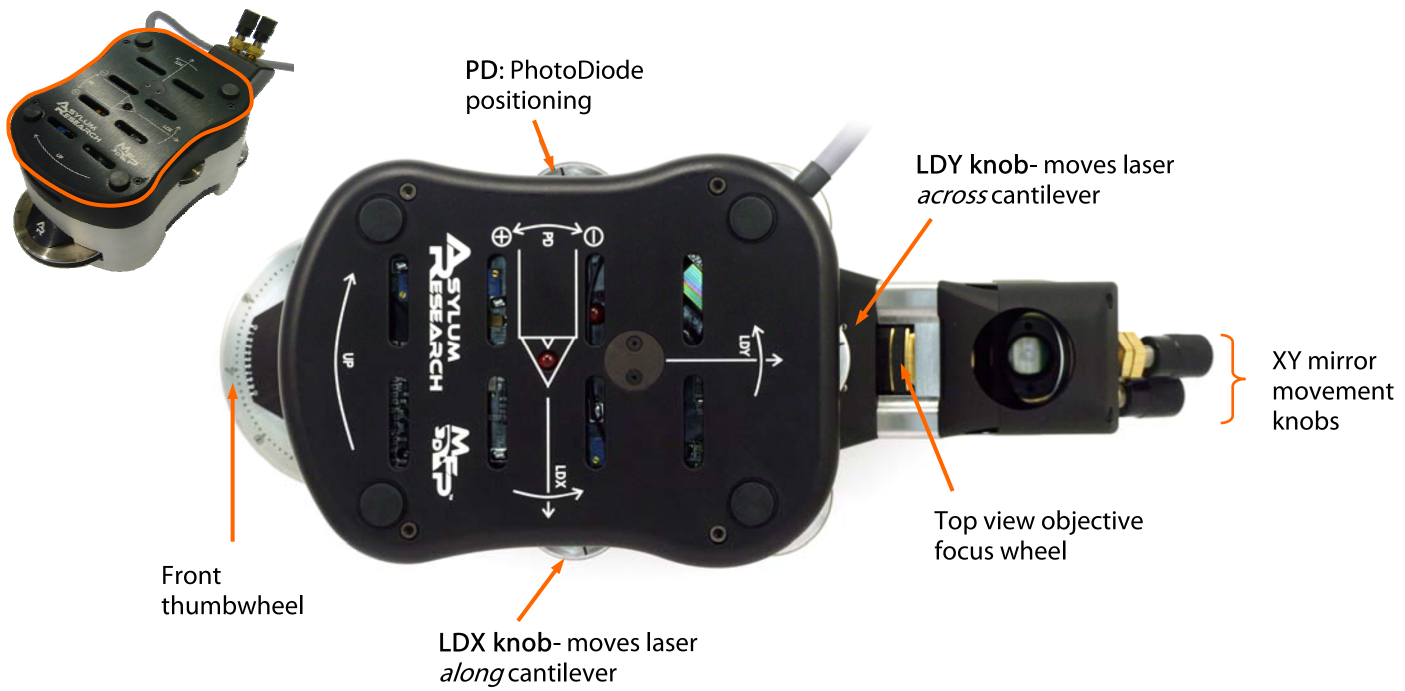


Figure 4.1: MFP-3D head with the various alignment thumbwheels labeled.

▪ The top of the MFP-3D™ head is conveniently labeled (Figure 4.1), including the orientation of the probe (with an LED being in the center of the triangular cantilever). The thumbwheel on the right (of head) moves the SLD along the length of the cantilever (LDX), while the thumbwheel on the back (of head) moves it across the cantilever (LDY). The head has arrows indicating which way the SLD will go as they are moved CCW. The left side of the head has a thumbwheel that adjusts the vertical deflection on the position sensitive photodiode (PD).

Figure 4.2: laser power on face of controller

✓ Turn on the SLD by turning the key on the front of the controller 90º clockwise (CW; Figure 4.2). Some activity in the Sum Voltage value on the S&D meter will also occur.

4.1A: SLD Alignment via IR card:

(For use with older heads without optics/ camera, or for the MFP purists; IR cards not shipping with systems as of summer 2009):

This is done on a stand that is kinematically machined for the legs, OR a table top- *BUT NOTON TOP OF THE SAMPLE.*

✓Place the IR card under the tip holder. Figure 4.3 (below) attempts to depict the procession needed to align the SLD. If the SLD spot isn't being blocked by anything, you can see it on the IR card. If you can't see it, move the SLD spot (using the LDX thumbwheel on the right side of the head, 'A' *in Figure 4.1*) along the length of the cantilever until you do- Figure 4.4A shows the visible SLD spot on the IR card. *If no spot is seen on the IR card, perhaps it may need charging for a few seconds under a fluorescent light.*

CAUTION: When adjusting any of the alignment thumbwheels, if you feel resistance turning them, ***DON'T FORCE IT***- It's probably at the end of its travel. If you over torque, it becomes very difficult to reverse its direction, OR the belt that is attached to the knobs to turn the pivot points on the optical assembly can get irreversibly screwed up, and it will have to be repaired at the factory. Use gentility when adjusting the LDX, LDY & PD knobs.

Then follow this procedure:

✓Move the SLD spot back onto the support chip via LDX thumbwheel (no spot visible- depicted in Figure 4.4B; *or* Action 1-Figure 4.3).

✓Move SLD spot until you see it on the card (spot visible- like Figure 4.4A; *or* Action 2-Figure 4.3);

▪*If the SLD spot is outside the transparent quartz window, it won't be visible on the IR card. This is indicated by the hatched area in Figure 4.3.*

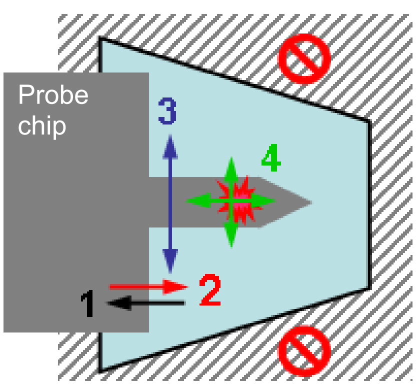


Figure 4.3: laser alignment pattern

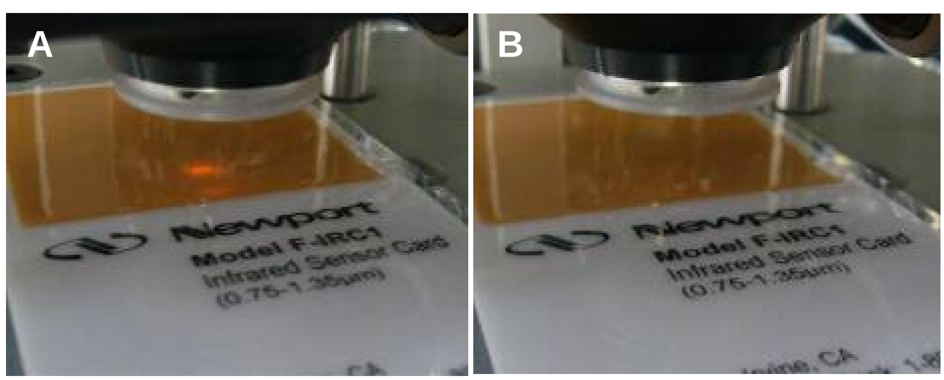


Figure 4.4: A) laser spot visible on IR card; B) laser spot not visible on IR card because it's being blocked by probe substrate

✓ Now use the LDY thumbwheel on the back side of the head ('B' *in Figure 4.1)* to adjust the beam perpendicular to the length of the cantilever (Sweep 3, Figure 4.3) WHILE MONITORING THE S&D METER.

There will be some activity (new value) in the 'Sum' Voltage on the S&D meter when the SLD crosses the cantilever.

⇨ Maximize/ optimize the 'Sum' voltage signal using both LDX & LDY thumbwheels (Sweeps 4, Figure 4.3)

✓ Move the LDX thumbwheel out (CCW) along the cantilever until the 'Sum' voltage in the S&D meter decreases to a small value again- this means the SLD spot is off the end of the cantilever.

✓ Move the LDX thumbwheel (CW) so the 'Sum' voltage reappears again; stop at a maximum value. *The goal is to place the SLD spot towards the end of the cantilever to maximize the optical lever sensitivity.*

✓ Move the LDY thumbwheel slightly to maximize the 'Sum' voltage in the S&D meter.

NOTE: for the most sensitivity in cantilever deflection, the SLD spot needs to be towards the end of the cantilever, *as opposed to the base where it meets the probe chip*. If the SLD spot is near the probe chip, the range of deflection won't be as large, regardless of how large the Sum signal may be.
See Figure 4.9 for visual example.

▪At this point, SLD should be properly aligned.

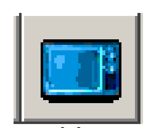
4.1B: SLD Alignment via top view CCD camera:

This method is a great way to actually see where the SLD spot is relative to the cantilever. It is also (generally) necessary when working in fluid, especially with functionalized probes.

▪ To actually see something with the CCD camera, the MFP-3D™ head needs to be over the sample. *It's a focus distance thing.*

BE SURE THERE IS ENOUGH ROOM BETWEEN SAMPLE AND TIP SUCH THAT IT DOESN'T CRASH AS THE HEAD IS PLACED OVER THE SAMPLE! If this does happen, the head won't be damaged (it's very robust), but the tip and sample will certainly be compromised.

✓ In the MFP-3D™ software, look for the CCD icon at the lower left tray of the MFP-3D software screen. If the fiber light is off, a black screen will come up in the software which is the video image.



ccd icon

✓ Turn on the power to the fiber light; using the variable potentiometer knob, increase illumination until you can see the tip in the CCD window.

FOR DUAL VIEW SYSTEMS ONLY: If the tip is not visible in the CCD image using a MFP-3D™ Dual View Stand Alone system, perhaps the top view optics and illumination shutter are set for bottom view- go to the Stand alone base and make sure both shutters are pulled out (See Figure 4.5). *The author commonly makes this mistake.*

Figure 4.5: Top view optics on Dual View Stand Alone base require illumination and camera shutters to be pulled out.

The default CCD image in the software is for a composite (RCA) video cable jack (yellow plug; below left)- If you are using a MFP-3D Bio, the CCD uses a S-video (4 pin jack; below right).

✓Click the '**Source**' button at the top of the CCD software window (red circle), which will change from Composite to S-Video. These jacks can be seen in the MFP3D base, and at the PC.

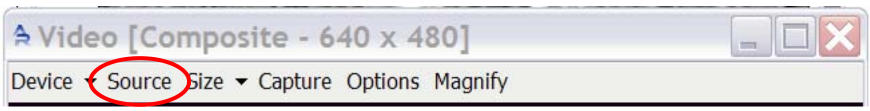
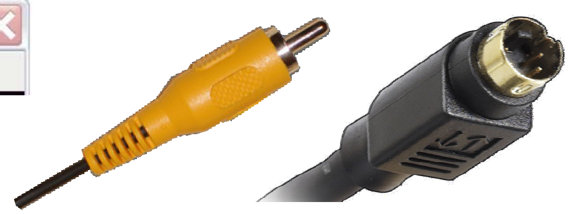


Figure 4.6: Click the 'Source' button to switch between composite/ RCA video (stand alone bases), or S-Video (MFP-3D- Bio).



Composite **S video**

▪ To adjust the CCD image focus, use the thumbwheel in the optics housing on the back of the head ('D' in Figure 4.1): CCW move the focus towards the sample.

▪ To adjust the location of the viewing field of the CCD camera, use the X and Y translation knobs on the back of the optics housing ('E' in Figure 4.1).

▪ Aligning the SLD using the CCD camera uses an approach similar as with IR card, but you can see the SLD spot with relation to the cantilever (Figure 4.7).

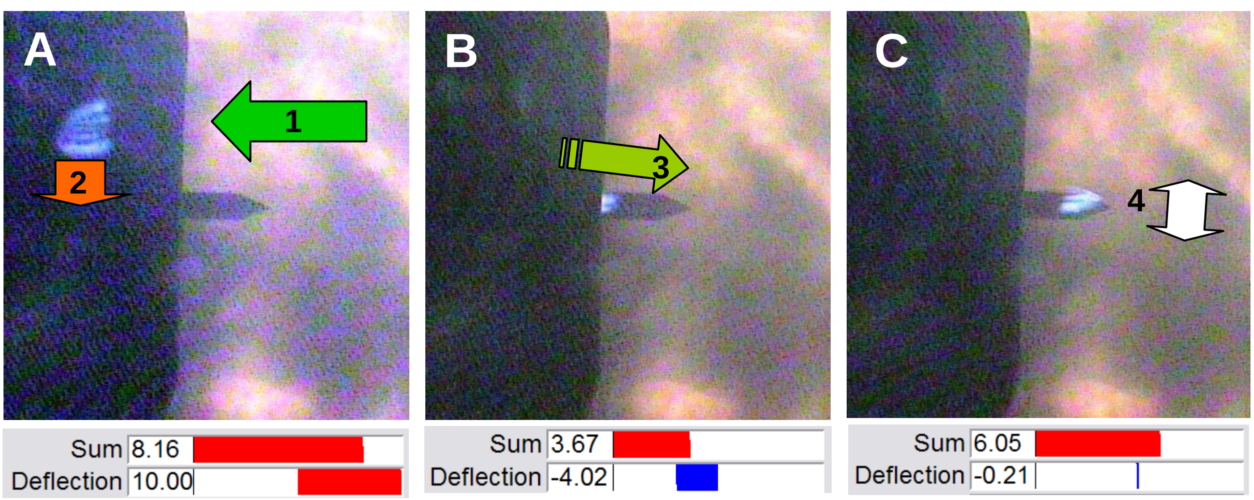


Figure 4.7: Aligning SLD with CCD (with concomitant Sum & Deflection values shown below): A) move spot back onto substrate with LDX knob (Action 1); B) using LDY, move SLD spot so it's inline with length of lever (Action 2), then use LDX to move out along cantilever (Action 3); C) once at end of lever, maximize Sum voltage with LDY (Action 4).

✓ Using the LDX thumbwheel, move the SLD spot onto the back of the probe substrate, following Action 1 in Figure 4.7A.

✓ Once the spot is visible on the probe substrate, use the LDY to move it such that it is in line with the length of the cantilever (Action 2 in Figure 4.7A).

✓ Turn the LDX CCW to move the spot to the end of theh lever- it should be visible during most the process unless spot is on anisotropic etch part of the probe substrate (Action 3 Figure 4.7B).

✓ Once spot is at end of lever, gently move the LDY side to side to maximize the sum seen in the S&D meter (Action 4 Figure 4.7C).

▪ The desired location of the spot is towards the end of the cantilever to maximize the optical lever sensitivity (Figure 4.8A). When the spot is at the base of the cantilever (Figure 4.8B), the optical lever sensitivity decreases (i.e., InvOLS value increases- see Section 9.1, page 9.5).

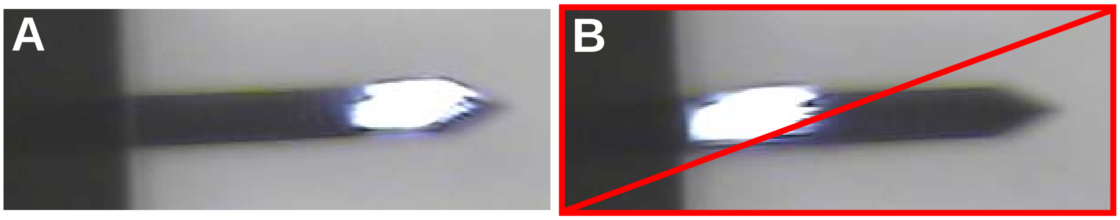


Figure 4.8: Align SLD spot towards end of cantilever to maximize optical lever sensitivity; B) shows spot at base of cantilever, which yield low optical lever sensitivity.

It is important that some elongation diffraction to the SLD spot is seen- this is the correct spot to use- see Figure 4.9*A*); If a spot <u>without</u> this elongation is seen (like the one in Figure 4.9*A* B), it is the wrong spot and will give no 'Sum' voltage in the S&D meter even though there appears to be a spot on the end of the cantilever.

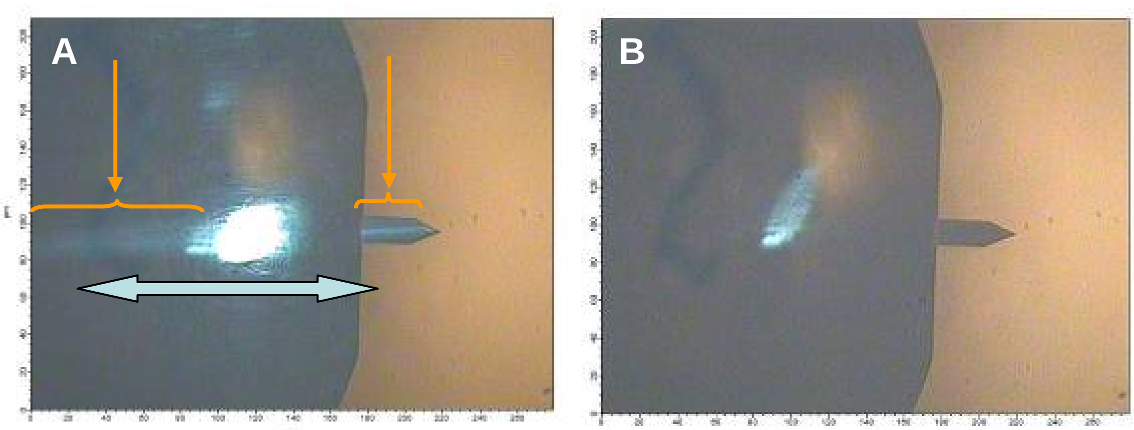


Figure 4.9: A) proper SLD spot with elongated appearance; B) SLD without elongation will give no appreciable Sum voltage. *NOTE: spot on probe chip for demonstration only.*

▪ Certain focal planes can also give the appearance that the spot is not on the cantilever in the CCD image, when in fact it is because the Sum voltage in the S&D meter.

If there is any question if the spot is aligned on the cantilever OR the probe substrate, there is a very easy way to tell- *do a thermal tune!* See Thermal Tuning (Section 5) for explicit instructions. If the SLD spot is on the Probe chip, there won't be any resonant peak (with significant amplitude or Q) in the power spectrum.

▪A ***ALTERNATIVE WAY*** to align the SLP spot using the CCD camera is to find the spot on the substrate- *Scott Maclaren (MRL-UIUC) taught me this protocol.*

✓ Turn the illumination down very low on the fiber light.

✓ Locate the SLD spot on the substrate- the author usually locates a diffracted spot that is along the same vector as the length of the cantilever. In Figure 4.10A, the actual SLD spot isn't vary apparent (red circle), but the diffracted spot is in line with the cantilever vector. *Please make exception to the author's careless probe orientation in this example.*

✓ Move LDX thumbwheel towards probe chip- the SLD spot is now reflecting off the cantilever (Figure 4.10B); there is a great deal of refracted light, presumably due to the low fiber light illumination level. Slight adjustment in LDY may also be needed to maximize 'Sum' voltage in S&D meter.

▪ When increasing the fiber light illumination, the spot is more apparent, and the amount of refracted light in the CCD image decreases (Figure 4.10C).

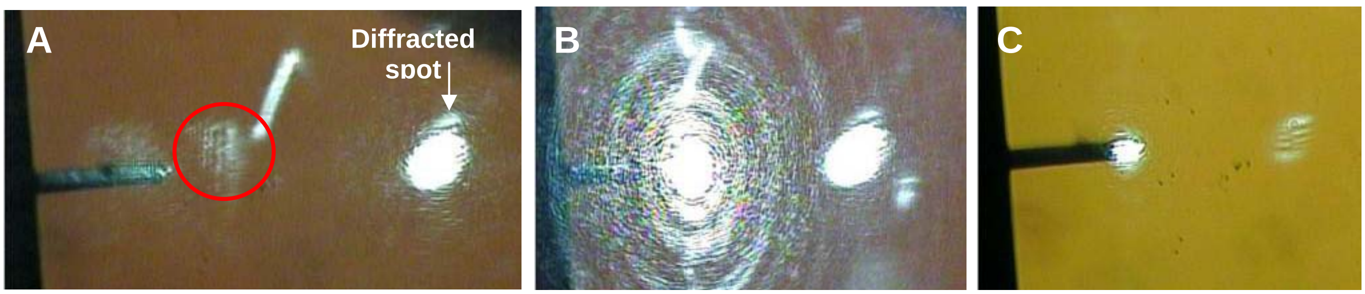


Figure 4.10: Aligning SLD starting with spot on substrate via CCD camera: A) using low fiber light illumination, locate SLD spot on substrate; B) move LDX and LDY thumbwheels until SLD spot is on end of cantilever with maximized 'Sum' voltage: C) increased fiber light illumination reduces refracted light seen in CCD image.

▪ *Alternatively, you can sweep the SLD spot until it crosses the cantilever; then maximize 'Sum' voltage.*

4.2: Zero Photodector (PD): *Now look at the 'Deflection' voltage signal in the S&D meter-*

This meter window is set up rather conveniently: If you have red in the deflection meter, you need to go negative to adjust to ~0 V (towards blue). The top of the head is labeled to tell you what way to go (positive or negative; see Figure 4.1; label 'C').

✓Use the PD thumbwheel ('C' in Figure 4.1) to do this: if the deflection value in the S&D meter is negative (blue), move thumbwheel CCW to get to ~0V; if the deflection signal is positive, move the PD thumbwheel CW to ~0V.

If you are working in fluid, there will be some thermal gradient causing the deflection to drift- use the PD thumbwheel to adjust this back to ~0V before beginning your AFM application. Typically, the first 20 minutes in fluid show the most pronounced amount of Deflection drift due to system equilibration.

4.3: TROUBLESHOOTING:

No significant Deflection voltage, or very low Sum voltages: typically either the cantilever isn't seated in the pocket well- perhaps some debris is acting as a fulcrum moving the orientation of the lever our of the proper plane;

- Sometimes the probe is bad, or worse, there is no cantilever on the probe (verify in CCD camera).

'Digital Wrapping' in the PD value: Say you are trying to zero the PD deflection from some 'railed' value in the deflection voltage in the S&D meter. No matter how much you turn the thumbwheel the way it's supposed to go, the deflection doesn't budge from that railed value (even if you go to the end of the travel and the thumbwheel starts to bind- AGAIN, DON'T FORCE IT!). What is likely occurring is something called '*digital wrapping*' of that deflection value.

So here's what to do-

➪Reverse the thumbwheel direction such that the turn is towards the same sign as your displayed deflection in the S&D meter (i.e, lets say the deflection value is railed positive- turn PD thumbwheel towards the positive, instead of toward negative)- what you'll find is at some point, the deflection sudden becomes railed negative; but the way it was displayed, it became wrapped to the positive. Then just keep thumbing the PD that way and it'll easily be zeroed. This is hard to explain in text, but easy to fix practically.

Reducing abuse on head cable:

Be careful not to torque/twist the cable to head: **always follow the same rotation path** as you took head off with. The head cable should only experience 180° worth of rotation in its regular on stage, off stage cycling.

Continual twisting of the head can:

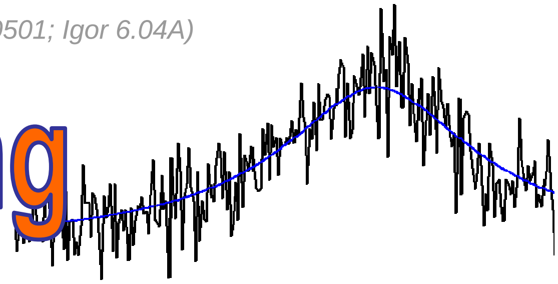
1) Break down the head cable over time (see Figure 4.10).

2) Torque the cable such that mechanical noise is more easily coupled into the head b/c of the torque. This can also add thermal drift into your system.

Figure 4.10: Excessive twisting of cable causes damage.

It is common that the system will experience some drift within the first 30 minutes or so- AR engineers think this may be mostly do to with the head "settling" into position in its kinematic mount. Internally acquired Drift vs. Temp graphs suggest the data from this period isn't very correlated with temperature, but more related to time, suggesting this mechanical settling. To attenuate this, gently place pressure on the head with slight rotation to the left- *it doesn't have to feel like you have moved the head- we're only talking microns worth of movement.*

5. Thermal Tuning



A Thermal tune is performed to determine the natural resonant frequency of the cantilever by monitoring the amplitude over a user defined frequency range. The MFP-3D™ uses the PD A/D converter to measure this, which can acquire frequencies up to 2.5 MHz.

Thermal Tunes are mostly used for a few specific tasks:

❶ To determine the frequency of a cantilever for drive frequency selection for AC mode in air or fluid; *OR* higher resonance eigenmodes for DualAC™ imaging techniques.

❷ Is the second step in determining the spring constant (see Section 9 for the complete cantilever spring constant, *k*, determination protocol).

❸ Determining resonant frequency changes if the tip has picked up material (or has become broken).

The Thermal tab is located in the Master Panel, seen in Figure 5.1.

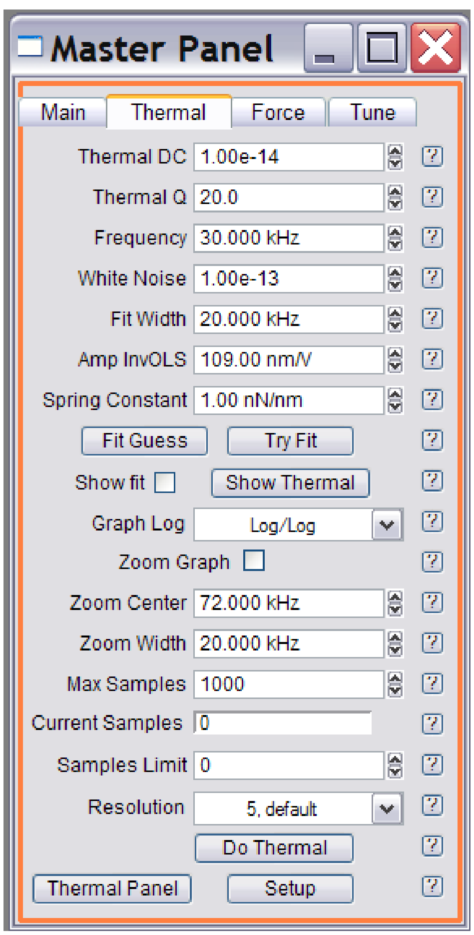


Figure 5.1: The Thermal tab.

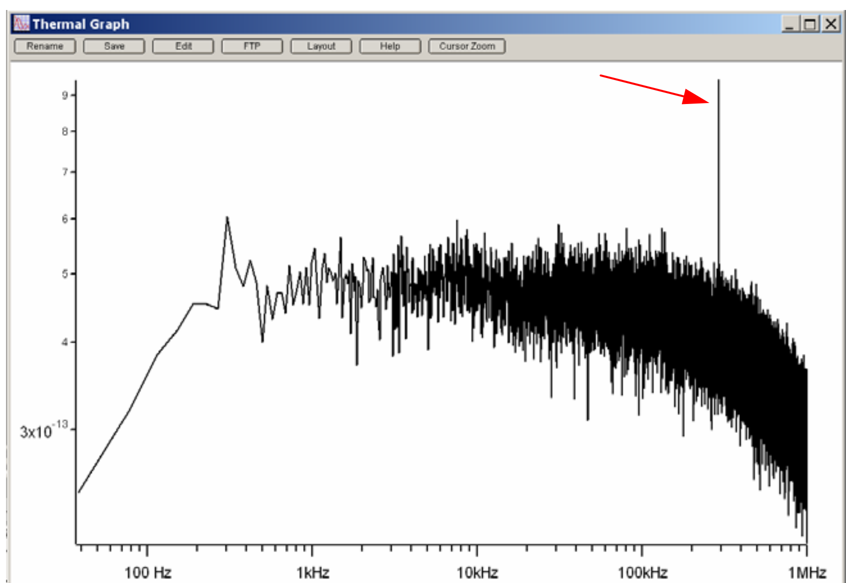


Figure 5.2: Power spectrum of typical AC 160 Si cantilever in air. Red arrow points to fundamental resonant frequency of cantilever.

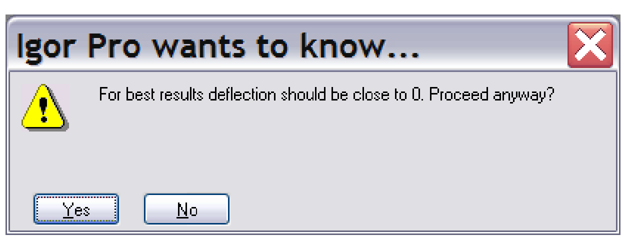


Figure 5.3: Pre thermal tune dialogue if PSD isn't zeroed.

Assuming a probe is properly loaded, SLD aligned and the PD zeroed, follow the below procedure to acquire a thermal tune of the cantilever-

✓Click the 'Do Thermal' button; a power spectrum plot similar to the one in Figure 5.2 will appear, continuously averaging spectrums in real time. In this example, an Olympus AC 160 Si cantilever in air was used. The higher Q of the cantilever causes the sharp peak in air.

▪ If the deflection isn't zeroed, a dialogue like the one in Figure 5.3, will come up- *don't be alarmed:* either click 'No' and zero the PD, or if precision isn't your top priority, click 'Yes' and continue. The reason to zero them is that PDs have more linear response in their center relative to their center. *The author typically zeros the PD.*

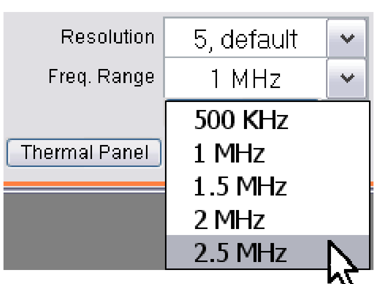
▪ The user can limit the number samples acquired; generally a couple dozen is sufficient for most tasks unless the S/N ratio really matters in the application. *Notice the baseline is mostly noise, but the one sharp line around 300kHz is the AC160's resonant frequency (red arrow, Figure 5.2).*

✓Click 'Stop' button to terminate the collection of power spectrums.

The resolution of the acquisition can be changed in the resolution pull down menu of the *Thermal* tab. Larger numbers go faster (less resolution); while smaller values acquire slower, but give less noise. The way the data is plotted (*i.e., linear vs. logarithmic*) can also be changed in the 'Graph Log' pull down menu.

The bandwidth of the Thermal tune can also be selected: the default parameter is 1MHz. To change this to a larger or smaller value, click the 'Setup' button in the *Thermal* tab, and activate the 'Show' checkbox for the Frequency Range at the bottom of the panel. Use the Freq. Range pull-down to select other Thermal Power spectrum frequency ranges (Figure 5.4A). Since the Thermal plot is default to only 1MHz, increase the scale on the thermal plot by clicking on the X axis to bring up Igor's 'Modify Axis' panel and manually increase the X axis to the desired range (Figure 5.4B).

A



B

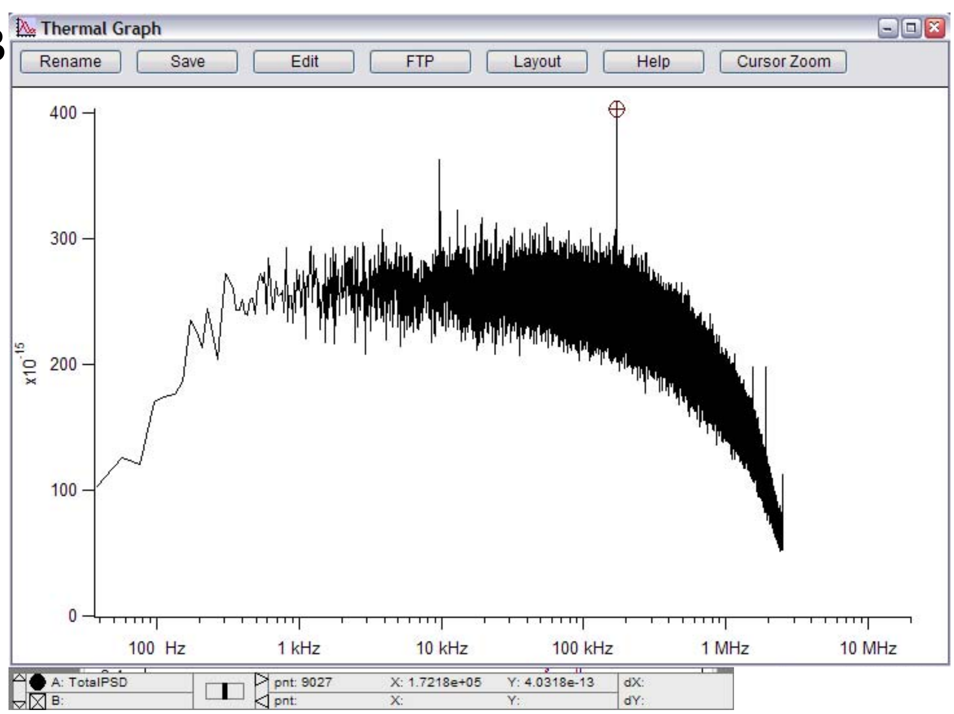


Figure 5.4: A) Selecting different frequency ranges for the power spectrum from the Freq. Range pull down menu; B) Use Igor Modify Axis Dialogue to manually increase frequency (X axis) range to display larger ranges, in this case, 2.5MHz.

Thermal tune plots can be saved by -

❶ Clicking the 'Save' button at the top of the thermal plot. Saves as Igor graph (.pxp)

❷ From File→Save Graph Copy… in the Igor main menu. Saves as Igor graph (.pxp)

❸ Sent to a Layout *as a graphic* (see Section 14.13).

✓ Zoom into the area of the peak by right mouse clicking around the peak, then right or left click to see the menu shown in Figure 5.5A.

✓ Choose 'Expand'; the result would look something like Figure 5.5B.

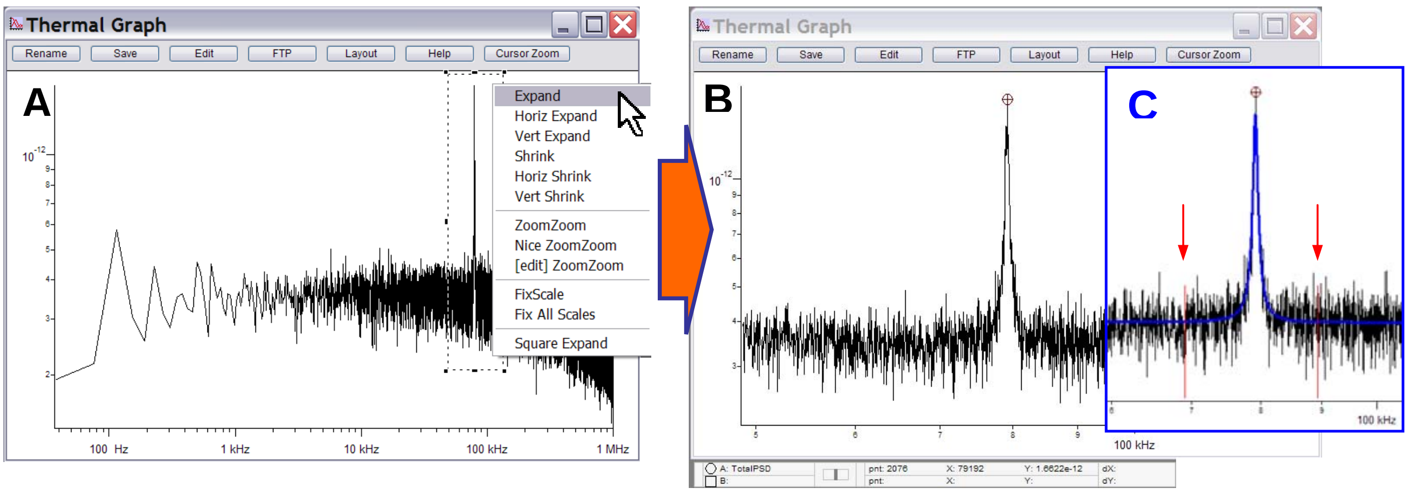


Figure 5.5: A) Full Spectrum thermal tune; B) Zoom of resonant peak; C) The 'fit' peak; Red arrows show 'fit width' cursors on power spectrum.

✓ Next, activate the Igor cursors (⊗,⊠), by using Ctrl + i; the cursors come up in the bottom tray of the plot.

✓ Drag one of the cursors to the peak. The example in Figure 5.5B, cursor ⊗ was placed on the resonant peak – 79.192 kHz.

✓ Type the 'X:' value (~79 kHz is sufficient) in the Igor cursor tray into the 'Zoom Center' parameter of the Thermal Tab. *Note the units in the tray are in Hz, while the units in the parameter box are in kHz.*

✓ Click the 'Show Fit' checkbox ☑; a blue fit line should appear on plot

✓ Click the 'Fit Guess' button; a blue Fit function curve appears somewhere in the vicinity of the resonant peak. If it doesn't, confirm that the 'Zoom center' setvar value is in a similar range as the resonant frequency peak.

✓ Click the 'Try Fit' button. The blue fit function curve should be fit to the resonant peak (Figure 5.5C); meanwhile the resonant frequency value and Q parameters in the Thermal Tab are updated.

▪ Thermal tunes work very well for determining the resonant frequencies (and ultimately drive frequencies) of cantilevers *in fluid*. See Section 6.2.2: 'AC mode imaging in-fluid' for this protocol.

The thermal tune is also a very convenient way to check whether the SLD spot is on the cantilever (when aligning SLD via IR card, sometimes a nice sum is given when the spot is on the probe substrate).

▪ Figure 5.6 (right) shows and example of a thermal tune when the SLD was on the back of the probe chip, yet a reasonable Sum voltage was displayed in the S&D meter.

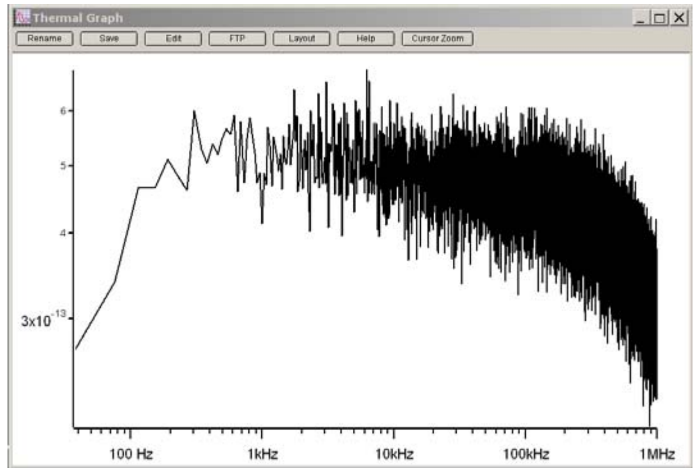


Figure 5.6: Thermal tune of SLD spot on back of probe chip - *no apparent cantilever resonant frequency.*

▪ Figure 5.7 shows two cases in which a thermal tune can tell you what has happened if the resonant frequency changes throughout an imaging experiment. *If the tip breaks (lowering mass, increasing* $\mathbf{f_o}$*; Figure 5.7A), or if there is an increase in mass on the cantilever/ tip from adhered debris (lowering* $\mathbf{f_o}$*; Figure 5.7B).*

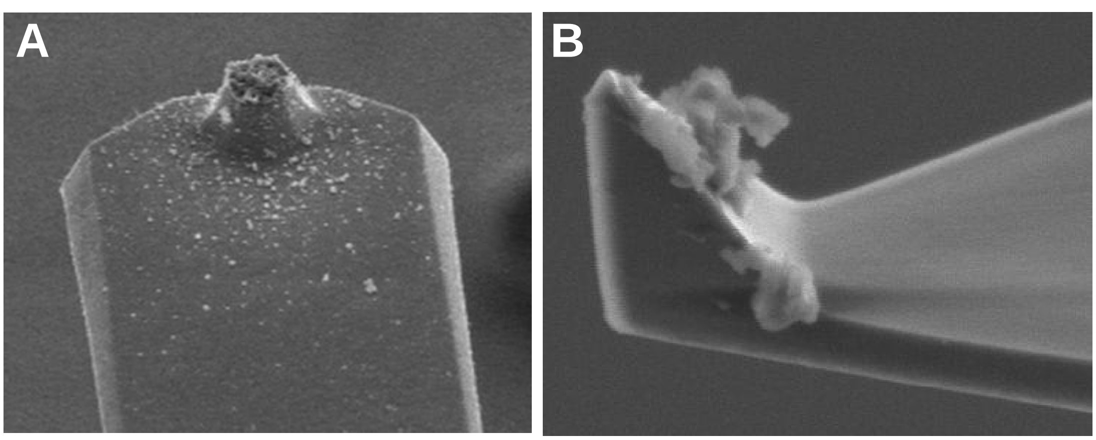


Figure 5.7: SEM images of compromised tips: A) broken tip, will shift f_o to higher value; B) debris on tip will add mass, shift f_o to lower value.

6. AC Mode Imaging

AC mode imaging is one of the most commonly used imaging modes in AFM. This section will discuss how to select a cantilever drive frequency, pre-engagement imaging parameters, tip engagement, and fine tuning the imaging parameters for artifact free images. See Section 7 for changing the appearance of the image windows and saving image files. Due to the different skills that need to be used during imaging in either air or fluid, this Section has been broken down into subsections:

6.1: AC Mode Imaging in Air:

The *Main* Tab of the Master Panel is where most of the parameters for imaging are found (Figure 6.1, right). The important parameters to tune will be Set Point voltage, Integral Gain, Drive Amplitude, Scan Rate (*highlighted in red*, Figure 6.1.1), and Scan Angle.

A description of each of the parameters in the Main tab are on page 6.2

✓ From Main tab in the Master Panel, confirm that 'AC mode' is selected from the imaging mode pull-down menu (Figure 6.1.1). AC mode is the default imaging mode.

▪ The '*Hamster*' wheel (on the front of the controller, Figure 1.5) can control/ precisely fine tune parameters activated by the radio buttons. Radio buttons can be seen in Figure 6.1.1 as the circular features to the riht of certain parameter setvar windows (i.e., Scan Size, X&Y Offsets, Set Point, Gain, Drive Amplitude, Drive Freq.); specifically, the Set Point voltage radio button is activated, indicated by a green center dot.

The first thing that needs to be done in AC mode imaging is to select the tip's oscillatory drive frequency during imaging- assuming a cantilever is loaded & the SLD properly aligned, follow the below procedure to obtain AC mode images **in AIR.**

✓Adjust the Deflection voltage (PD thumbwheel on the head; Figure 4.1) to ~ 0V (no color in deflection voltage S&D meter) for AC mode.

The Main Tab: Whether in AC mode or Contact mode, this tab in the Master Panel controls most parameters needed during imaging. The default panel looks like the one in Figure MainTab3. A brief description of the parameters and the relation to others is described below-

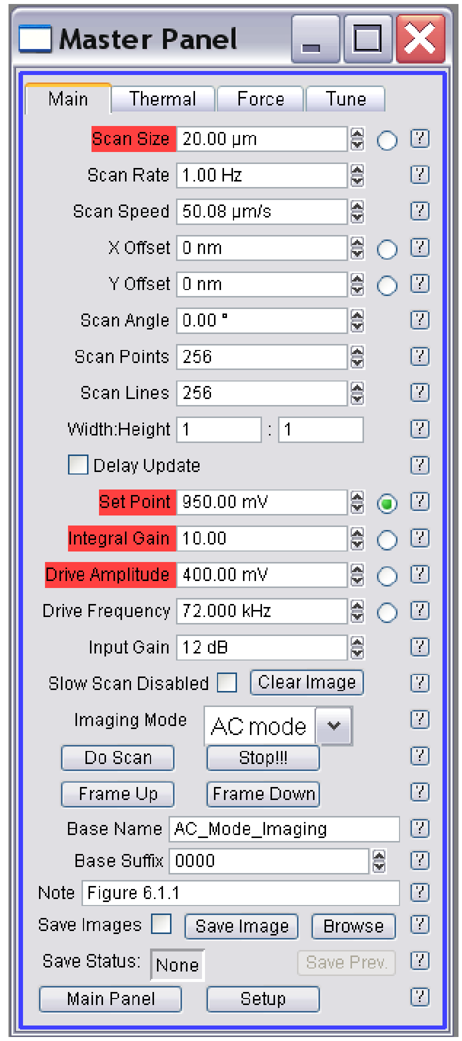


Figure 6.1.1: Main Tab for imaging parameters

Scan Size: scan area; 90µm x 90 µm maximum in closed loop (100 µm open); Area depends on amount of X, Y offsets

Scan Rate: Frequency to complete a trace retrace cycle

Scan Speed: analogous scan speed relative to Scan Size and Scan Rate

X, Y offsets: offsets center of image; affected by scan size

Scan Angle: positive angles rotate image *counter clock wise*

Scan Points/ Lines: amount of data points lines in image; can be asymmetrical; in multiples of 64

Width/Height: image aspect ratio

Delay Update checkbox: This will delay any parameter changes made until the scan reaches the end of its area;

Set Point: This is the setpoint voltage on the PSD the feedback loop uses (contact mode is deflection; AC mode is peak to peak amplitude)

Integral Gain: This is the main gain used for the feedback loop

Proportional gain: This is a feedback gain often used with very large Z range changes in
a sample; typically not useful for most imaging applications on the MFP3D

Drive Amplitude: the voltage applied to the shake piezo in the cantilever holder to obtain the peak to peak amplitude seen in S&D meter. *for use with AC mode only;*

Drive Frequency: The frequency that the cantilever is driven at (driven by shake piezo) *for use with AC mode only;*

Input Gain: a 20db analog amplifier on the 5MHz PSD; has adjustable gain values.

Slow Scan Disabled checkbox: when activated, only scans the same line; great for tuning a feedback loop

Clear Image button: clears all image data in the channels.

Imaging Mode pull down: Select imaging mode here: Contact, AC, PFM.

Do Scan/ Stop Buttons: Do scan will raster tip according to parameters; stop will withdraw tip

Frame Up/ down buttons: will begin scanning from top or bottom of image; good for activating the delay up date, or scan rate parameter changes made during a scan.

Base Name: File name; 17 characters or less; cannot start with a number

Base suffix: increases by one with every additional frame completed

Note: imaging notes can be put here

Save Images checkbox: activating this will ask where to save data; if not designated, will dump in My Documents>MFP3D data with a new folder for everyday

Save Status: lets one know if data is being captured; Save Partial, etc.

Setup: Expands window to allow user to show limited imaging parameters, or all of them.

6.1.1: AUTO TUNING:

✓ Open the *Tune* tab of the Master Panel (Figure 6.1.2).

✓ Choose a Target Amplitude of 1.0V. This means what it takes to drive the oscillate the cantilever so there is 1.0V worth of sine wave on the PSD.

NOTE- For sharp tips (i.e., spikes or other expensive probes), 1.0V worth of free air amplitude may be too much because the tip will be hitting the surface with more force then it might prefer- *INSTEAD,* choose a Target Amplitude of 300 mV or so. Proceed with the following directions, but adjust the set point (amplitude) voltage ratio accordingly.

✓ Change the 'Target Percent' to -5.0 %; however, depending on your application, you can assign it to any other user defined value within reason. *The description below was written with -5% in mind.* The minus sign indicates that the drive frequency will be on the left side of the resonant peak, which helps ensure the tip will remain in repulsive mode when engaged *(see Section 6.1.4, 6.1.4A for further description of repulsive mode).*

✓ The default Auto Tune 'High' and 'Low' values are ~50 kHz to 400 kHz, respectively, accommodating most (fundamental) drive frequencies for common commercially available AC mode cantilevers (in air).

✓ Click the 'Auto Tune' button; the frequency sweep commences.

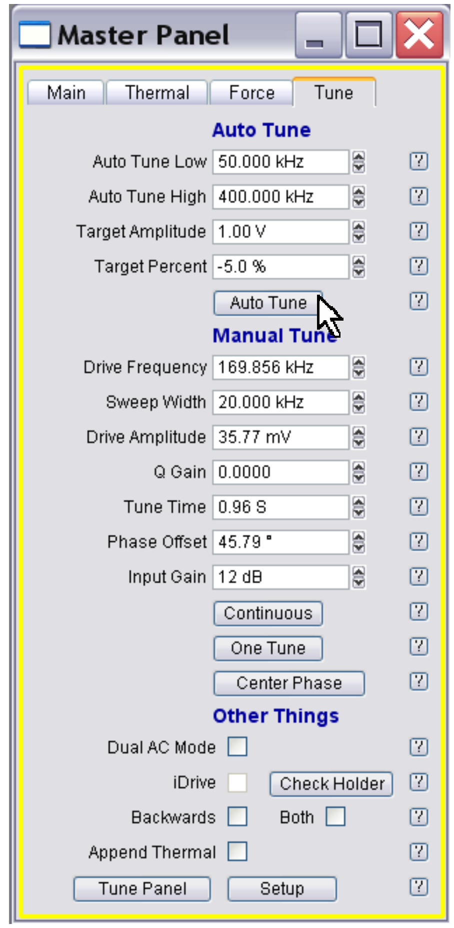


Figure 6.1.2: Tune Tab.

▪ An Auto Tune plot similar to the one(s) in Figure 6.1.3 appears- initially one appears momentarily that is similar to Figure 6.1.3A; then updates to one similar to Figure 6.1.3B. The software automatically picks a Drive Frequency at a Target Percent of -5% (left side) of peak maximum at the (*minimum*) Drive Amplitude needed to make a 1.0 V amplitude, displayed in the S&D meter. The left side of the peak will ensure that the tip experiences net repulsive forces with the sample as it interacts with the surface.

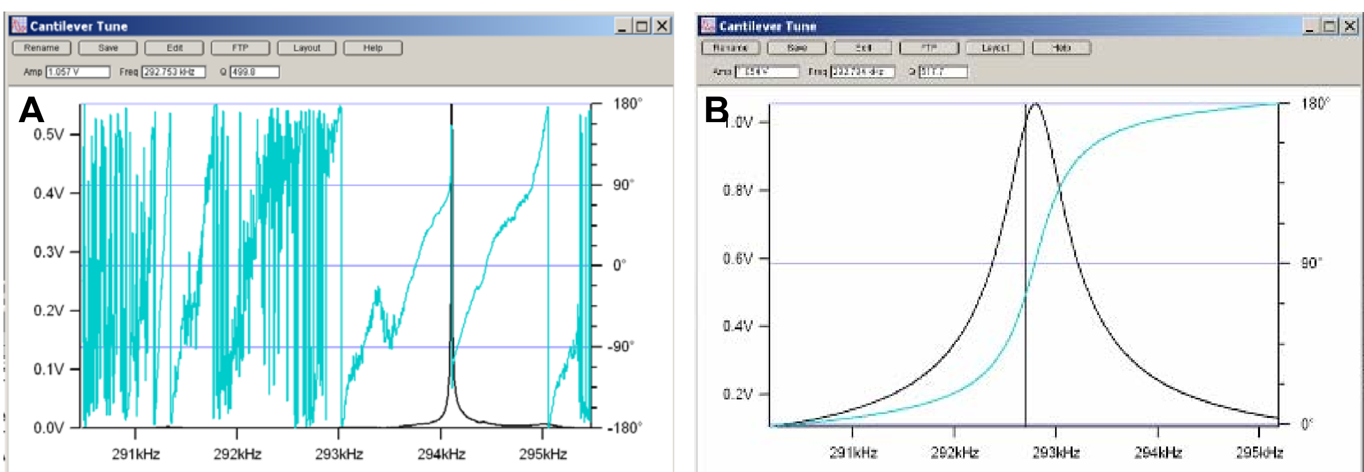


Figure 6.1.3: Auto tune of an Olympus AC160 Si (*f*~300kHz; *k*= 40N/m) cantilever in air: A) Early stages of Auto Tune; B) final result of Auto Tune.

▪ The 'Target Amplitude' & 'Target Percent' setvars can be defined by the user, depending on the application *(i.e., repulsive or attractive mode imaging, see Section 6.1.4).* The Drive Frequency value will automatically be updated in the *Tune* and *Main* tabs of the Master Panel, and the Q (quality factor) of the cantilever is determined, displayed at the top of the Cantilever Tune plot in and the *Tune* tab.

Tune plots can be saved by -

❶ Clicking the 'Save' button at the top of the thermal plot. Saves as Igor graph (.pxp)

❷ From File→ Save Graph Copy… in the Igor main menu. Saves as Igor graph (.pxp)

❸ Sent to a Layout *as a graphic* (see Section 14.13).

If using high aspect-ratio tips, there is a chance the Q (Quality factor) of the cantilever will be so high that the drive amplitude will be very low, and an error message comes up during the Auto Tune that says it's having difficulty completing the Auto Tune. There are a few different things you can do to get around this:

❶Manually increase the Drive Amplitude in the *Tune* tab, click the '**One Tune**' or '**Auto Tune**' button and it should work. Also click '**Center Phase**' if using 'One Tune'.

❷use Negative Q gain: a description of this is seen in section 6.1.4B.

❸the coupling to the tip may not be sufficient most likely due to the probe chip being seated improperly in the pocket under the tongue clamp.

▪ The teal colored curve is the Phase angle of the cantilever, which is set to 90 º at the resonant frequency value. Notice the Amp, Freq and Q values at the top of the panel are the values calculated for the largest peak within the Sweep Width (in Figure 6.1.3B's case, 5 kHz, defined in the Tune tab), and are updated in the *Tune* tab of the Master Panel. Also *notice the black vertical line represents the Target Percent amplitude (in this case: -5% of the resonant frequency, at 1.0V free air amplitude voltage).*

No idea what the resonant frequency is of your cantilever is?

NO problem! (although this is not normally necessary, unless doing some higher eigenmode application)- ***Perform a Thermal Tune*** first to (roughly) determine the resonant frequency of the cantilever, *as described in the Section 5.* ***In brief:*** ① Perform a Thermal Tune; ② Bring up the Igor cursors (⊗,☒) with Crtl + **i**; ③ Select the frequency of the fundamental peak; ④ Read value from Igor cursor tray, confirm this frequency value falls within Auto Tune 'High' and 'Low' values in *Tune* tab; ⑤ Click 'Auto Tune' button, as described above.

▪ At this point, the S&D meter has a 'free air' amplitude of 1.0 V (~ +/- 0.02 V), and the Phase value in S&D meter gives the 'free air' value of the phase- *this latter value will be important when tuning the parameters to avoid mode hopping artifacts.* A post auto tune example of what the S&D meter looks like is shown in Figure 6.1.4. If the 'Engage' button hasn't been activated since starting the software, there usually isn't any Z voltage color meter present, although it may say 70V (Figure 6.1.4- *don't be alarmed by this*).

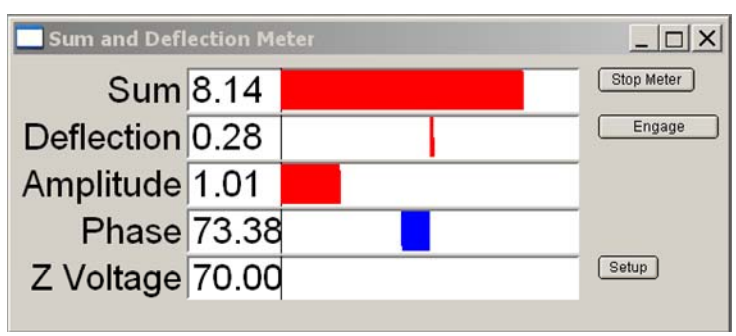


Figure 6.1.4: S&D meter after an Auto Tune; before tip engagement

6.1.2: TIP ENGAGEMENT:

Once a drive frequency has been selected, the next step is to select the pre-engagement imaging parameters (mainly the Set Point voltage ratio to Free Air amplitude voltage) that the feedback loop will maintain during imaging. There are two approaches to this- the 'Hard' engage; or the 'Soft' or gentle engage. The latter is the preferred methods among the Asylum inmates.

▪ *When doing a* ***Hard engage****, pick some percentage (like 20%) of the 'free air' amplitude voltage (i.e., the pre-engagement amplitude value found in the S&D meter) that will engage the tip on surface. Although it is quick and easy, there are some disadvantages to the 'Hard' engage, like truncating the tip to some degree as it snaps into contact with the surface during engagement.*

▪ *When doing a* ***Soft engage****, a very small percentage of the free air amplitude is used as the Set Point voltage (like 5%), falsely engaging the tip (on the water and compressed air layer between tip and sample); the Set Point voltage is then finally lowered using the setvars or Hamster wheel to some smaller percentage to induce a 'Hard' engage.*

⇨ *THE ADVANTAGE OF THE SOFT ENGAGE is the tip engages at the absolute lowest Set Point FORCE, preserving the tip's apex. Both will be discussed below-*

BUT FIRST, here's something to give an idea about choosing Set Point Voltages-

▪ A qualitative relationship between Free Air Amplitude voltage, Set Point Amplitude voltage and relative force applied by the tip amplitude *vs.* tip-sample distance in AC mode is depicted in Figure 6.1.5 below. The graph shows that as the Set Point voltage is decreased (*at constant Drive Amplitude*), the tip applies more force to the sample. The Y axis can be thought of a Set Point voltage percentage of the free air amplitude, where 0% would be the 'free air' (cantilever not dampened) amplitude voltage, and 100% would be 0V.
The two different approaches to engaging the tip are described below-

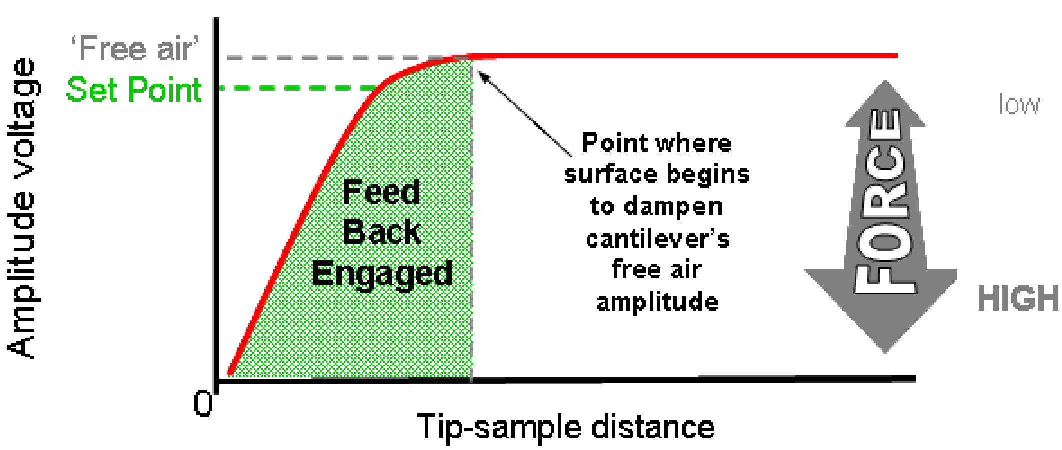


Figure 6.1.5: A qualitative conceptual amplitude voltage *vs*. tip-sample distance for AC Mode imaging. Lower set point values result in higher tip-sample force; higher set point values result in lower tip-sample forces.
NOTE: This plot is not to scale, nor is actual data.

6.1.2A: Hard Engage:

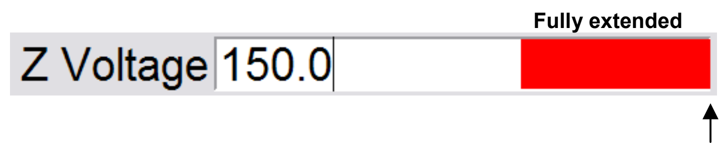
✓ A good Set Point voltage to use is ~20% of the free air; For example, if the free air amplitude is 1.0V, 20% of that is 800mV (coincidentally, that is the default Set Point used). With larger Set Point percentages, you'll notice more of a 'Snap' to contact upon engagement with the Z piezo voltage in the S&D meter, which is likely to truncate the tip's apex (to some degree), ultimately compromising the lateral resolution of the imaging.

✓ Choose an Integral gain of 8 to 10. This allows the feedback loop to be plenty responsive enough as the tip engages with the surface.

✓ The Drive amplitude was chosen by the software in the Auto Tune step. *Different cantilevers will give different voltage values to achieve this Target amplitude during the Auto Tune.*

✓ Choose a scan rate of ~ 1Hz. If it's a soft / delicate, or very rough sample, choosing a slower scan rate is a good idea.

✓ Click the 'Engage' Button on the S&D meter; you'll notice the Z piezo voltage will increase to 150V, meaning it's fully extended anticipating the Set Point voltage to be reached (activating feedback loop).



✓ Wheel the thumbwheel on the front of the MFP-3D™ head counterclockwise. As the tip nears the surface, the Amplitude voltage value on the S&D meter will decrease until it reaches the same voltage as the user-defined Set Point. At this point, there is a concomitant chime sound (if speaker volume up), and the Z piezo voltage will go from 150 V to some lesser value (less red on the S&D meter). As the thumbwheel is further turned CCW, the Z piezo voltage will decrease (i.e., piezo is retracting) because the feedback loop is maintaining the user defined Set Point voltage relative to Piezo position in its range. *Adjust the Z piezo to ~ 70V such that the piezo is in the middle of its travel.*

▪ *If the sample is reflective, the shadow of the cantilever can be seen in the top view CCD camera image as it approaches the surface. See Section14.5 for an example of this.*

▪ If you have a surface with deep holes on a plateau, adjust the Z piezo voltage such that it is more retracted upon engagement, allowing the tip to have the range to plunge into the holes during scanning (presuming the tip apex is small enough). The opposite approach can be used for surfaces with very large features.

▪ The Z voltage value is the way to qualitatively monitor how far the piezo is retracted or extended in its overall travel range (e.g. 70V is in the middle of its range).

▪ At this point, the tip is just sitting on the surface, with the feedback loop maintaining the user defined Set Point voltage/amplitude/force.

▪ To begin imaging: See Subsection 6.1.3 for how to fine tune the imaging parameters (Set Point, Drive Amplitude, Scan Rate, & Integral Gain) while monitoring the images and scan (re)traces to obtain best possible, non-artifact laden image.

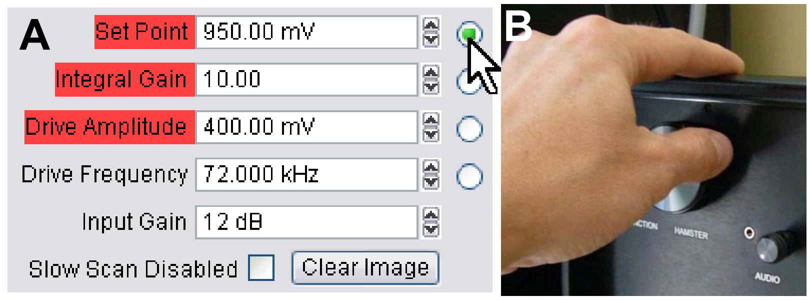


Figure 6.1.6: A) Activating radio label in Main tab; B) using Hamster wheel.

6.1.2B: <u>SOFT/ Gentle Engagement:</u> If you want to *<u>engage at the very lowest Set Point force possible</u>* (because you are using a modified/ functionalized tip; or an expensive, super-sharp tip; OR just want the best XY resolution achievable with a given tip) the MFP-3D™ is perfect for it. Start with a false engagement, then lower the Set Point voltage to truly engage on the surface.

Here's how to do it-

✓Perform an Auto Tune (Section 6.1.1).

✓ Set the Set Point voltage for about 5% of the free air amplitude voltage (i.e., for a 1.0 V free air amplitude, use a Set Point voltage of 950 mV; Figure 6.1.6A).

✓ Click 'Engage' button, as you normally would. Slowly turn the front thumbwheel CCW while monitoring the amplitude voltage (S&D meter), waiting for it to equal the Set Point voltage, at which point the feedback loop is engaged.

▪ Now the feedback loop is activated- *However,* this engagement is a false engagement- the tip is engaging on the (inherent) water layer and compressed air between the tip and sample.

✓ Adjust the Z piezo voltage to ~30 to 50 V (halfway blue). *You'll notice the Z piezo voltage seems to float/drift as you move the thumbwheel- that's because it's falsely engaged!*

✓ With the radio label for the Set Point voltage activated (use mouse cursor in Figure 6.1.6A, or use toggle switch on front of controller), use the 'Hamster' wheel on front of controller to increase the force (i.e., decrease Set Point voltage value- Figure 6.1.6B); the Z piezo voltage will move to some more positive voltage value (more red). At some point, you will see a 'hard' engagement in the Z piezo; continuing to decrease the Set Point value, you'll notice the Z piezo will no longer move to some more positive value; additionally, there usually is some concomitant phase decrease.

▪ At this point, the tip is 'hard' engaged on the surface, It has been engaged at the lowest possible Set Point force possible, therefore preserving the tip shape. It is *stationary* on the surface, waiting to be told to scan.

The stiffer the cantilever, the harder it is to do a gentle engage. When this occurs, you can engage in attractive mode with a low amplitude, then switch it back to repulsive mode once the surface is found. See section 6.1.4B for attractive mode description and parameter setup.

Is the acoustic hood hatch still open?- Probably. Follow this to preserve the tip's apex while shutting the door:
❶ Use the Hamster to *<u>increase</u>* the Set Point voltage to pop the tip off the surface (apparent in the Z voltage value(S&D meter);
❷ Close the hood door;
❸ Decrease the Set Point voltage with the Hamster again to hard engage;
❹ Proceed scanning. Publish Data/ acknowledge AR in experimental.

6.1.3: TUNING IMAGE PARAMETERS:

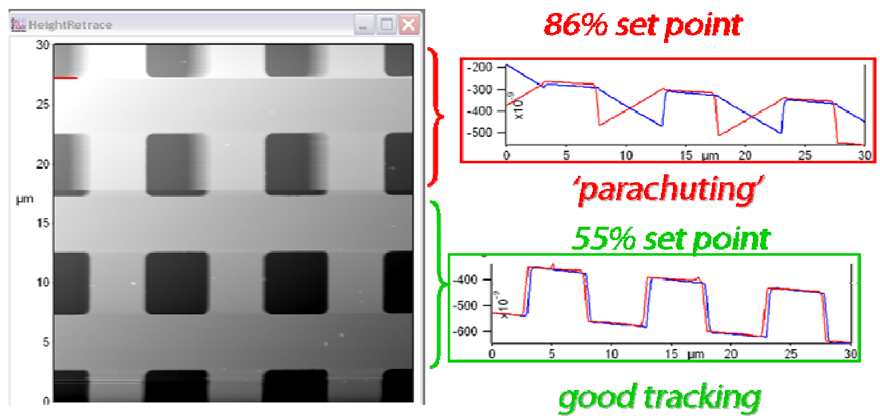
Once the tip is engaged on the surface, imaging can commence-

✓ Click the 'Do Scan' button, or 'Frame Up' or 'Frame Down' button on the *Main* tab of the Master panel. This will move the tip to the corner of the scan area, and begin scanning. There will be an initial time out while Igor is busy executing the command. (*Notice that the parameter increase/decrease arrows are not present while Igor is busy executing a parameter change*).

▪ Once scanning commences, the tip's tracking of the surface tip is generally very poor. This can be seen in the individual Trace and Retrace fast scan lines below each of the image channels, sometimes called 'parachuting'- where one side of the feature has very poor tracking; notice the opposite trace has the parachuting occurring on opposite side. Figure 6.1.7 shows this poor tracking of a calibration grid, and what is adjusted to improve the tracking.

▪ Typically, three parameters should be adjusted first, in some combination depending on preference:

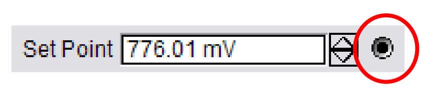
➊ The **Set Point voltage** generally must be adjusted, especially after doing a soft engage (because of the low set point force being maintained by the feedback loop). Decreasing the Set Point voltage value increases the force applied to the sample (recall Figure 6.1.5). Higher Set Point voltages (i.e, lower force), will help preserve the tip apex, but may not allow proper tracking of the surface.

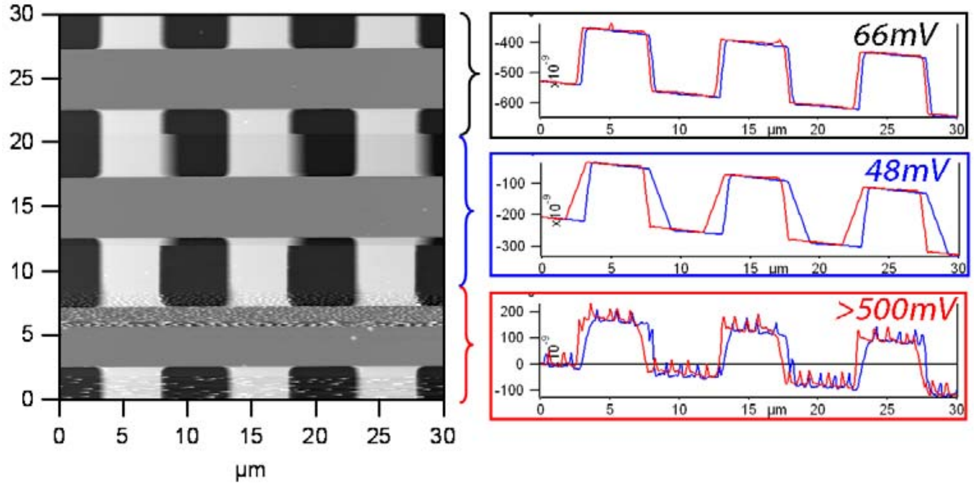


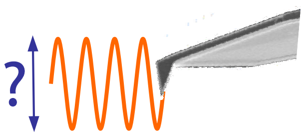
Adjusting the Set Point Voltage to more than 50% of the free air amplitude voltage doesn't really help apply more force. Beyond that, the cantilever isn't really acting far from a simple harmonic oscillator anymore.

▪ The **Drive Amplitude** can also be adjusted to increase the amount of Drive Amplitude applied to the shake piezo (and hence, cantilever); advantages of increasing this can be maintaining the tip in repulsive or attractive mode (See section 6.1.4), or when imaging sticky samples- the larger amplitudes help the tip escape the forces of the sample.

✓ Use the setvar arrows (to the right of a parameter setvar window), or the 'Hamster' wheel with activated radio button (red circle, right), to fine tune any of these tuning parameters in real time while the tip is scanning.







Want to know the actual (peak to peak) amplitude that the cantilever is oscillating?
⇨ the InvOLS of the cantilever must be known (*see Section 9.1, page 9.5).* For example, say you have the imaging parameters all tuned up to your liking and the Drive Amplitude setvar is set at 43 mV for a cantilever with an InvOLS of 56.43nm/V; then

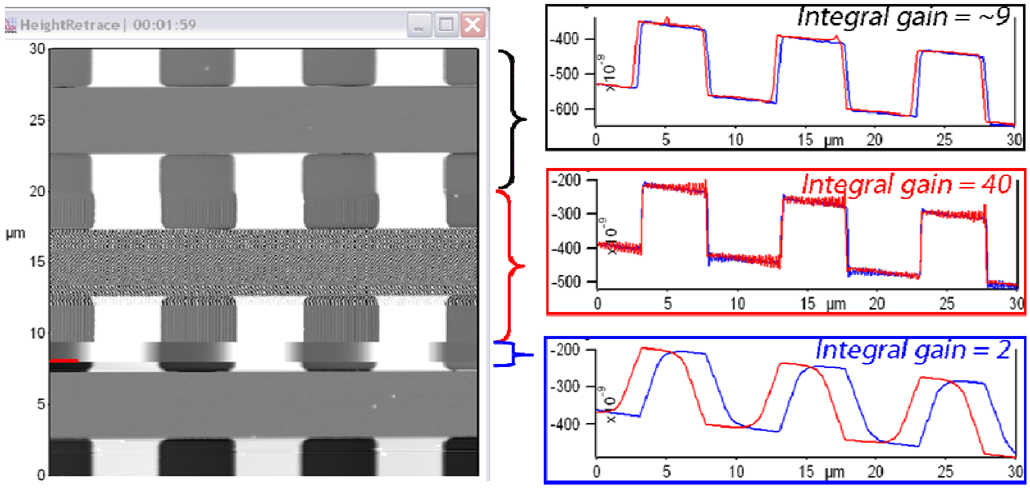
Actual Cantilever Amplitude= (0.043V)(56.43 nm/V)= 2.43 nm.

▪ *Some AR applications scientists adjust Drive Amplitude first- it's a personal preference. The trade off by increasing the Drive Amplitude is beating the tip apex harder against the surface* causing oscillations in the image (especially at feature edges)*; The tradeoff of lowering the Set Point voltage is applying more force to the sample (refer to Figure 6.1.5).*

▪ The **Integral Gain** should be adjusted such that the surface is tracking well; things to avoid are decreasing it to a value that doesn't allow the feedback to track the surface well, *OR* too high that oscillations are apparent in the image and trace/retrace scan lines below the images. When adjusting the Integral gain, monitor the amount of noise in the Amplitude Image- feedback oscillations are easily seen in the image since because it's an image of the feedback loop error.

▪ One of the best approaches to adjusting the Integral gain is to increase it until there is a 'ringing' seen in the (re)Traces lines below the height image. Then decrease it until this ringing goes away.

▪ Some adjust the Integral gain first before adjusting drive amplitude or Set Point Voltage.



Adjusting the 'Proportional' gain doesn't generally improve the imaging- this is because the frequency range of the scanner is below the range where the proportional gain contributes. It's best to keep proportional gain at zero. The *exception* to this rule is when very large sharp topography features are present where the feedback loop needs to react very quickly to the surface.

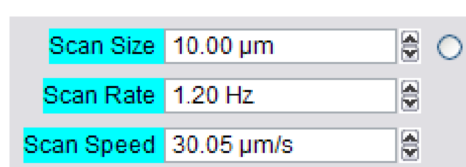
During tuning-
✓ Monitor how the tip's tracking improves by looking at how well the trace and retrace line scans compare to each other. Note that they do not have to overlap exactly (because they are slightly offset in the software display), but they should have similar shapes/slopes per given surface feature.

The following imaging parameters can be used to further improve tuning parameters-

▪ The **Scan Rate** is a parameter you can adjust to also improve image quality. Slowing the Scan Rate down can help the feedback keep up with the image features. The Scan Rate cannot be updated on the fly (during imaging) like the aforementioned imaging parameters. You must click 'Frame Up' or 'Frame Down' buttons to initiate the newly entered scan rate. *Keep in mind that too slow a scan rate can introduce image artifacts due to drift (if system isn't higher equilibrated, which is typically unlikely unless a day of equilibration has occurred in a very stable room).*

▪ The **Scan Angle** is something that can be also be adjusted. Sometimes changing the scan angle can reduce the havoc imparted on a cantilever; or changing the scan angle slightly to scan along or orthogonal to a high aspect ration surface feature. This parameter can be changed on the fly.

The **Delay Update** ☑ checkbox allows the user to change the parameters during a scan, which will take effect at the end of that scan. During the period before update, the parameters changed will be highlighted in a light blue color. Notice that setvar without radio labels, these highlights also occur because these values only take effect when the frame is finished (e.g. scan rate).



▪ *It's also great for changing the number of scan lines and points on the fly.*
-Without the Delay Update ☑ checkbox activated, the tip has to be withdrawn to change either the Scan Lines or Scan Points setvar values. The author is superstitious enough, and has had experiences where the tip is somehow changed during this withdraw step.

-However, with the Delay Update ☑ checkbox activated, these parameters can be changed, then click the '**Frame Up**' or '**Frame Down**' buttons to activate this new change- *Notice the tip stays on the surface* (S&D meter- Z piezo voltage). This makes scanning the surface at a lower value of scan points and lines to look for something more efficient (quicker), then increase for higher resolution imaging.

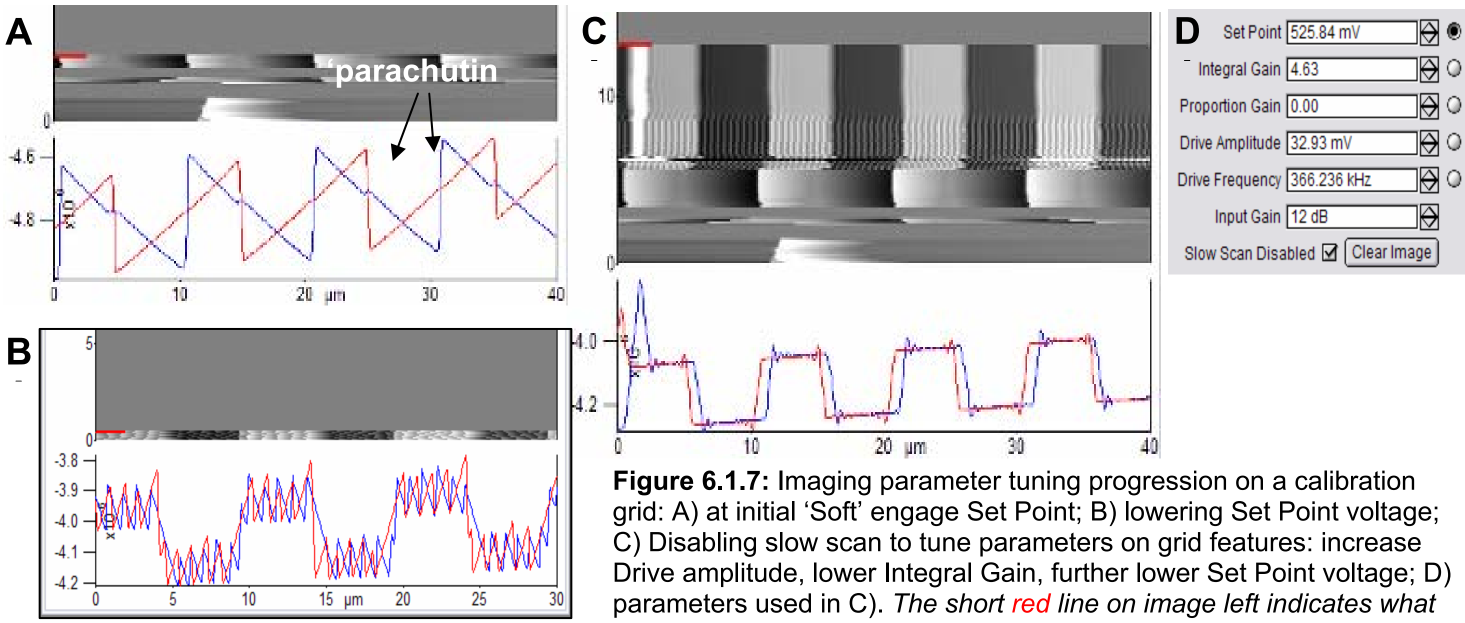


Figure 6.1.7: Imaging parameter tuning progression on a calibration grid: A) at initial 'Soft' engage Set Point; B) lowering Set Point voltage; C) Disabling slow scan to tune parameters on grid features: increase Drive amplitude, lower Integral Gain, further lower Set Point voltage; D) parameters used in C). *The short red line on image left indicates what slow scan line the tip is at.*

▪ Figure 6.1.7 shows an example of a typical imaging tune progression of a calibration grid (5µm x 5µm x 200nm depressed features with 10µm pitch).

Panel A) The image shows the tip scanning at the Set Point voltage needed for the 'Hard' engage after performing a Soft engage- *notice the very poor tracking in the trace and retrace;* and poor image quality.

Panel B) The Set Point voltage was slightly lowered to 890mV (while maintaining Integral gain=10 & Drive Amp= 30.9 mV) - *notice the tracking improves, yet there are great oscillations due to the Integral gain was too high*;

Panel C) The slow scan was 'disabled' so the parameters could be tuned along the same scan line (this feature is in the *Main* tab of the Master Panel)- it's an easier comparison since you expect the features to be the same from scan line to scan line; the drive amplitude was increased and the Integral gain was decreased, most noticeable in the oscillations in the image. At the point where this screen shot was obtained, the imaging is pretty close to very good.

Panel D) shows the parameters at the slow scan line (red bar at left side of image).

▪ Another common imaging artifact occurs when going over asperities with an insufficient amount of Set Point force because there isn't sufficient dampening of the cantilever. This can be easily tuned up with an increased Set Point force (i.e., lower set point voltage, Figure 6.1.8). Notice the asperities and upper large feature in A) have a tailing (parachuting) on one side due to a low set point force. This is because the feedback loop isn't tuned well enough to track those features efficiently. In B), the set point force was increased to improve tracking.

Figure 6.1.8: Increasing Set Point force (i.e., lower set point voltage) to improve tracking over large surface asperities: A) before adjustment- 'Gentle' engage Set Point force (i.e., lowest set point force to engage); B) increased Set Point force increases cantilever dampening to reduce poor tracking

Another image parameter tuning approach is to determine the integral gain ***before*** adjusting Set Point voltage or Drive amplitude voltage. Advantages of this are using low Set Point forces and or lower drive amplitudes (AC mode), further preserving tip and sample. *Nicholas Geisse, AR Bio apps scientist taught me this- he does this for imaging cells using contact mode in fluid.*

Here's another useful image tuning habit-

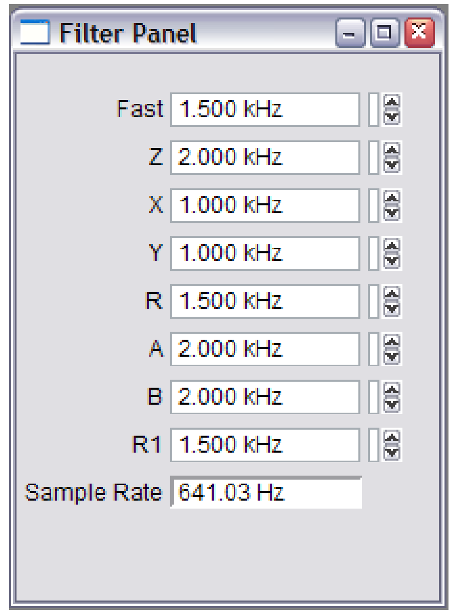
✓ With the slow scan disabled checkbox ☑ enabled (so the tip scans the same lines, i.e. same features for better comparison (Figure 6.1.7C)), increase the integral gain until oscillations are seen in the fast scan Trace/ Retrace lines below the height (or Z sensor) channel;

✓ Then back it off until they disappear.

✓ At this point, adjust Set Point voltage (and / or Drive amplitude) until good tracking occurs.

✓ Scan rate and angle can also be adjusted to further improve image quality.

Filter Panel:



In some instances, there is need to adjust the low pass filters (LPF) on the ADCs to quell some image noise.

✓Go to Programming → Make Filter Panel; the Filter Panel (right) appears.

▪ Depending on whether imaging in contact mode or AC mode, the Fast and R LPF setvars can be adjusted- there is a LP filter one would want to adjust initially. This LPF on the PSD is called the ‘Fast ADC’ (because its 5MHz, 16 bit); It also has a 1 to 10x gain on it- this is the ‘Input Gain’ setvar in the *Main* tab of the Master Panel that adjust the amount of bits used in the S&D meter (see Section 6.1). Anyway, this LPF goes into either the FPGA, or DSP depending what imaging mode is activated.

For Contact mode-
Fast applies this LP filter to the FPGA in the controller. The author suggests slowly stepping this from default 1.5 kHz in 50 or 100 Hz increments.

For AC mode-
R applies the LP filter to the Amplitude signal *(i.e., Rcosθ)* that goes into the DSP. As it is decreases, you can notice the sharpness of the features to decrease (i.e., get smoother). If too high, the features seem jittery or noisy- step down in 100 Hz increments until satisfied the spurious noise is getting clipped appropriately.

Z, X & Y are the LP filters on the LVDT sensors LPs- located on the inputs. *The author would only change X,Y or Z to improve imaging in special cases, which escapes the author at the time of writing this manualette.*

A, B are auxiliary LP filters; these LPs are located on the inputs

R1 is for second amplitude techniques such as the Bimodal stuff (Dual AC- see Section 6.1.5); also located on the DSP.

Sample Rate is a result of the scan rate and pixel resolution.

6.1.4: MONITORING PHASE in AC MODE:

This section explains the basic approach to preventing a common image artifact known 'mode hopping' (i.e., when the phase switches between intermittent contact (net repulsive forces) and non-contact modes (net attractive forces)), See Figure 6.1.9 for examples of artifacts). The MFP-3D™ software allows to better control whether the tip is in a net attractive or repulsive force regime (with respect to the surface) by monitoring the phase signal of the oscillating cantilever during imaging. *The Phase is always on.*

Mode hopping occurs when the Phase flips between greater and less than the free air Phase value (θ). This can be easily seen as off-scale z data in the Phase channel (Figure 6.1.9). *This concept works well in air, but doesn't no seem to hold true in fluid when driving the cantilever with the shake piezo due to the complication of the resonant cavity peak created between tip and sample from the oscillating cantilever holder.*

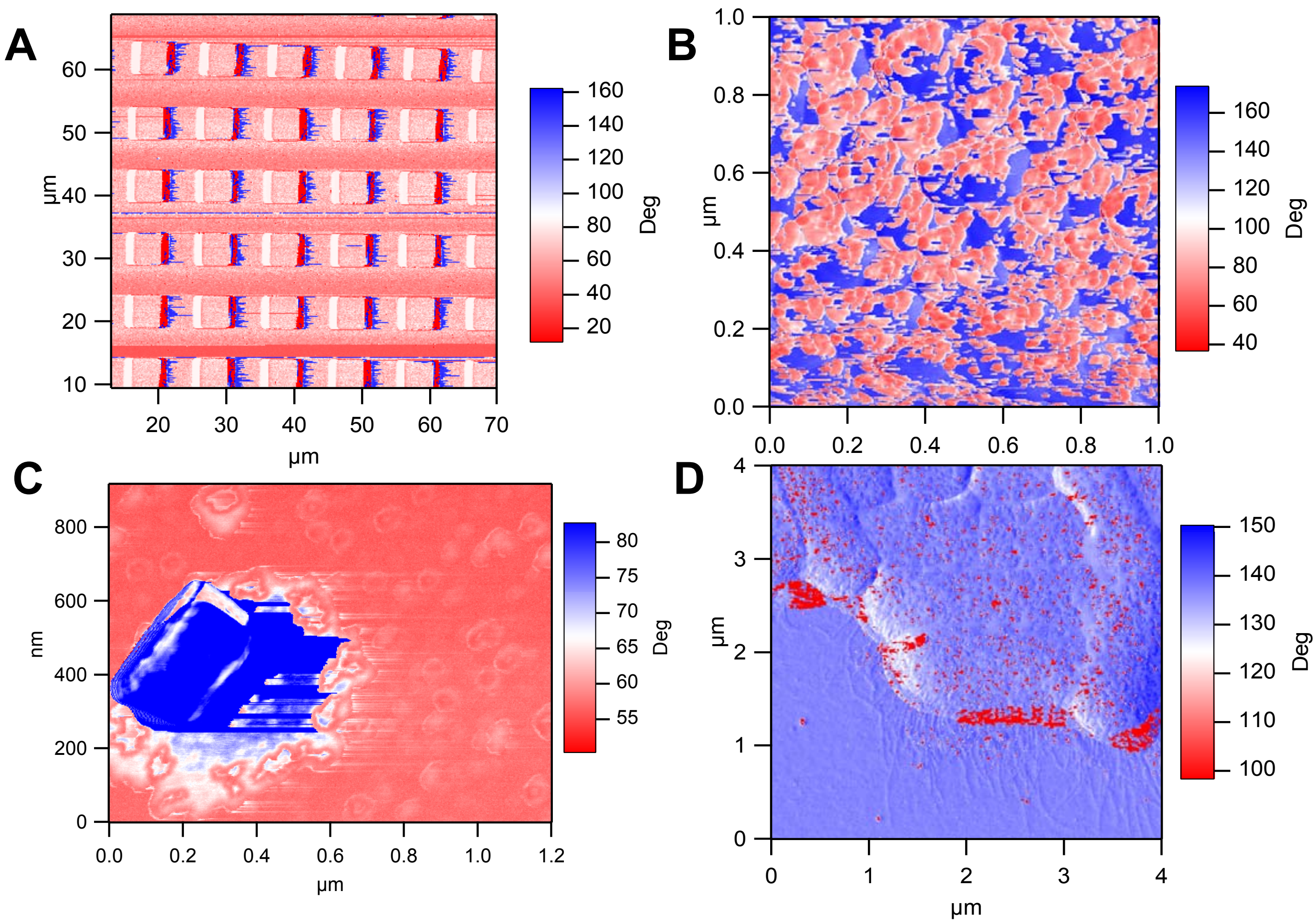


Figure 6.1.9: Examples of mode hopping in the Phase Channel: A) edge effects (calibration grid); B) streaking (polymer film); C) polymer particle; D) bacterial film.

IN THEORY for a simple harmonic oscillator (SHO):

▪ The sine of the phase (at constant amplitude) is the tip-sample dissipation. But sine is symmetric: sin(80°) = sin(100°). Thus, there are always two phase angles consistent with a given dissipation. So the second important part is whether the phase is above or below 90° (driving cantilever at its resonance frequency)- which one wouldn't do during normal AC mode imaging- usually done off resonance- see below.

The TAKE HOME MESSAGE for SHO THEORY applied to cantilevers is qualitatively depicted in Figure 6.1.10: As the tip interacts with the surface, a shift in the cantilever's resonant frequency also results in a phase shift of the AC signal (*notice* drive frequency is at resonance; i.e., 0% Target Percent in *Tune* tab):

-A frequency **decrease** (i.e., *lead*) shifts the phase **above** 90º, putting it in ATTRACTIVE MODE (low amplitude mode);

-A frequency **increase** (i.e., *lag*) shifts the phase **below** 90º, putting it in REPULSIVE MODE (high amplitude mode).

The colors represent what color the phase value regime will be in the S & D meter (driving at resonance).

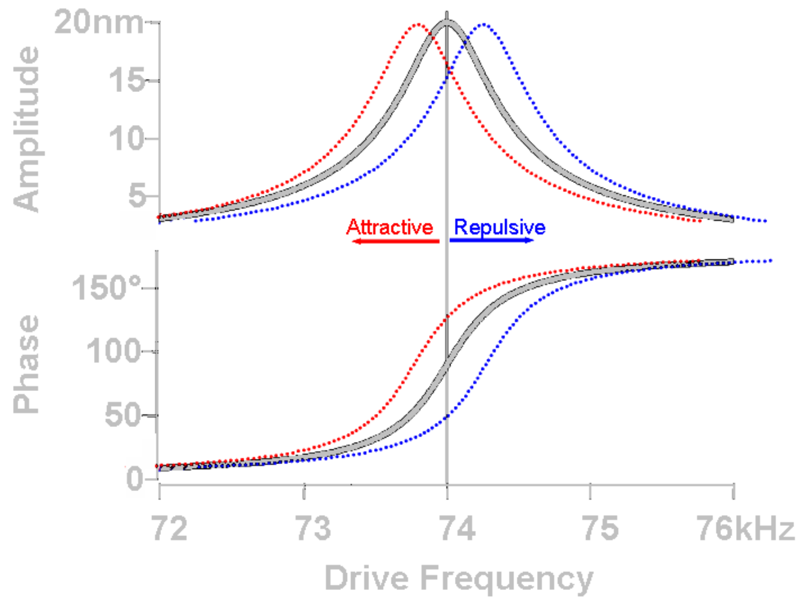


Figure 6.1.10: A shift in resonant frequency, f_o, (at constant amplitude) as the tip interacts with surface results in phase shift of the AC signal: Decrease in f_o results in phase increase (attractive mode); while increase in f_o results in phase decrease (Repulsive mode).

Notice from the amplitude plot above that if you choose a negative Target Percent amplitude is chosen during an Auto Tune (Section 6.1.1), it helps keep the tip in repulsive mode during AC mode imaging (black vertical line crosses blue dotted line; see Section 6.1.4A). Likewise, positive Target percent amplitudes help the tip stay in attractive mode (black vertical line crosses red dotted line; see Section 6.1.4B).

In reality (empirically), use the 'free air' Phase value as the demarcation value between attractive and repulsive modes instead of an absolute value of 90º. *This is commonly the case when using a non-zero Target Amplitude Percent during auto tunes (see Auto Tune, Section 6.1.1).*

★Sections 6.1.4A & B describe how to image in Attractive or Repulsive modes. ★

▪ When AC mode imaging *in AIR* on the MFP-3D™, you can monitor if the tip is experiencing net attractive or net repulsive forces with the surface via the phase value in the S & D meter. To display the real time Phase signal, go into 'setup' window in the S&D meter, use the pull-down menu next to Phase and select 'On' rather than the default 'Auto'. *See Section 7.7C for further description.*

For more on this topic, see the following references (but there are more):

-J.P. Cleveland, B. Anczykowski, A.E. Schmid, V.B. Elings, *Appl. Phys. Lett.*, **1998** *72*(20) , p2613.
-J. Tamayo, R. Garcia, *Appl. Phys. Lett.*, **1998** *73*(20), p2926.
-A. San Paulo, R. Garcia, Biophys. Journ., **2000** *78*, p1559.
-R. Garcia, R. Perez, Surface Science Reports, **2002** *47* p197-301.

▪Table 6.1.4 was prepared to help you adjust imaging / experimental parameters to (help) stay in repulsive or attractive mode, and to eliminate mode hopping.

TABLE 6.1.4	
Repulsive mode	**Attractive mode**
< 'free air' phase	> 'free air' phase
Low Q	High Q
Use **Higher** free air amplitude (Increase A_{fa})	Use **Lower** free air amplitude (Reduce A_{fa})
Decrease A_{sp}/ A_{fa}	Increase A_{sp}/ A_{fa}
Smaller tip radius	Larger tip radius
Set $f < f_o$ **(left side of Resonance)**	Set $f > f_o$ **(right side of Resonance)**
where A_{fa} is the free air amplitude (in volts); and A_{sp} is the Set Point (in volts); f is drive frequency; f_o is resonant frequency of cantilever; Q is the cantilever quality factor.	

NOTE: This attractive/ repulsive Phase stuff gets more complicated in fluid- this author notes that this behavior is being studied internally with magnetically actuated cantilevers (iDrive™).

6.1.4A: REPULSIVE MODE IMAGING: *Hints to get a good repulsive mode image-*

Imaging in repulsive mode can accentuate the phase contrast-

Following the steps before should achieve a nice repulsive image to be obtained- there are some samples in which mode hopping (to some degree) seem unavoidable.

✓ Perform an Auto Tune (*Tune* tab; as described in Section 6.1.1,) with a -5% target amplitude, and 1.0V Free Air amplitude. This will set the Free Air amplitude at 1.0V, with the minimum amount of Drive amplitude voltage to obtain this- this value should be in the range of tens to hundreds of mV, depending on the cantilever (and how well the probe is coupled to the shake piezo).

✓ Before engaging the tip, take notice of the 'free air' phase value- this is the value you will want to be *below* to keep the tip in repulsive mode.

✓ Engage tip (it's best to do a 'Soft' engage to preserve the tip apex); At the point of the hard engagement, the Phase should decrease a few degrees (*although this can be sample dependent*). If it doesn't, increase the Drive amplitude voltage slightly, and/ or decrease the set point voltage slightly (*NOTE: Smaller free air amplitudes can also be used*). If the Phase is already 'hopping', try increasing the Drive Amplitude voltage such that the Phase seems to stay stable. This will be apparent in the S&D meter.

If the Phase isn't staying below the initial free air Phase value upon contact, or flipping all over the Phase scale (i.e., mode hopping because sample –tip forces are acting crazy), try increasing the Drive Amplitude. To preserve the tip's apex (*a sharp tip helps keep the tip in repulsive mode*), the author has found this can be done by:

① Withdrawing the tip.
② Increasing the drive amplitude (by 1.5x or 2x, for starters).
③ Adjust (decrease) the 'Input Gain' on the Main tab of the master panel (*see Figure 6.6.11*).
④ Re-engage tip to evaluate if the Drive Amplitude increase was sufficient (i.e., *is it still flipping between attractive and repulsive?*). If not, repeat with larger Drive amplitude; or make target percent more negative.

A note on item 3 mentioned in the above Note-

▪ By decreasing the Gain value (in dB), the dynamic range is increased (in volts), while the bits/V is decreased. Figure 6.1.11 shows an example of this- decreasing the input gain, causes the scale of the amplitude range to become larger (B).

▪ Once the tip is scanning, increasing the Drive Amplitude voltage, (and/or decreasing the Set point voltage) can lower the Phase angle value, further pushing into the repulsive regime, which can help ensure to prevent unexpected mode hopping.

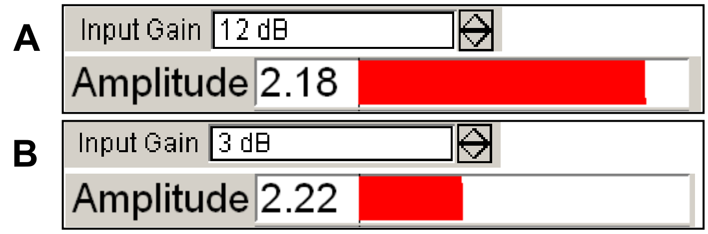


Figure 6.1.11: decreasing the 'Input Gain' to increase the dynamic range.

Adding **Q gain** on the drive can help stabilize repulsive mode imaging; This process is also described in the attractive mode protocol below, but will be described here as well.

✓ Auto Tune to 5% to 10% on the left side of the peak

✓ Increase the Q gain by only a couple hundredths place per manual tune

✓ Click the 'One Tune' button which sweeps once the frequency range once, and displays the updated plot- notice the Q increases, the resonant peak look sharper.

✓ Click the '**Center Phase**' to center the phase signal with the resonant frequency value.

✓ Repeat process until top of curve starts to looking round and/ or ringing on right side of resonant peak, or Phase signal is displayed.

▪ Notice with additional Q gain, the amplitude response will go up. Keep this in mind if you have need to attenuate the drive amplitude to get back to a desired few air amplitude before engagement.

▪ Figure 6.1.12 shows this process with an AC 240 (2kHz Si lever) with some common visuals to look for, although ever lever shows different degrees of response relative to amount of Q gain. Panel A) shows a post auto tune- no Q gain is added. Panel B shows the response after a few Q gain increases/ phase centering executions- notice the top of the peak is starting to look more round than sharp; this means the ringing is about to start. … As in Panel C). A this point, decrease the Q gain until the ringing goes away. Panel D) is shown as a Q gain and Phase freakout.

▪At any point, clicking the 'Auto Tune' button will reset the Q gain to zero.

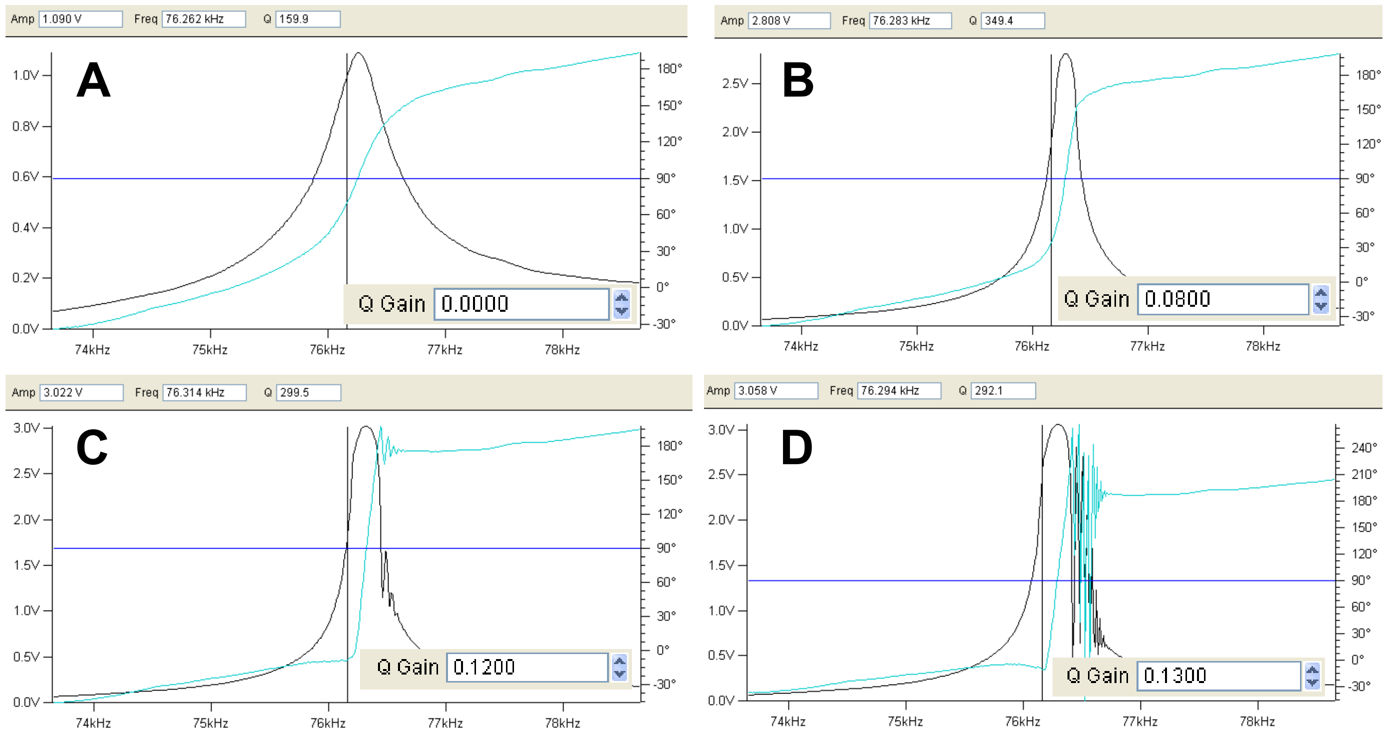


Figure 6.1.12: Q gain and repulsive imaging: A) post auto tune- no Q gain; B)almost ringing; C) ringing; D) freakout.

If all else fails, the tip could be too blunt (recall larger surface areas promote attractive mode- *see Table 6.1.4*)- *CHANGE THE TIP*; or the sample could possess charge that needs to be quelled/ dissipated using a 'Static Master' (*see Figure 6.1.12*).

TROUBLESHOOTING-

If you are still having trouble with mode hopping, it could be due to the sample - here are a couple things that may be contributing to it-

1) If you are experiencing difficulty keeping the tip in repulsive mode, perhaps the tip is too dull (see Table 6.1). If you are re-using the tip, this is a strong likely hood.

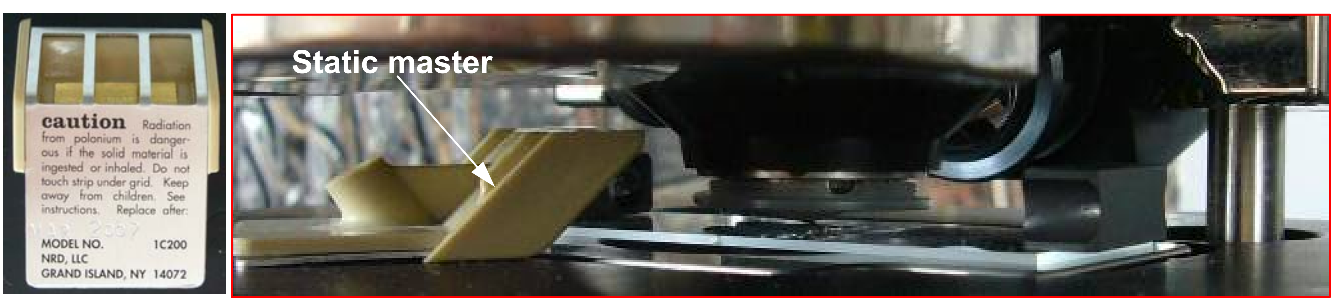


Figure 6.1.13: Placing static master near sample to dissipate charge.

2) Surface charges- use the Static Master device to dissipate the charge. Place the Static Master in the vicinity of the sample (see Figure 6.1.13). Sometimes the glass slide the sample is glued to is the culprit of the excess charge- in these cases, mounting the sample on a magnetic puck and placing it on a metal sample holder can help quell this charge (see Figure 6.1.14).

3) the Q gain needs to be increased slightly- See above and Section 6.1.4B for a description of increasing the Q gain and performing subsequent Manual tunes to confirm no ringing is occurring (i.e., Figure 6.1. 15).

4) The tip could be sticking to the sample- Keith Jones (AR apps scientist) has found that a Pt coated Si cantilever can sometimes work well with 'sticky' samples. We use Electrilevers™ (Olympus AC 240s coated with Pt), but if you have access to a sputterer, perhaps you can make your own. Try 3 nm of an adhesion layer such as Ir, Cr, Ti; and 5 to 10 nm of Pt.

Figure 6.1.14: eliminating charge by removing any glass components in sample mounting. Here, the sample (graphite) is fixed to sample puck via silver paint, and placed on an AR magnetic sample holder.

6.1.4B: ATTRACTIVE MODE IMAGING: *Hints to get a good attractive mode image-*

Imaging in attractive mode is great for soft samples that shouldn't be perturbed as much in repulsive mode, however can sometimes be tricky when you actually want it to occur- here are some simple steps to help keep the tip in attractive mode:

In the Tune tab of the Main panel-

✓ Set the Target Amplitude to 1V; this will have to be adjusted (decreased) later, but for the initial Auto tune, it will give the cantilever enough amplitude to have a successful Auto tune.

✓ Set the Target Percent to +10 to +20%; this will set the Drive frequency on the right side of the cantilever's resonant peak- a position more likely to keep the tip in attractive mode while imaging.

✓ Click the 'Auto Tune' button. This will sweep the piezo frequency and find the Drive Frequency based on the setvar parameters just entered. A plot similar to Figure 6.1.15A should be seen.

- *This will give a good starting point for the next step-*

✓ Slightly increase the Q gain from 0.00 to 0.01.

✓ Click the 'One Tune' button. Notice the FWHM value of the new resonant peak will decrease. These values are also calculated and displayed in the Auto Tune plot (Figure 6.1.3).

✓ Repeat this process until the resonant peak & Phase start to show ringing (Figure 6.1.15B).

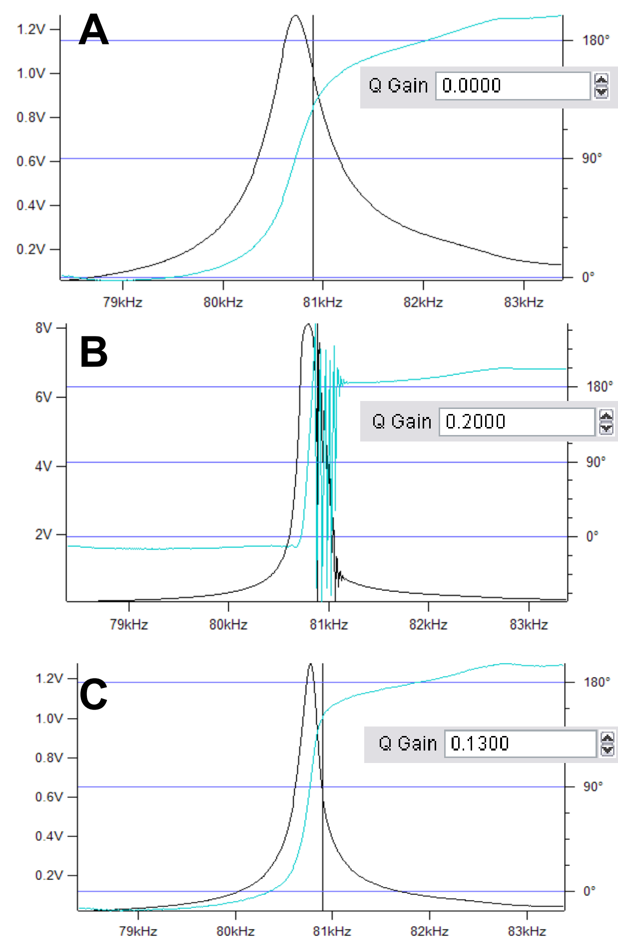


Figure 6.1.15: Attractive mode drive frequency and amplitude selection with an Olympus AC 240 Si cantilever. A) after auto tune; B) increase Q Gain until Phase and Drive has ringing; C) Decrease Q gain until ringing disappears

✓ Now decrease the Q gain until this ringing ceases in the Drive or Phase plots (Figure 6.1.15C).

Lastly, click the 'Center Phase' button to center the inflection point of the phase signal with the resonant peak of the cantilever.

NOTICE THE FREE AIR AMPLITUDE IS MUCH LARGER NOW (look at the S&D meter)

▪ *Since attractive mode imaging works best with **smaller free air amplitudes**, this amplitude must now be decreased-*

✓ Decrease the free air amplitude (manually) to less than 0.5 V - the author usually goes to ~0.3V.

✓ Choose a Set Point voltage that is about 95% of this new free air amplitude, such that you perform a 'gentle' / soft engage- this is important since you are attempting to image in attractive (non-contact) mode.

✓Click the 'Engage' button; engage tip by wheeling thumbwheel down (CCW), as described in Section 6.1.2.

▪ Watch the free air amplitude during the hard engagement- if it flips to repulsive mode (i.e., Phase value less than free air phase value), increase drive amplitude, increase Q gain slightly, or decrease free air amplitude.

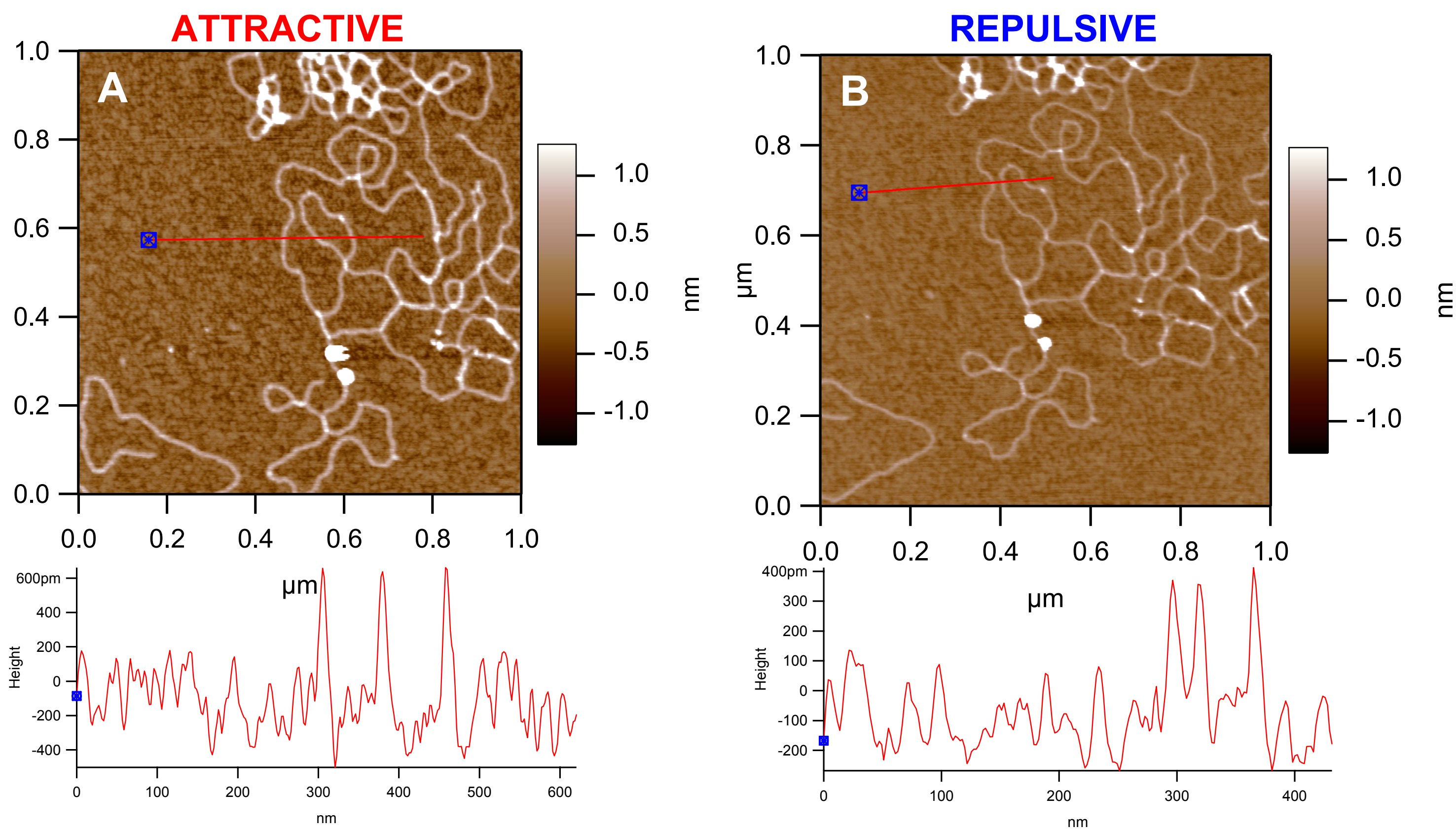


Figure 6.1.16: Consecutive AC mode Images of DNA in air with same tip and scan area: A) Attractive mode, and B) Repulsive mode.

✓Once scanning, decreasing the Set Point voltage, and/or increasing the Drive amplitude will (should) increase the Phase value, further pushing the tip – sample interaction into the attractive regime.

▪ Figure 6.1.16 shows an example of AC mode imaging lambda DNA on mica with attractive and repulsive mode imaging (obtained consecutively), performed with the same tip and sample region. In A), the target percent was +20%, a 'free air' amplitude of ~350 mV, and a Q gain of 0.013. Notice high contrast in attractive mode image. In B), the target percent was -5%, a free air of 1.0 V, and Q gain of 0.00V. Notice the same area of the DNA give ~ 1nm of height in the attractive mode, and ~ 0.5 nm in repulsive mode.

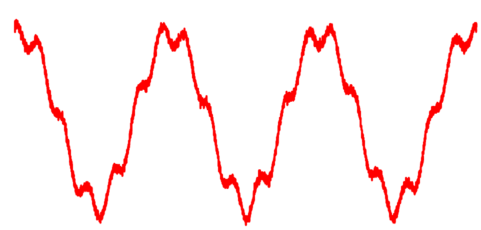
6.1.5: DualAC™ Imaging:

DualAC™ is a relatively new AC imaging mode offered on the MFP-3D. The concept is simultaneously driving the cantilever at two of its flexural resonances, giving an AC waveform similar to the one depicted in Figure 6.1.5.1A. The technique requires two independent lock-in amplifiers to accomplish this, which the MFP-3D controller has internally. The fundamental cantilever resonance frequency (i.e., the first eigenmode) is typically locked-in for the feedback loop, which ultimately constrains the movement of the cantilever at this mode. What's interesting is that the second eigenmode is not constrained by the feedback loop, quite often offering increased image contrasts in the second amplitude and/ or second phase channels. Since this is based on the flexural resonant frequencies of a cantilever, these higher eigenmodes are not integers (as with harmonics), but rather follow the relationship:

$$C_{first} = 1; C_{second} = 6.1; C_{third} = 17.5$$

Thus, cantilever selection for this technique is limited to the bandwidth of the system (i.e., the second or third eigenmode of stiffer cantilevers is often at too high a frequency to be driven by the MFP-3D- maximum is 2.5MHz).

A



B

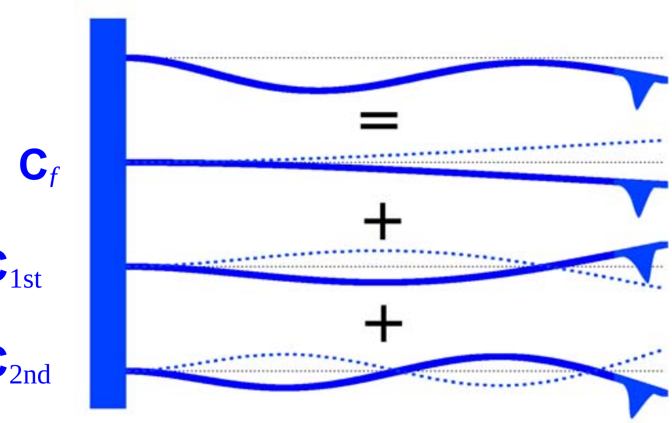


Figure 6.1.5.1: A) resulting Dual AC waveform; B) higher mode behavior of cantilevers, where C_f is the fundamental resonant frequency, C_{1st} is the first eigen mode and C_{2nd} is the second eigen mode.

▪ If the resonant frequencies of the cantilever are not known, just do a thermal tune. In some instances, the range of the thermal tune will have to be increased, and the thermal plot's Frequency axis manually increased (see Section 5)

For more reading on higher harmonic imaging, see the following references:

J.P. Cleveland et al., Applied Physics Letters 72, 2613 (1998)
A. San Paulo, R. Garcia, Biophys. Journ., 78, 1559 (2000)
R. Proksch, Applied Physics Letters 89, 113121 (2006).
T. Rodriguez, and R. Garcia, Appl. Phys. Lett. 84 (3), 449 (2004).

Keep in mind that not all samples will show good contrast in the second mode- likewise, some samples show good contrast between the first and second amplitudes, while showing little difference between the first and second Phase; or vice versa. Heterogeneous samples seem to show good contrast with this technique.

▪The same attractive / repulsive imaging mode rules seem to apply to the second mode as does the first. The author suggests using the protocols described in Section 6.1.4 to avoid mode hoping in the second mode.

▪ For this protocol, the author has found that some water based latex paint gives excellent contrast in the Amplitude & Phase images, and can easily be made by most people wanting to practice/ learn how to do this technique. The sample is a small amount of Sherwin-Williams ProMar200 water based latex on a glass slide (*as a former professional carpenter, the author's of the opinion that Sherwin Williams is one of the best paintson the market*). Water based paints are heterogeneous because of the small emulsion polymer particles and other fillers used as the ingredient that gives the paint its opaque character.

For in-air DualAC imaging-
✓ To get started, determine the fundamental resonant frequency of the cantilever (if not known) by performing a thermal tune (see Section 5); to determine the second eigenmode, roughly multiple by 6.1- this will be important for entering high and low tune values for the DualAC Auto Tune.

An example of a thermal tune of an Olympus AC 240 silicon cantilever (*f*~70kHz; *k*~2N/m) is shown in Figure 6.1.5.2. Notice the second 'eigenmode' is roughly 6 times the fundamental frequency (inset).

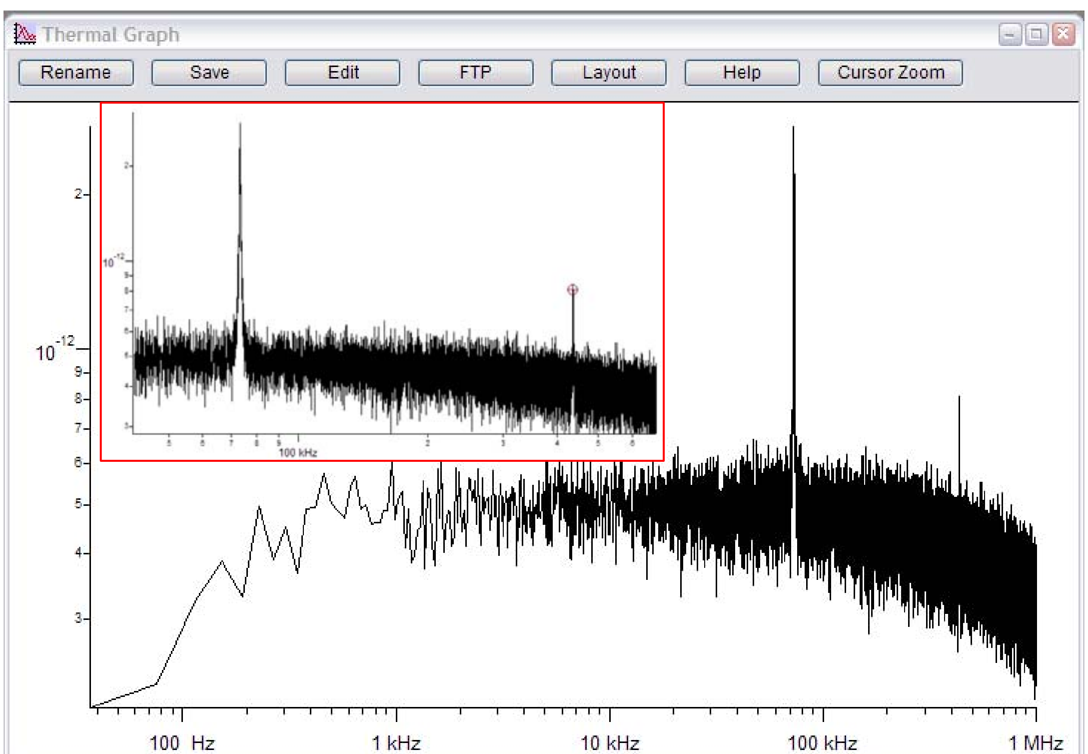
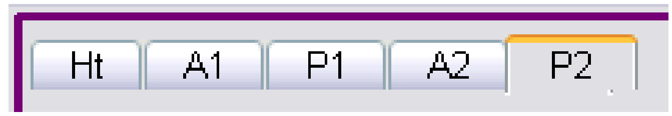


Figure 6.1.5.2: Thermal tune showing fundamental and second eigen mode peaks

✓Confirm that Height, Phase1, Amplitude1, Phase 2 and Amplitude2 are the activated image channel tabs in the Master Channel Panel (*see Section 7*).



✓ Go to the *Tune* tab of the Master Panel.

✓ Click on the DualAC Mode ☑check box in the 'Other Things' portion of the panel; this will expand the panel to include the second mode parameters (Figure 6.1.5.3).
✓Confirm that the Auto Tune 'High' and 'Low' setvar values are in the range of the cantilever being used.

✓Choose a Target Percent and Target Amplitude for the fundamental resonant peak (i.e., first eigen mode)- The author usually goes with 1V and -5% to be in repulsive mode (*see Section 6.1.4*).

✓Click on 'Auto Tune' in the first column (left) which is for the the fundamental frequency of the cantilever. This will tune the cantilever is it normally does in standard AC imaging (*see Section 6.1.1*).

For second mode tuning-

✓Confirm that the 'Auto Tune High' and 'Low' setvar values will be sufficient to include the desired second mode frequency.

✓Choose a Target percent and Target Amplitude for the second mode- The author usually goes with the default 100mV and -5% to be in repulsive mode.

✓Click the 'Auto Tune' Button; after a moment, the software should choose a frequency.

▪ Notice the Frequency Ratio setvar value is updated to the multiplier value of the fundamental peak.

NOTE: If the Auto Tune for the second mode doesn't happen to be successful, in certain instances the coupling of the probe chip to the cantilever holder may be too good, causing the Drive Amplitude to be too small to achieve the defined Target Amplitude- when this happens, increase the target amplitude and step it down later once imaging commences.

✓Perform a 'gentle engage' *as described in Section 6.1.2*. once the tip is hard engaged on the surface, adjust the Drive amplitude appropriately to keep it in the imaging mode desired (i.e., repulsive or attractive).

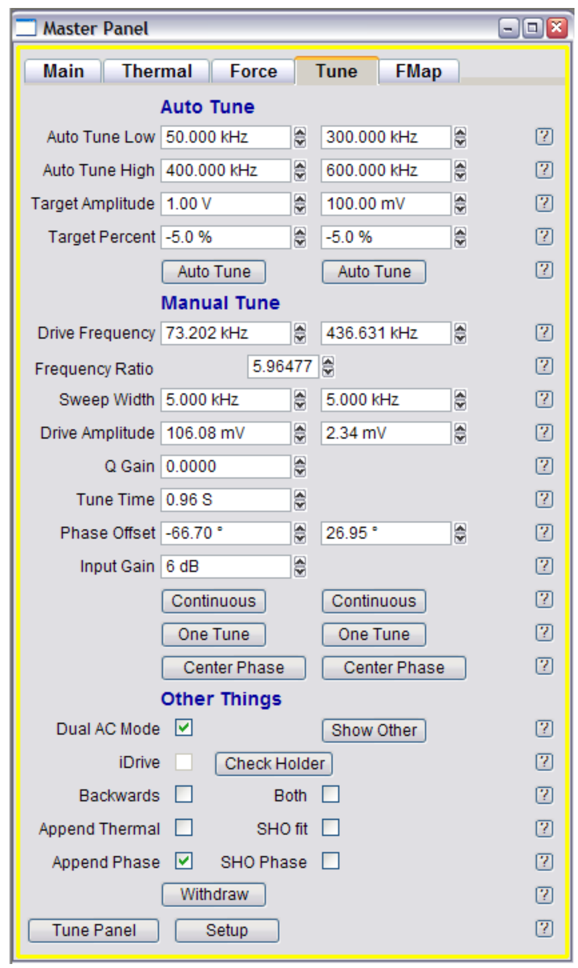
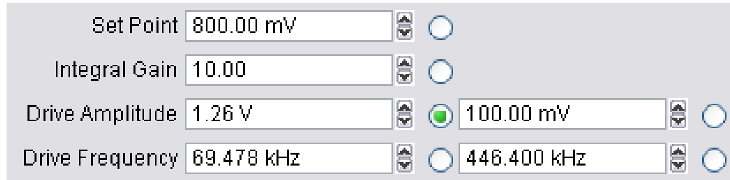


Figure 6.1.5.3: Expanded Tune tab for Dual AC.

✓ Start imaging. Notice that the *Main* tab of the Master Panel now has an expanded panel offering Drive Amplitude and Drive Frequency setvars with radio button for image tuning.



✓ Save the data if you want.

▪DualAC™ allows mixed modes to be done (i.e., repulsive- repulsive; repulsive- attractive; attractive- repulsive; attractive- attractive). Since this is a new area of AFM, there is a lot that is still left to be learned.

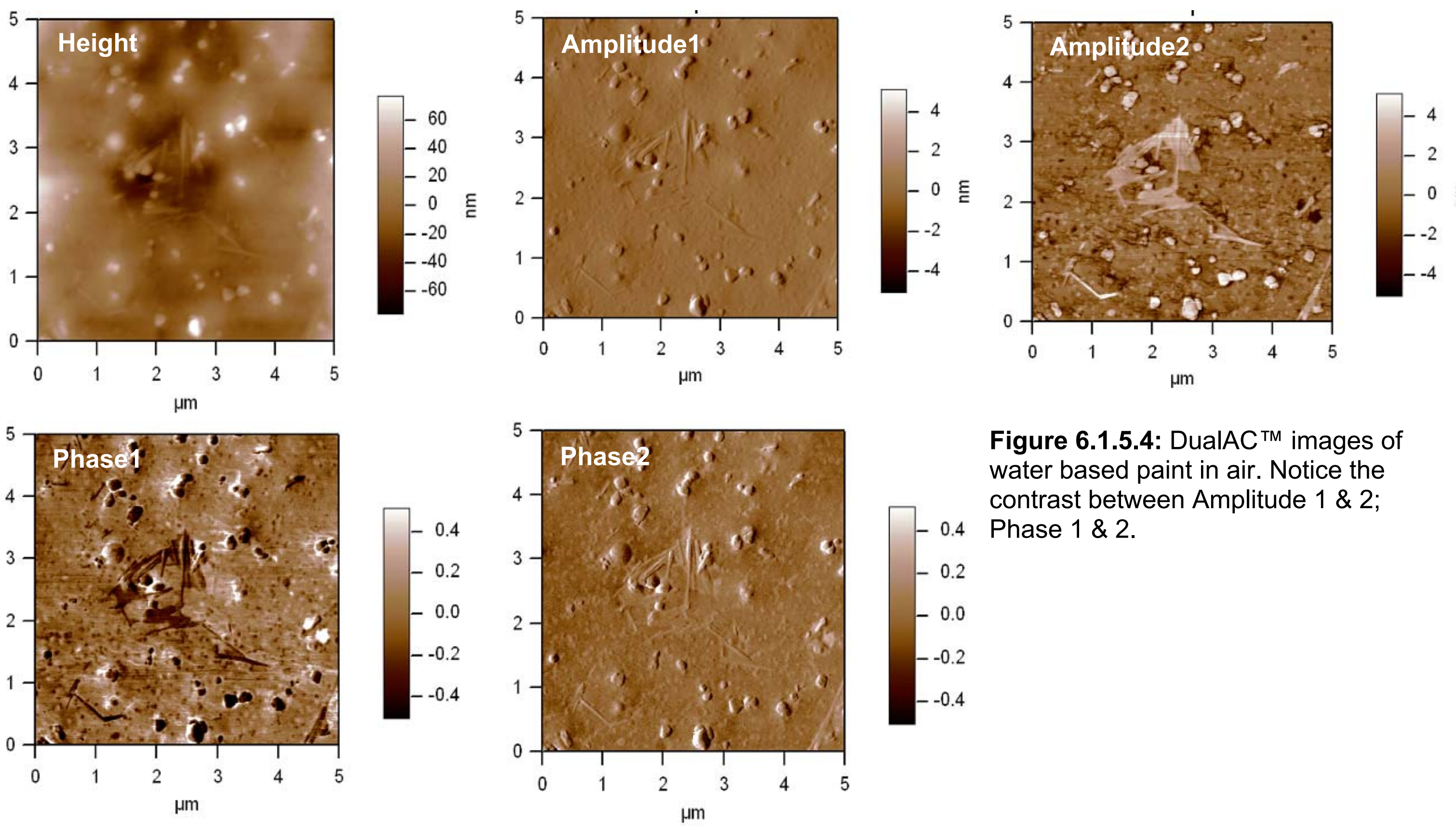


Figure 6.1.5.4: DualAC™ images of water based paint in air. Notice the contrast between Amplitude 1 & 2; Phase 1 & 2.

▪Figure 6.1.5.4 shows an example of a Dual AC™ mode image of some dried water based latex paint, imaged in air with a Nanoworld NCL-50 silicon lever (*f*~190kHz; *k*~48N/m). The author was able to keep both modes in repulsive modes.

DualAC™ in-fluid – thus far, DualAC™ in-fluid while driving the cantilever acoustically (i.e., with the shake piezo) hasn't been all that successful. The main reason is likely because in fluid, the entire cantilever holder is ultimately driving the fluid between the tip and sample, creating a resonant cavity.

Recently, Asylum Research scientists have discovered that DualAC™ has been successful using the magnetically actuated iDrive™- with this module, just the cantilever is actuated, eliminating all the other crazy waves that normally occur in solution when driving via acoustic mode.

6.2: 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*....

FIRST- It's a good idea to have an extra layer of protection between the sample slide and the scanner. See section 6.2.6 for simple, cost effective precautions to prevent scanner damage due to fluid spill.

6.2.1: 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. Figure 6.2.1 shows a few 10s of µLs 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! *Figure 3.5 shows an example of this.*

Figure 6.2.1: Wetting the cantilever
Photo courtesy of Dr. Irene Revenko

DO NOT SUBMERGE CANTILEVER HOLDER BEYOND KEL-F/ PEEK BODY. If this does occur, fluid can wick into the circuit board of the cantilever holder and cause deleterious effects.

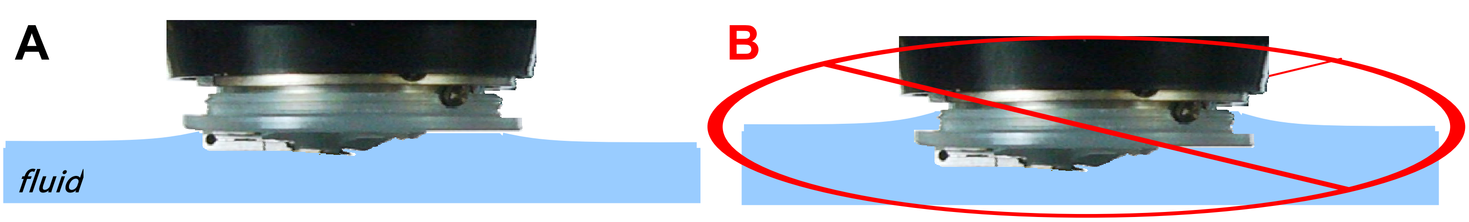


Figure 6.2.2: Do not submerge cantilever holder beyond polymer body: A) fluid level / meniscus should not go beyond edge of cantilever holder; B) is the fluid level surpasses the edge of the Kel-F polymer body, the fluid can wick its way into the printed circuit board in the holder and short out voltage lines, and compromise pogo pins and other components.

✓ Open CCD camera; turn on fiber light illumination so you can see the cantilever(s). (*see Section 4.1B*).

▪ Sometimes the cantilevers are very floppy such that they bend back on the probe chip upon meeting the fluid interface; *OR* an air bubble (*see* Figure 6.2.3) will get trapped somewhere by the cantilever, which will eventually compromise the deflection signal because the bubble may migrate- it's best to nip it in the bud before imaging.

There are two ways to alleviate the occurrence of bubbles:

❶ Gently lift the front of the head up, pivoting on back legs, such that the cantilever holder comes out of the fluid; then plunge it back into the fluid(Figure 6.2.2). Sometimes this process must be repeated. Figure 6.2.4 shows an example of a bent cantilever as seen in the CCD camera image.

❷ If the bubble isn't removed after trying step 1 multiple times, lift the head up off the stage, place on its side and dispense/ flow some imaging fluid to remove the bubble with shear force of the fluid under gravity; re-wet the tip and place back on stage. *Try again.*

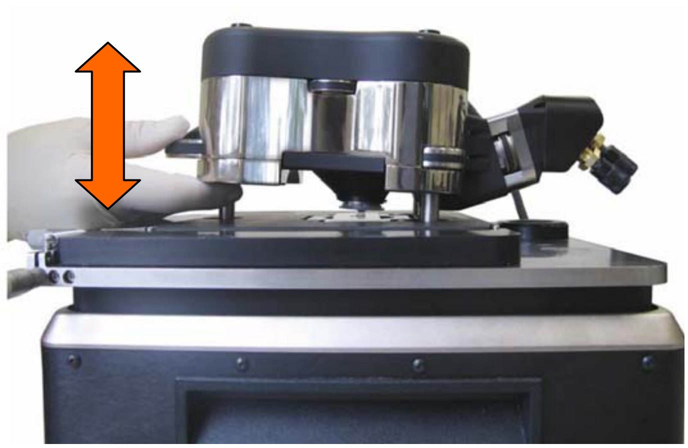


Figure 6.2.2: Lift head in and out of the fluid on sample to remove air bubbles.

▪ *Figure 6.2.3 & 6.2.4A,B show examples of monitoring the removal of an air bubble trapped between the legs of the legs of a triangular cantilever (A), and after removing the bubble as (B) described in Figure 6.2.2.*

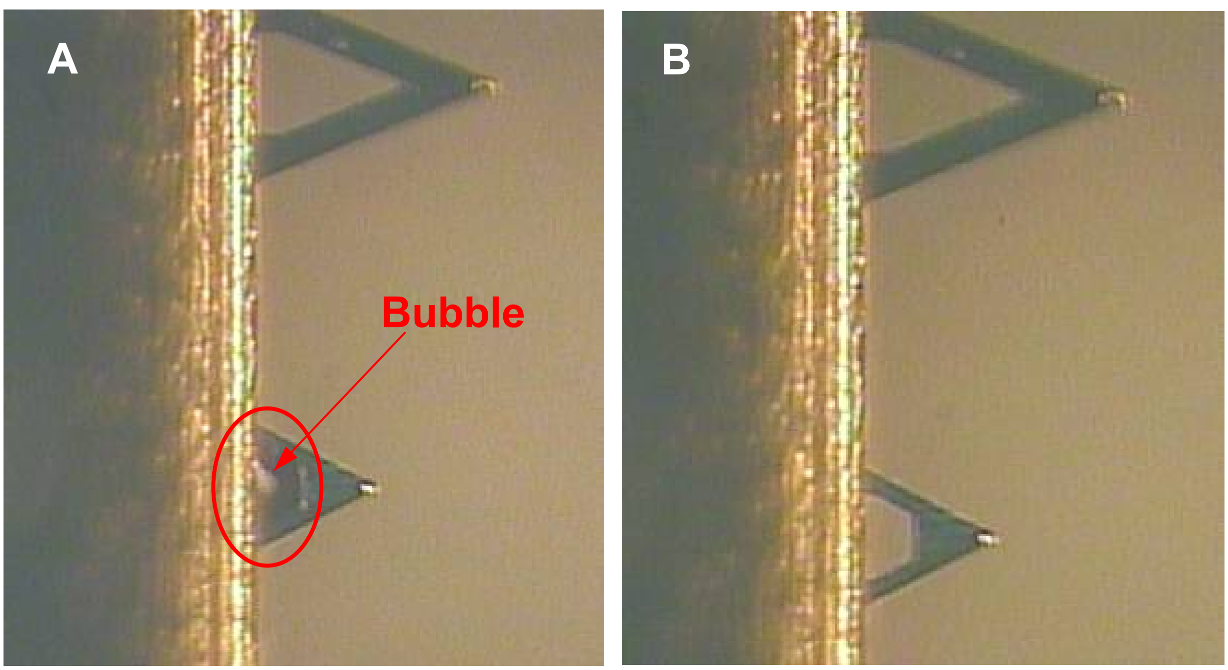


Figure 6.2.3: Using the CCD camera to determine the presence of and air bubble trapped near the cantilever in fluid. A) BEFORE: trapped air bubble (in red circle); B) AFTER: no more air bubble.

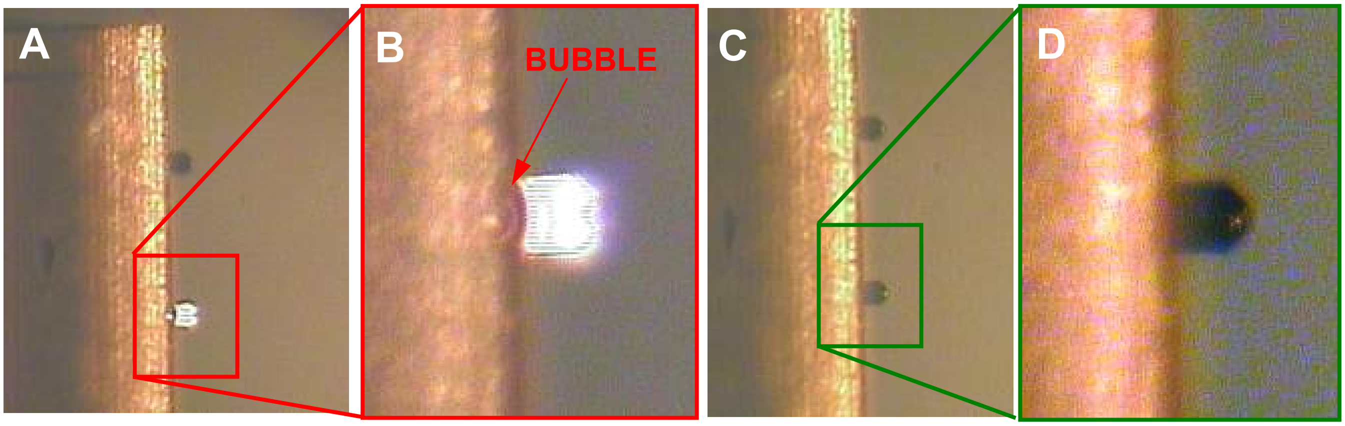


Figure 6.2.4: Floppy/ soft Si_3N_x cantilevers can be bent by surface tension of fluid as seen in CCD camera images. A) cantilevers appear bright when bent/deflected improperly; B) CCD zoom of cantilever in red box- notice small bubble at base, and deflection of cantilever; C) properly oriented cantilever; D) CCD zoom of cantilever in green box. The cantilever in above images is an Olympus Biolever™ (30 µm x 60 µm; *k* ~0.03 N/m).

▪ A properly oriented cantilever should look like a silhouette of the cantilever in the CCD camera image (similar to Figures 6.2.3B & 6.2.4C,D). If it appears brighter than that, there is probably something wrong (seated in pocket improperly, deflected, broken, etc.).

6.2.2: 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. Refer to Section 4.3 for this procedure.

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, Figure 6.2.1). 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.

6.2.3: 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 (*see Section 5*)); a window of 5 to 10kHz is a good place to start.

✓ In the *Tune* Tab, click the 'Append Thermal' ☑ checkbox.

✓ Using the Igor (Crtl+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 6.2.5D will result.

✓ Click the 'Center Phase' button once a drive frequency has been determined. This will center the phase at 90º, 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 (*recall Section 6.1.4*).

✓Select imaging parameters (i.e., set point to free air voltage ratios) similar to the description in Section 6.1.2 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 ~ 95 % of the 'free air' amplitude voltage.

✓ Proceed to Section 6.2.4 for tip engagement in fluid.

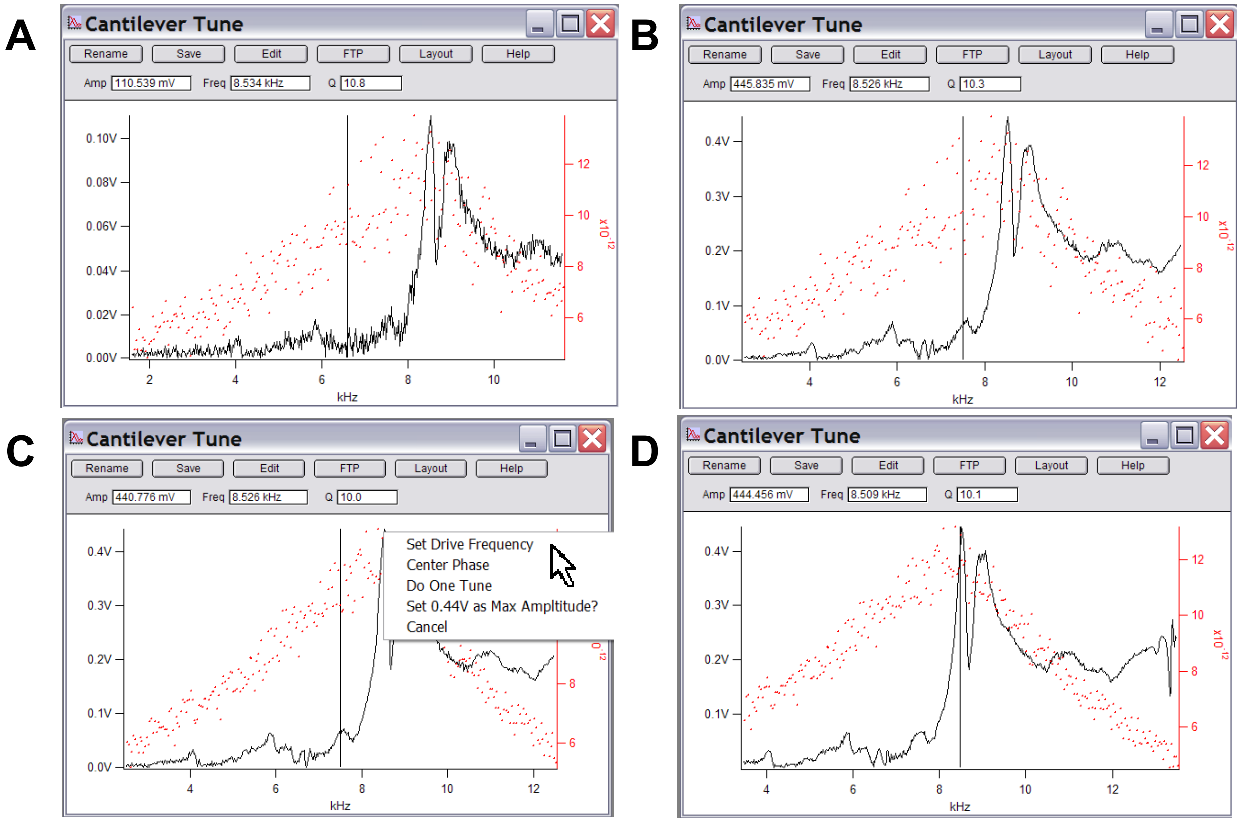


Figure 6.2.5: Performing a 'One Tune' to select Drive Frequency in AC mode: A) 'One Tune' Drive Frequency spectrum (black) with 'Append Thermal' spectrum (red scatter plot); B) Drive amplitude increased to increase S/N: C) Setting Drive Frequency with right mouse click on curve of interest; D) resulting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

2) The *HACK* way-

Alternatively, you can follow this procedure if you have difficulty getting a decent image from the above protocol. *Although the author used to follow this procedure before the above procedure was learned and practiced, it certainly isn't a refined way to go about it; that being said, it can be effective.*

✓ From the Thermal (power spectrum) plot, place an Igor cursor on the left side of the first or second peak, about 5 to 10% of the maximum amplitude. The areas indicated by the red arrow (Figure 6.2.6A) are where you can manually pick the drive frequencies by entering the 'X' cursor value in the drive frequency window of the *Tune* tab. Alternatively, the author has had some imaging success by driving near the first Eigen mode (blue arrow, Figure 6.2.6A).

▪ To aid this process, you can Zoom in on the first fundamental resonance peak (Figure 6.2.6B).

✓Place the Igor cursors some where on the left side of the resonant peak, enter that frequency value into the Drive Frequency setvar parameter window in the *Tune* Tab.

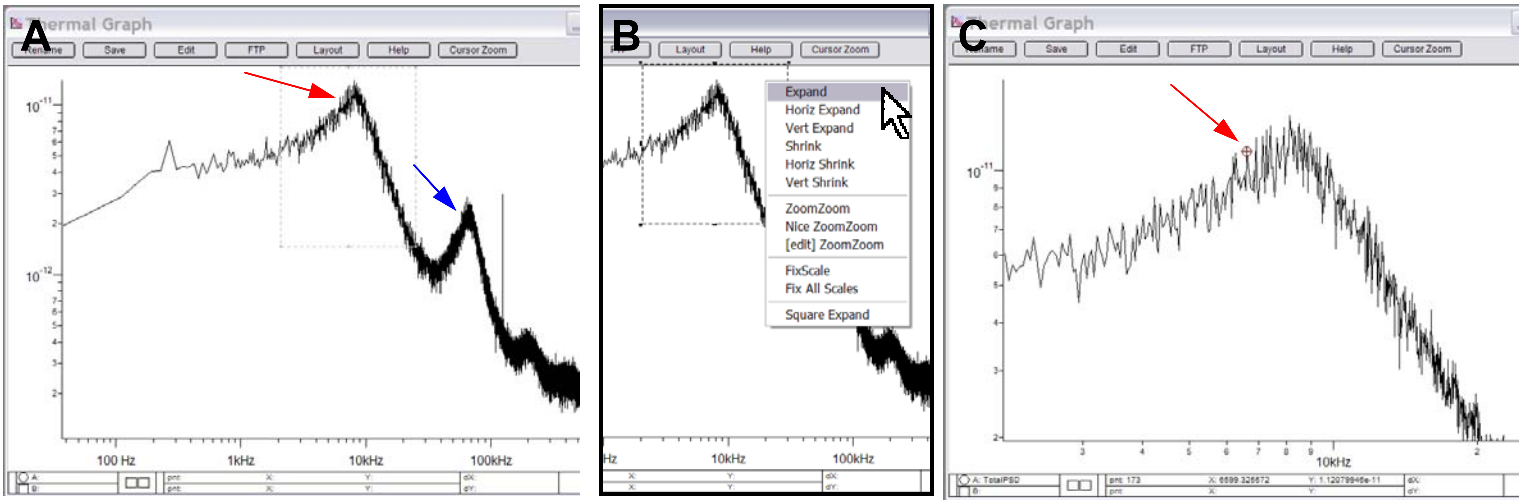


Figure 6.2.6: Thermal tune freq vs. amplitude plot *IN FLUID*. Peaks become broader due to lower 'Q' value, and resonant peaks shift to lower frequency (generally 1/2 to 1/3 the in air resonant frequency.

✓ Pick a Drive amplitude – the author's experience is that it must be several hundred mV's before the tip won't think its false engaged. *This sometime can be a trial and error process, increasing the Drive Amplitude or lowering the Set Point voltage until the Piezo extends fully when clicking the 'Engage' button.*

✓ Engage as you normally would in the 'hard' engage technique (Section 6.1.2). Notice the amplitude will increase as the tip approaches the surface, up to a certain point, then decreases drastically when the tip is very close to surface- increasing the likelihood of truncating the tip (*to avoid this, see Section 6.2.4*).

6.2.4: 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 (as described above in Section 6.1.2).

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 (*see Section 6.1.2B*).

✓ Move piezo into middle of Z range (~70 V).

✓ Start scanning.

✓ Tune parameters as described in Sections 6.1.3 & 6.2.5. With some samples, do not surprised if you cannot get the tip to track the surface as well as it does in fluid.

6.2.5: 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.

Although calibration grids are also great for learning how to image, It is NOT a good idea to put water (or fluid) onto the provided 10µm calibration grid- they are never the same after that.

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. Figure 6.2.7 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).*

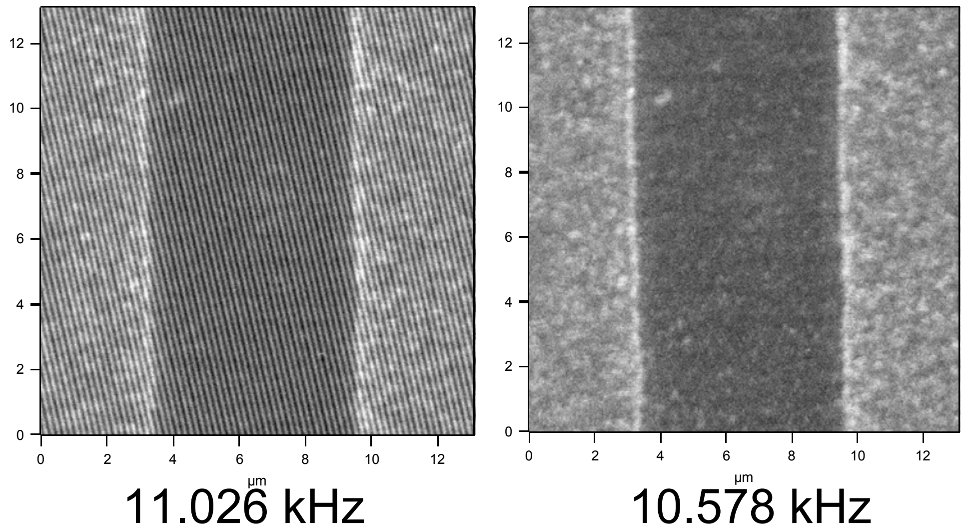


Figure 6.2.7: Monitoring the Phase image while changing the Drive Frequency on the fly.

Post Fluid imaging:

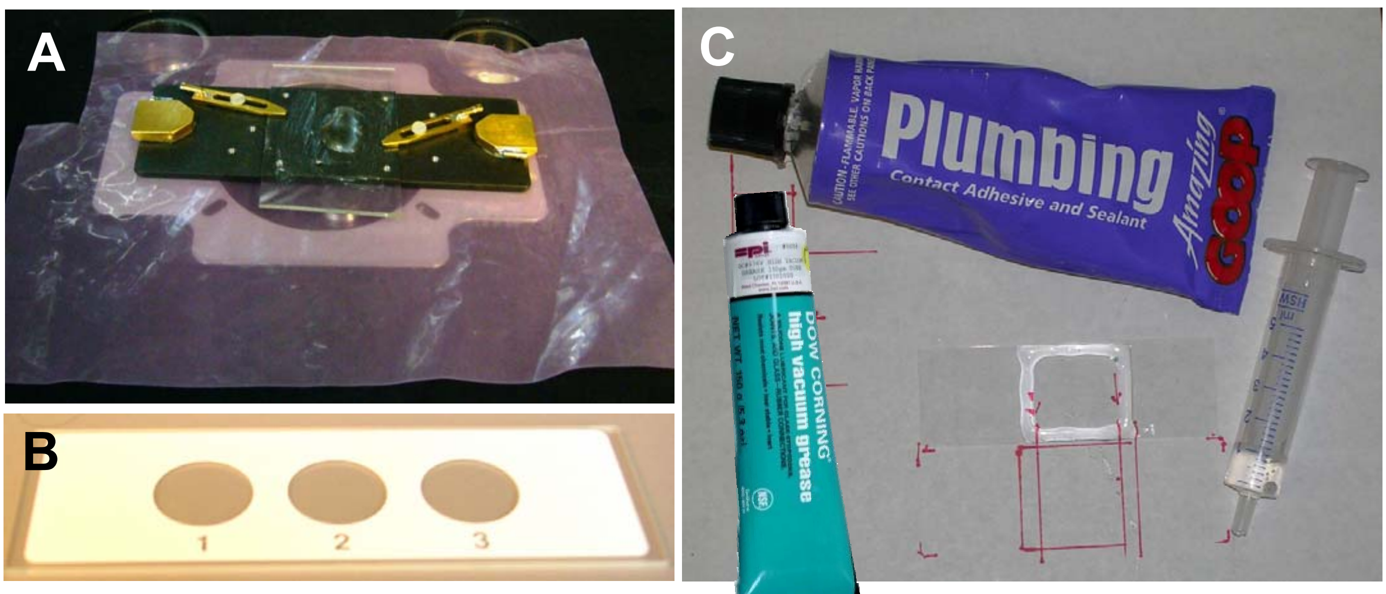
The author typically sets the head on its side immediately after imaging to allow the excess fluid to drip off the cantilever holder. It's based out of habit from the post-doc days, and continues today out of superstition (which often accompanies SPM). Figure 6.2.8 shows the head places on its side on the head stand in the acoustic hood, allowing gravity to let fluid fall to head resting stand.

Figure 6.2.8: Place head on its side after use in fluid to allow gravity to remove fluid safely.

6.2.6: Imaging In Fluids- PRECAUTIONS:

When imaging in fluids, aside from the precautions described in Figure 6.2.2, it is important not to allow buffer (or other corrosive fluids) to get to the scanner, or you'll have a $10,000 problem, minimum. What can happen is that excess fluid can drip down onto the top of the stage, get sucked into the bottom of the scanner through the back plate via capillary action, then into the flexor channels via capillary action. Fluid can ruin the LVDT sensors and compromise (corrode) the flexor channels from moving properly. The following is an easy precaution to take, especially if you have a 'Stand Alone' top view base.

IF YOU DO NOT HAVE A CLOSED FLUID CELL: To protect the scanner, place a piece of plastic under the sample that has the droplet on it (see Figure 6.2.9A below). There are Teflon coated standard microscope slides with circular bare glass regions that could also be used (Figure 6.2.9B). A variety of these slides can be purchased from the EMS website under Figure 6.2.9B.



www.emsdiasum.com/microscopy/products/histology/slides.aspx#63414

Figure 6.2.9: Precautions to exercise while working with fluids without an AR closed fluid cell: A) Place piece of plastic (or parafilm™ under sample; B) get some teflon screen printed slides from a vendor; C) make hydrophobic barriers around sample with silicone or a PAP pen.

▪ A reservoir around sample can also be made. This can be done a variety of ways: the author has found that a silicone based adhesive, or vacuum grease (Dow Corning 976V) works very well to fabricate a fluid barrier on a glass slide. Figure 6.2.10 shows the cantilever holder above a droplet on a sample surrounded by vacuum grease.

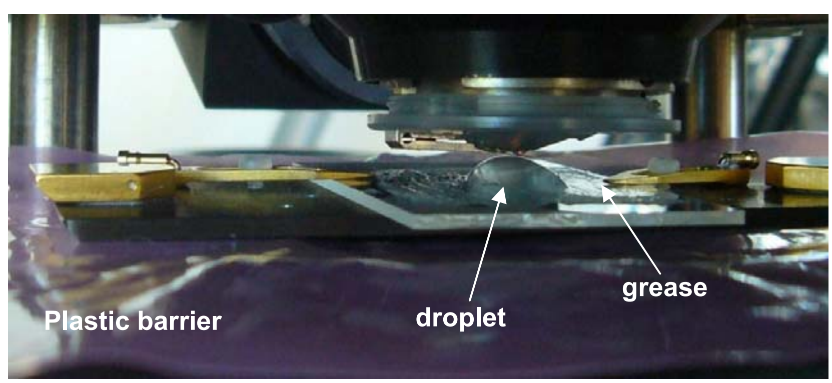


Figure 6.2.10: Droplet containment with vacuum grease.

✓ Put a small amount of the silicone product into a 1 to 5 mL syringe to control the amount coming out, much like what is used when piping frosting onto a cake, or a caulking tube. Templates can be drawn onto a piece of paper to get consistent sizes during application (Figure 6.2.9C). When choosing a silicone adhesive, check the ingredients to insure the solvents won't interfere with your sample. If choosing any of these approaches, make sure the thickness of the barrier won't prevent the cantilever holder from getting the tip to the surface (i.e. it doesn't get hung up on the barrier), or you'll never get the tip won't engage, indicative of lack of hard engage in Z piezo meter, and ridiculous Deflection voltage variations (*See Chapter 15 - Troubleshooting*).

▪ PAP pens can also be used to create a hydrophobic barrier around your sample; but they are more expensive.

Another product that is pretty useful - inch wide adhesive stickers that act as a hydrophobic barrier. They are actually sold as spacers (called SecureSeal™), but they polymeric material they are made from is sufficiently hydrophobic to use similar to the product in Figure 6.2.9B, yet comparably priced. Figure 6.2.11 below shows the package and one installed on a cheek cell sample (hence the sharp mark in the image which is the area the cells were put).

http://www.gracebio.com/Products/Imaging__Microscopy/SecureSeal_Imaging_Spacers/

Their CoverWell™ imaging chambers also look cool, and made of red silicone.

http://www.gracebio.com/Products/Imaging__Microscopy/CoverWell_Imaging_Chambers/

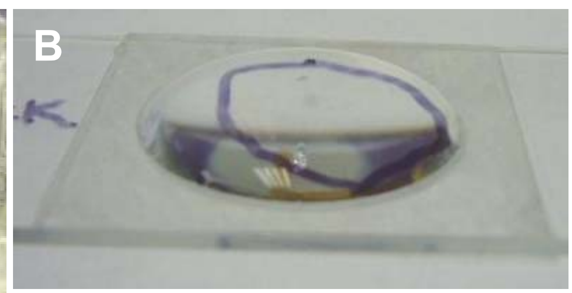


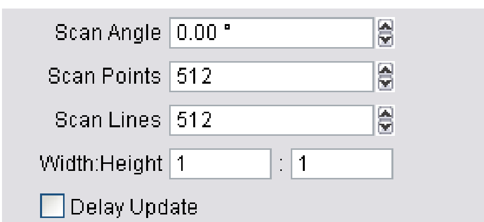
Figure 6.2.11: A) Adhesive hydrophobic barriers for standard microscope slides; B) installed with water drop on biological AFM sample.

7. Managing Images Channels

This section describes how to manage image channels: from how to turn on / capture additional input channels, or adjusting data scales during imaging. Topics include: Zooming on image features; save/ capture images; adjusting the Z scale of the image; real time section analysis, among others.

7.0: Selecting image pixel density:

The Scan Line and Scan Points (i.e., pixel density) is selected in the *Main* tab of the Master Panel using the respective setvar values. Note that the tip must be withdrawn to change these values, unless the Delay update checkbox is activated (See Sections 6.1.3 or 8.3 for more on the Delay Update function).



▪ The aspect ratio of the image can also be changed in factors of 2, whole integer numbers. This will automatically update the number of scan lines or points – for example, say a 1:1 image at 512 x 512 was being collected, then changed to 1:4- the scan lines will be changed to 128 while the points will remain at 512.

One important calculation to keep in mind:

$$\text{Pixel Size} = (\text{Scan Area} / \text{\# of pixels})$$

For example, for a 512 x 512 pixels in a 5µm scan area, each pixel is 9.76nm²; while in a 90µm scan area, each pixel covers 175.78nm².

⇨So make sure the feature of interested has a high enough resolution such that it isn't pixilated, but isn't so high that it doesn't take 2 days to collect the image.

▪ The absolute number of simultaneous data channels at high pixel resolution is difficult to quantify, but is directly related to computing power. For example, twelve channels at 2k x 2k can be collected with the stock PC, or three channels at 4k x 4k. At lower pixel resolution (512 x 512), up to twenty images can be simultaneously collected. Obviously the higher the pixel density, the longer it takes to collect that data.

Some notes on selecting pixel densities-

▪ Unless the Delay update checkbox is activated, the tip must be withdrawn to change these values. If the Delay Update ☑ checkbox is activated, the user can change the pixel density and have it take place once the scan reaches top or bottom, or the '**Frame Up**' or '**Frame Down**' button is clicked.

▪ Since Igor is a memory based program, collecting images with very large pixel densities (i.e, 2 to 4k x 2 to 4k), is most likely to occur with a fresh experiment / template (as opposed to one that has a lot of information already stored in it). To help accommodate this, saving images to memory is not recommended.

The author often uses a low pixel density to scan quickly to find an area of interest, then increase it to get the good image.

7.1: Master Channel Panel-

This panel is where to select data channels to display & capture; adjust Z scales; color tables and flattening.

OPENING IMAGE CHANNELS:

▪ To open/ activate additional image channels **before tip is scanning**, go to the Master Channel Panel to do this. The default MFP-3D™ software is set for AC mode with Height, Amplitude and Phase channels (*Figure 7.1.1*A) to be open in the Master Channel Panel, *but it's a good idea to always capture the Z sensors data too, especially when working with topography features larger than 500 nm (Figure 7.1.1B). Why?? Because the closed loop sensors are very linear and quiet over their range, whereas the piezo voltage (i.e., height channel suffers from piezo creep & hysteresis).*

What ever the first channel tab is selected as, it will be 32 bit; all others are 16 bit.

Additional image channels *CANNOT* be activated during scanning.

▪ Pull-down menus in the Master Channel Panel are self explanatory- and the help menus (question marks) to the right explain features further: Color Map, Live Display (Section 7.1B), and Live Flatten (Section 7.1C) should be selected to the user's preference.

▪ (*The author suggests…*) Save both Trace and Retrace images! - just in case there is sample slope or some debris stuck to one side of the tip that will give an imaging artifact). *Capturing the trace and retrace data usually increase your chances of obtaining that 'publication quality' image.*

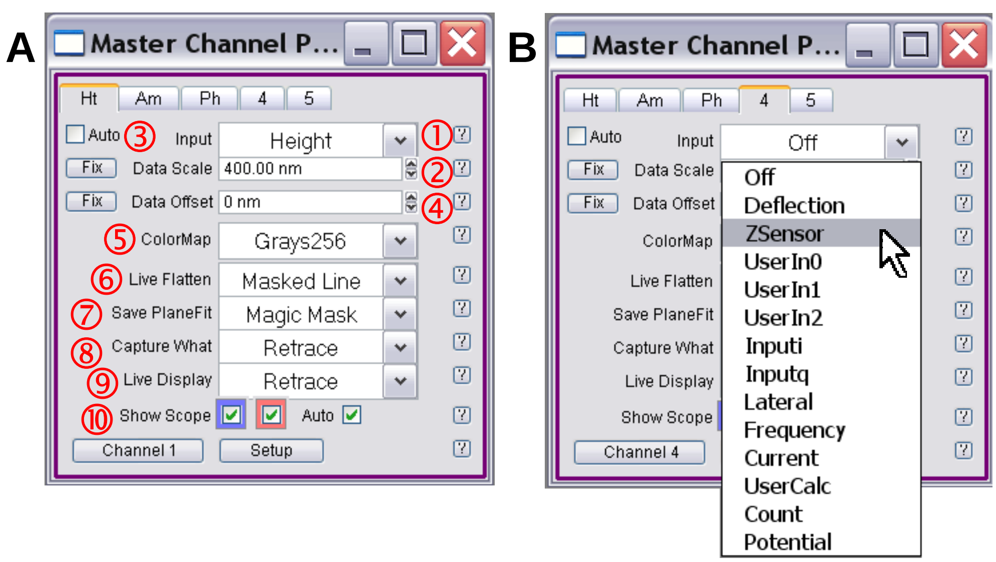


Figure 7.1.1: A) Master Channel Panel; B) Selecting additional channels from Input pull down menu in the Master Panel.

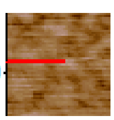
①The **Input** pull-down menu- choose what data channel to display per respective tab; Note: only activated when tip is not scanning.
② The **Data Scale** setvar- adjust the Z scale of the image here; or click the 'Fix' button to left of setvar to autoscale (*see Section 7.1A*).
③ The '**Auto**' checkbox autoscales the Z scale data during imaging (*see Section 7.1B*).
④ The **Data Offset** setvar- adjust where the center of the (Z) Data scale of the image here, or click the 'Fix' button to left of setvar to autoscale.
⑤ The **ColorMap** pull-down menu offers many color tables.
⑥ The **Live Flatten** pull-down menu (*see Section 7.1C).*
⑦ The **Save Planefit** pull-down menu (*see Section 7.1D).*
⑧ The **Capture What** pull-down menu (*see Section 7.1E).*
⑨ The **Live Display** pull-down menu (*see Section 7.1E).*
⑩ Show Scope (*see Section 7.1B).*

7.1A: ADJUSTING IMAGE Z SCALE-

There are many ways to adjust the Z scale in the image channel:
❶ Click Auto ☑ check box in top left hand corner of image channel tabs in the Master Channel Panel.
❷ Click the 'Fix' button to the left of the Data Scale setvar parameter window.
❸ Draw a quadrangle onto the image window, right (or left) mouse click inside the selected area and select 'Fix Scale'. This will fix the scale according to the Z values within the selected area.
*Alternatively, you can select 'Fix All Scales' which will rescale the Z data **in all imaging data channels**. It's a very nice feature.*
❹ Manually type a value, or use setvar arrows, in the Data Scale setvar parameter window.

7.1B: ADJUSTING TRACE/ RETRACE SCALES:

The individual Trace and Retrace images can be viewed *and saved* by selecting them in the respective pull-down menu on the Master Channel Panel. To view Trace or Retrace, confirm proper check box is selected.



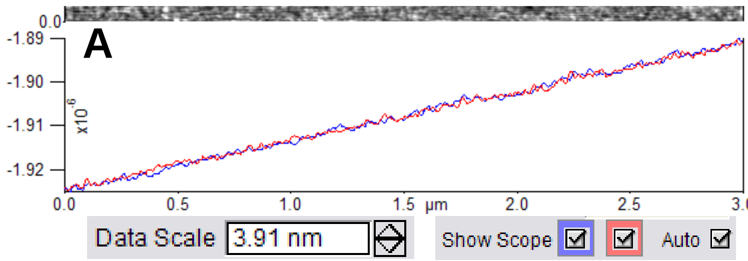
▪ The red bar/cursor to the left of the image indicates which scan line the tip is located (i.e., which slow scan line it's rastering).

▪ Trace or Retrace oscilloscope scan lines below the image can be turned ON or OFF with the 'Show Scope' check boxes at the bottom of the Master Channel Panel.
Trace is Red; Retrace is Blue.

▪ The Auto ☑ check box (if checked) auto scales the trace/retrace lines (below the image) with every slow scan line added. Typically, it shows the slope of the sample relative to the tip, because it's auto-scaled- even if the slope (Z) is sub-nanometer over several tens of microns (XY), its still going to display slope because its auto-scaled (Figure 7.1.2A).

▪ If the Auto checkbox is unchecked, it scales to the Data Scale setvar value. For example, if the Data scale value is too low, then trace/ retrace will be clipped (*as seen in Figure 7.1.2B*); this also depends on the Data Offset value.

▪ Figure 7.1.2 shows an example result in the trace/ retrace lines from changing the Data Scale setvar value. Notice in 7.2B, the larger peaks are clipped because the Z scale is lower (red ellipse). By increasing the Z scale, the entire traces can be scaled down (Figure 7.1.2C).



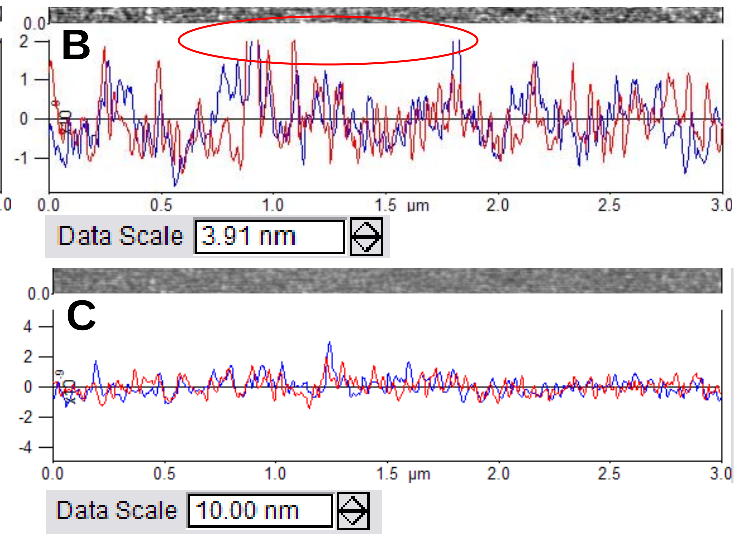


Figure 7.1.2: Effects of adjusting the Data Scale: A) 'Auto' checkbox activated; B) Auto checkbox unchecked, smaller Data Scale defined in setvar window- Notice larger features clipped in the Trace/Retrace lines; C) 'Auto' checkbox unactivated, larger Data Scale defined in setvar window.

▪ The Data and Offset Scales of the Trace *and* Retrace image channels can be separately controlled by clicking the 'Setup' button in the Master Channel Panel (Figure 7.1.3). The upper set of scale setvar applies to the Trace image, while the lower will apply to the Retrace. From the author's understanding, if *just* the top is open, it will apply to both Trace and Retrace images of the respective channel.

▪ It's a good habit to always save the data as raw, unflattened data- to do this, select None in the Save Planefit pull-down menu (see Section 7.1.3D).

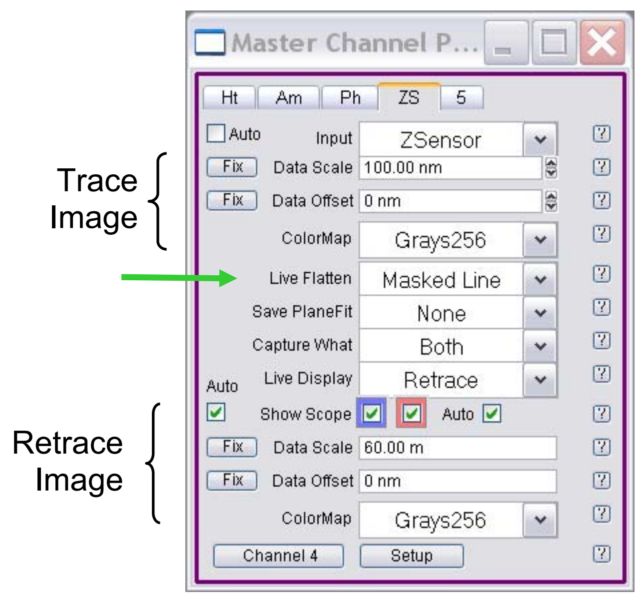
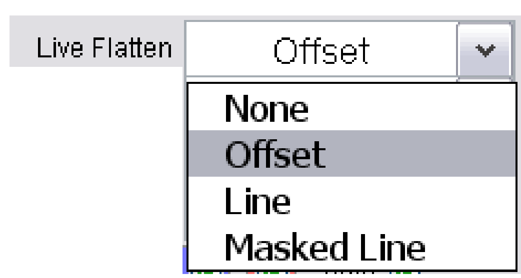


Figure 7.1.3: Activating Data Scale and Offset setvar windows for Trace and Retrace images. Green arrow: Choosing live flattening

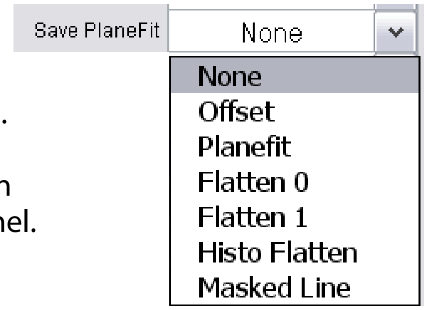
7.1C: REAL TIME FLATTENING:

The Live Flatten pull-down menu allows the real time scan to be flattened via plane, or line, Masked Line, or not at all. *This modification is only applied to real time data, but do not apply to saved data.*



None: leaves you with your raw data.
Offset: takes the average value of the scan line, and subtracts that from the data.
Line: Fits each scan line to a straight line, subtracts this line out of the scan line.
Masked Line: (*New feature!)* this fits a line to each scan line, but doesn't use any data on the line that is more than 1/4 of the Data Scale away from the line. If you change the Data Scale the lines after the change are affected, but not the lines already done. If you do want to redo the current lines, reselect Masked Line in the popup. *NOTE: The author shamelessly copied that text from the help menu.*

7.1D: SAVE PLANEFIT:



Caution: This will modify the Saved data.
None: saves the raw data.
Offset: takes the average value of the Image, and subtracts that from the data.
Planefit: subtracts a first order XY Plane from the Image.
The rest of these options modify your data quite a bit more. But you can return the data to its raw state with the Ultra Restore Layer button on the Modify panel.
Flatten 0: this removes the offset from each individual line.
Flatten 1: this removes the offset and slope from each individual line.
Histo Flatten: this removes the offset from each line by looking at a histogram of each line to determine the offset. The slope is removed in the X direction by a planefit on the whole image.
Magic Mask: this does a first order flatten on each line, then calculates a mask and redoes the flatten. This process is reiterated until it's satisfied. This process is similar to flattening offline images (See Section 12.2.1)
Magic Mask (Pits): Use this if the sample happens to be imaging something with pits. This algorithm can't tell if the features are pits or bumps.

7.1E: REALTIME OR SAVED DISPLAYS:

Use the pull-down menus to display or save the trace, retrace or both images. The author suggests saving both traces, but rarely displays both, unless the sample is expected to give some interesting result on the trace and retrace.

7.2: CAPTURING IMAGES:

The process of capturing image files occurs in the *Main* Tab of the Master Panel (*see Figures 6.1, 8.1*).

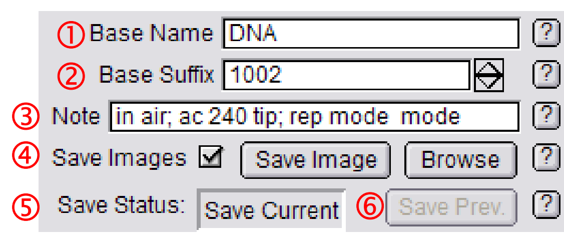


Figure 7.2.1: Activating the Save Image ☑ checkbox in the *Main* Tab.

① **Base Name**- type a 17 character (or less) filename. *The filename can't start with a number-Igor doesn't seem to like that.*

② **Base Suffix**- type; The software is designed to do continuous capture, increasing the 'Base Suffix' by one with every additional captured image.

③ Image '**Note**' line allows an unlimited length of text to describe imaging / experimental conditions (Figure 12.1.1); this can later be recalled in 'Show Note' option under in commands pull-down menu of the Display Window (offline analysis -*see Section 12.1*)

④ **Save Images** ☑ checkbox; a dialogue will appear to choose where to save the image data. Use 'Browse' button to change folders, or type a new folder name. Alternatively, the path can be typed in using colons (:) or \ to separate folders.
During imaging, the button will change to '**Save Partial**' -a function that allows the user to save a partial image the tip has withdrawn before the entire image has been collected.

⑤ The '**Save Status**' tells you if it's saving current scan or the next.

⑥ 'Save Prev.' saves the image scan previous if you didn't initially save it to disk (it's temporarily stored in experiment memory). This is a nice feature for the temporarily distracted.

- 'Browse' lets you look at stored images (*see Section 12 regarding more on introductory data analysis*).

Save Graphics File (standard Igor panel):

▪ If you happen to just a want a screen shot of one of the windows, you can make it the forward most window, and goto File→ save Graphics… the dialogue shown in Figure 7.2.2 will appear. There are many things that can be user-defined in this panel.

① Size: the size of the saved graphics image can be adjusted here.

② File Format- choose what type of file (TIF, JPEG, etc.)

③Resolution: choose 1, 2 or 4x the resolution- larger values good for larger presentations to reduce appearance of pixilation.

④File Name:

⑤Path: the 'Path' needs to be defined: do this by going to the main menu and choosing:

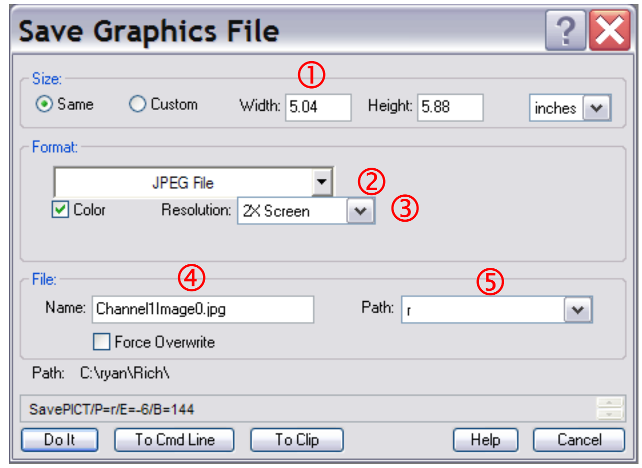


Figure 7.2.2: Saving graphics in Igor- allows size, file type, screen resolution, filename, path to be defined by user.

✓ Misc → New Path…; this will bring up the 'New Symbolic Path' dialogue (Figure 7.2.3)

✓ Name the Path; the author generally uses one letter because it's easy.

✓ If the path needs to be changed, click the '**Path**' button; this will bring up the 'Pick a Folder' dialogue-

A

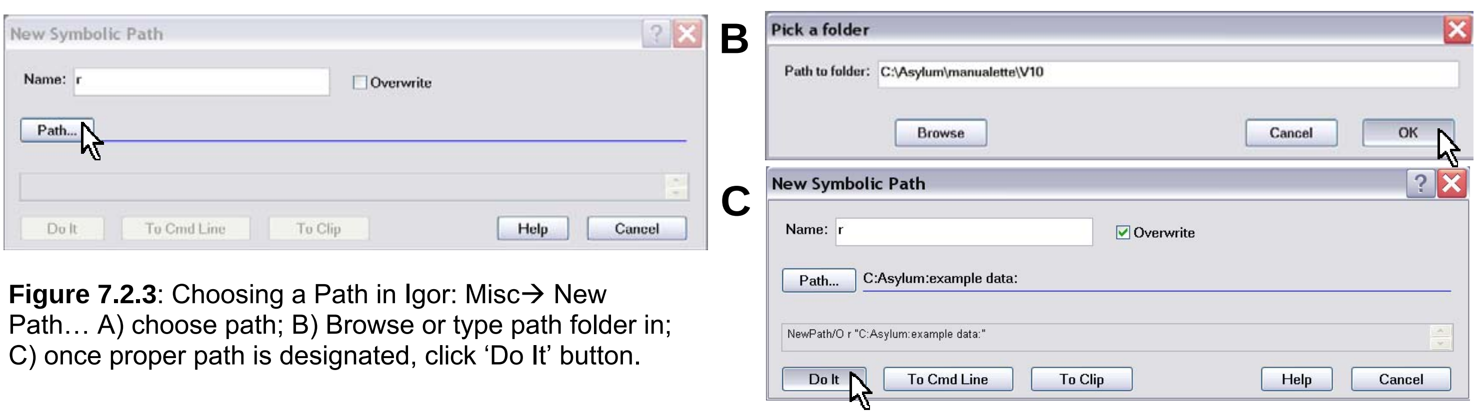


Figure 7.2.3: Choosing a Path in Igor: Misc→ New Path… A) choose path; B) Browse or type path folder in; C) once proper path is designated, click 'Do It' button.

✓Click the 'Browse' button to select the folder you want the data dumped; Click 'OK' button when complete.

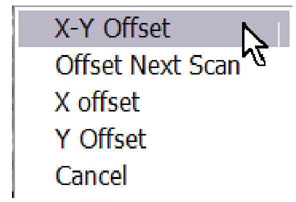
▪This will update the 'New Symbolic Path' dialogue with the proper path; Click the 'Do It' button, which will install the new path choice into the Path of the Save Graphics File window (Figure 7.2.2).

7.3: Offset Scan Area:

Images can be offset a number of ways:

❶ Manually typing in a value into the X, Y offset setvar parameter windows. For more precision, pull up the Igor cursors (Ctrl + i) and use one to get the coordinates of the center of the offset image.

❷ Using the Hamster wheel (with activated radio buttons) to move. The author doesn't really recommend this since the Igor's update time is the rate limiting step in the process.



❸ Place the cursor somewhere in the image, or trace/ retrace line, left click the mouse button and choose X, Y or X-Y offset, or Offset Next Scan from the pop-up menu that appears.

❹ Use the Igor Crtl + i cursors to determine and exact XY coordinate of the center of the offset; type these values into the X & Y offset setvar windows. See section 7.5 for similar example.

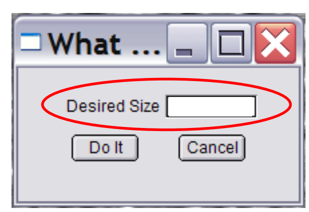
❺ During the same imaging session, the tip can be offset by performing a similar operation as described in 3, but on saved data. Since the closed loop sensors are so good in the MFP-3D, the tip will go to that area. *See Section 7.5 for more description and example. NOTE: if the head has been touched for some reason between the time the image was captured and this type of offset is selected, there is a great possibility that the head had been displaced by a couple microns in the kinematic divots on the baseplate.*

7.4: *Z●●MING* images:

There are multiple ways to zoom on an image feature:

❶ Manually type in a value into the Scan Area setvar parameter window; This will zoom with the same image center as the previous scan area- unless you manually offset, which would be a separate step (*see Section 7.3*).

❷ Use mouse cursor to draw a box around a feature of interest on image; the Igor mouse cursor will change shape to indicate a pop up menu is available; right mouse click and select 'Zoom Zoom', this will pick the *smallest* side of the (imprecise) rectangle that was created. Figure 7.4 shows an example of this to zoom.



[edit] Zoom Zoom dialogue

❸ To be more precise, select '[edit] Zoom Zoom' and manually type in the Desired Size value. Click 'Do It' to Zoom to the desired location.

❹ 'Nice' Zoom Zoom rounds to a nice number for the scan area.

▪ An example of Zooming can be seen in Figure 7.4 - using the left button of the mouse, a square/ rectangle is drawn around feature of interest; move the mouse back inside the quadrangle- a special cursor should appear that brings up a menu after right or left mouse click. Select 'Zoom Zoom' to decrease the scan area relative to the smallest side of the quadrangle; To make a more precise scan area, select '[edit] Zoom Zoom' ; which will present a dialogue allowing you to manually type in a value (i.e., instead of 35.61 µm, 35 µm was selected).

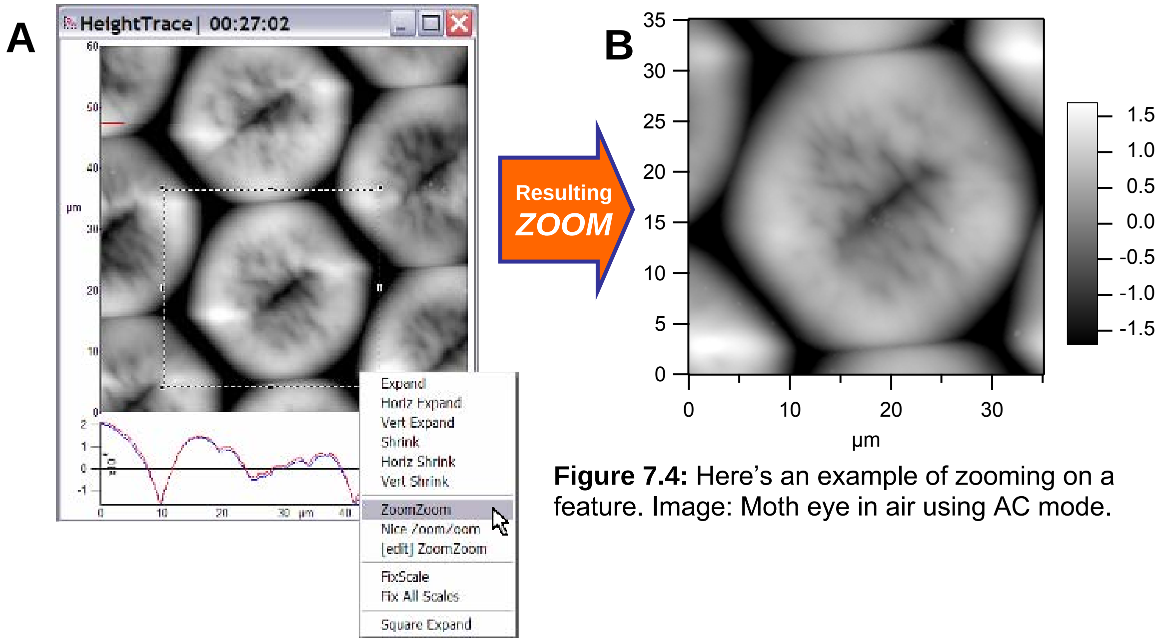


Figure 7.4: Here's an example of zooming on a feature. Image: Moth eye in air using AC mode.

7.5: Determining multiple Offset/ Zoom parameters within scan area:

There are two ways to do this-

❶ Use Igor cursor Ctrl + i to determine XY coordinates before offset zoom.

❷ From a larger scan area image, zoom on one area, then use larger scan area saved data to zoom on second feature.

In some instances, it is desirable to zoom into two separate areas/ features of interest from a larger scan area- *The only problem is knowing the exact coordinates of the scan area image centers will be before hand so they can be typed into the Offset setvar values later…*

This is also easy to accomplish because of Igor.

✓ Click Ctrl + i to bring up the Igor Cursors.

✓ Place the cursors in the areas of interest, to determine the X,Y coordinates within the scan area.

▪ It's often a good move to record these numbers so you remember them, especially for the second image zoom/ offset. In the command line, type a comment using two // forward slashes. You can easily retrieve these values by scrolling up into the history window (see Figure 7.5.1B).

2) Zooms/ offsets can also occur from a saved image, NOTE: To be of significance, the saved image must be viewed while the MFP is still scanning within that 90 um XY scan area. Figure 7.5.2 shows an example of this.

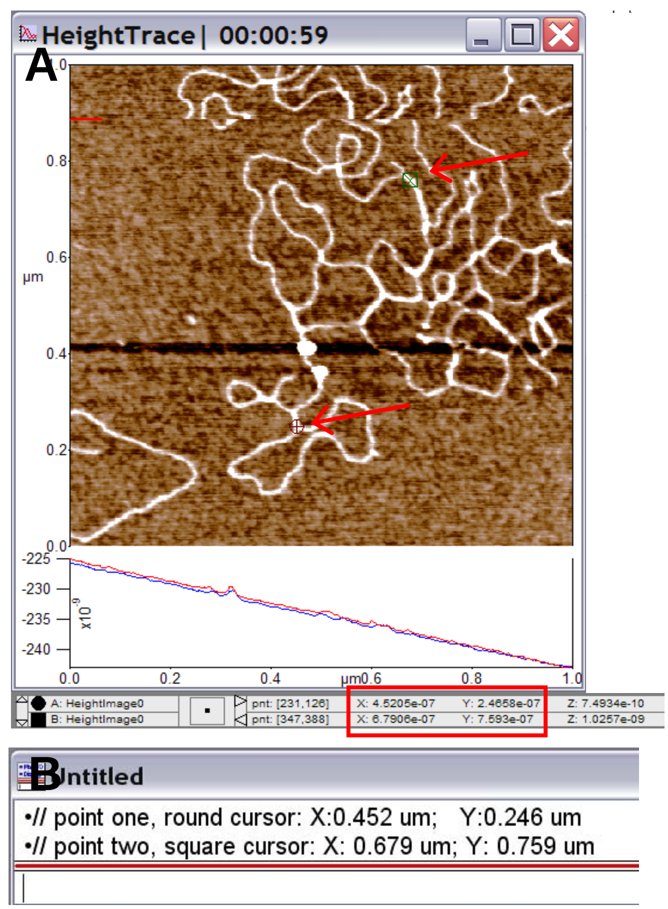


Figure 7.5.1: A) Using the Igor Ctrl + I cursors to find XY coordinates within a scan area during real time imaging; B) copy coordinates in command line as comments.

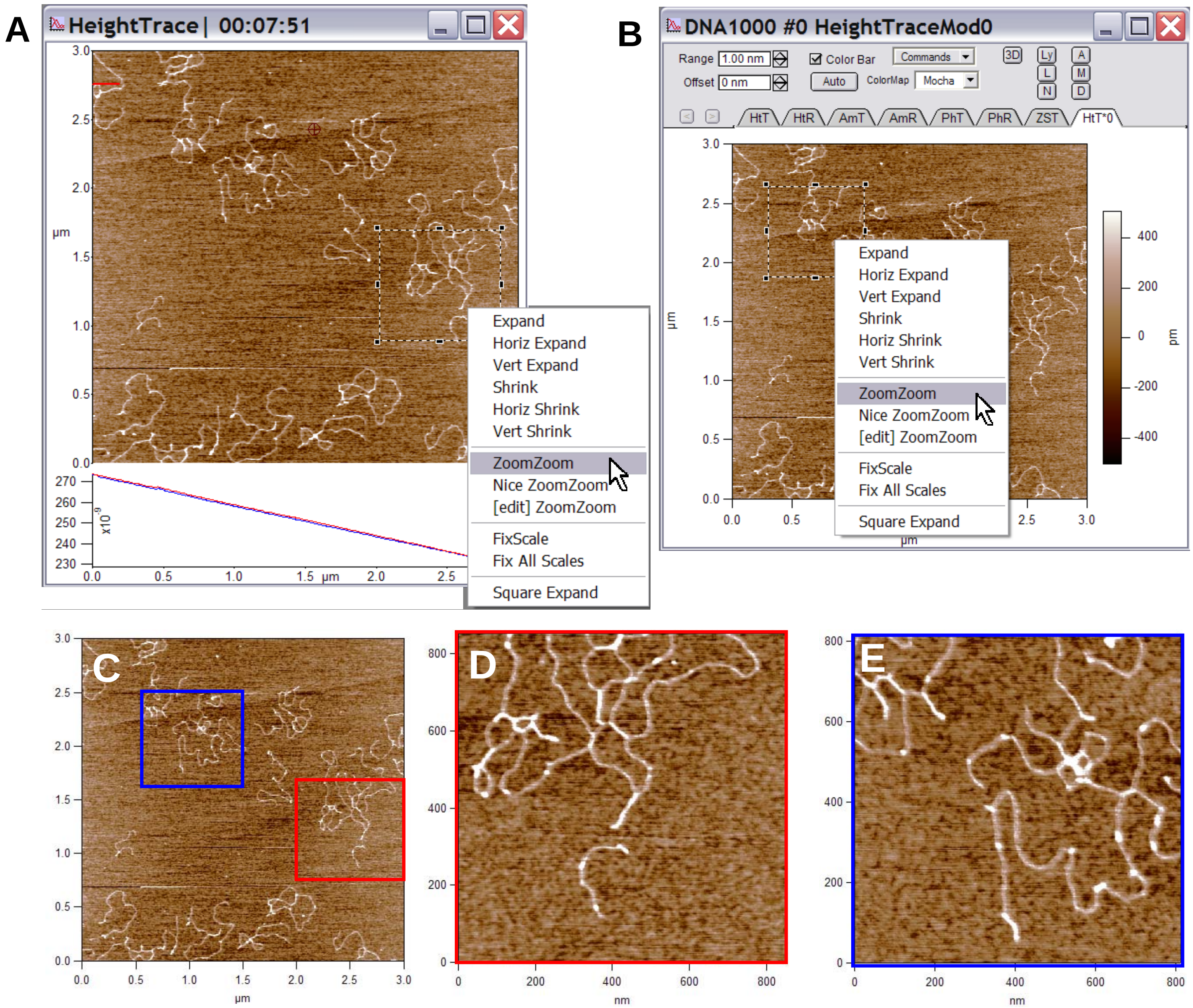


Figure 7.5.2: Zoom/ offsets from saved data: A) real time scan to zoom in on one area; B) Using saved data of initial real time data to zoom on another area of interest; C) large saved image; D) real time zoom image (panel A); E) zoom from saved image blue box in C).

7.6: Real Time Section Analysis:

7.6A: Basic Real-Time Line Section Analysis:

To measure a section line analysis during a Real Time image acquisition, open the Analyze panel (Figure 7.6.1).

✓ Go to MFP IP → Analyze Panel.

✓ Click 'Include RealTime' checkbox ☑.

✓ 'Line' will be selected from the Mode pull-down menu because it's unable to do 'Free Hand' lines in real time. *See Figure* 12.7.2.7 *for examples of Free Hand line drawing sections.*

✓Click the 'Draw' button; pull cursor over image feature to draw a line on the feature of interest in the image (Figure 7.6.2A).

✓ Select Full Width checkbox ☑ for sections to traverse to the edge of scan areas along the line vector drawn (figure 7.6.1).

▪ Multiple scan lines can be averaged to eliminate noise or give more statistical relevance to the sectioned line.

▪ Once the line is drawn, a Section plot similar to Figure 7.6.2B will appear. The Igor cursors (Ctrl + i) can be placed on the curve to measure points on the line (*notice blue cursors on of feature in image being measure at FWHM*), which are displayed in the lower half of the Analyze panel (Figure 7.6.2B).

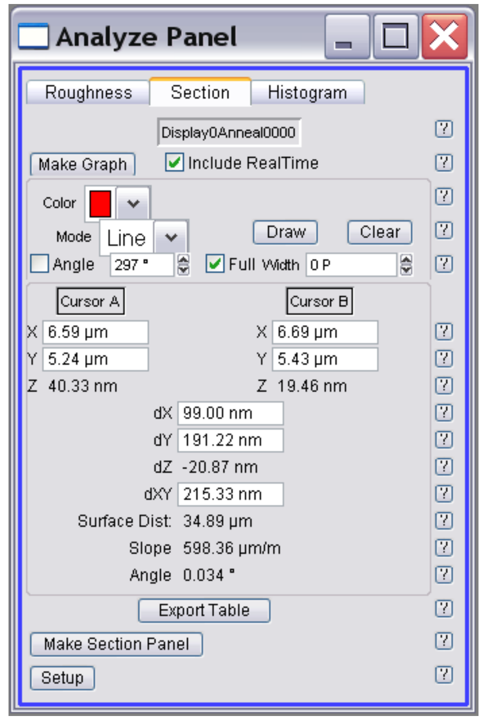


Figure 7.6.1: Section tab of the Analyze panel.

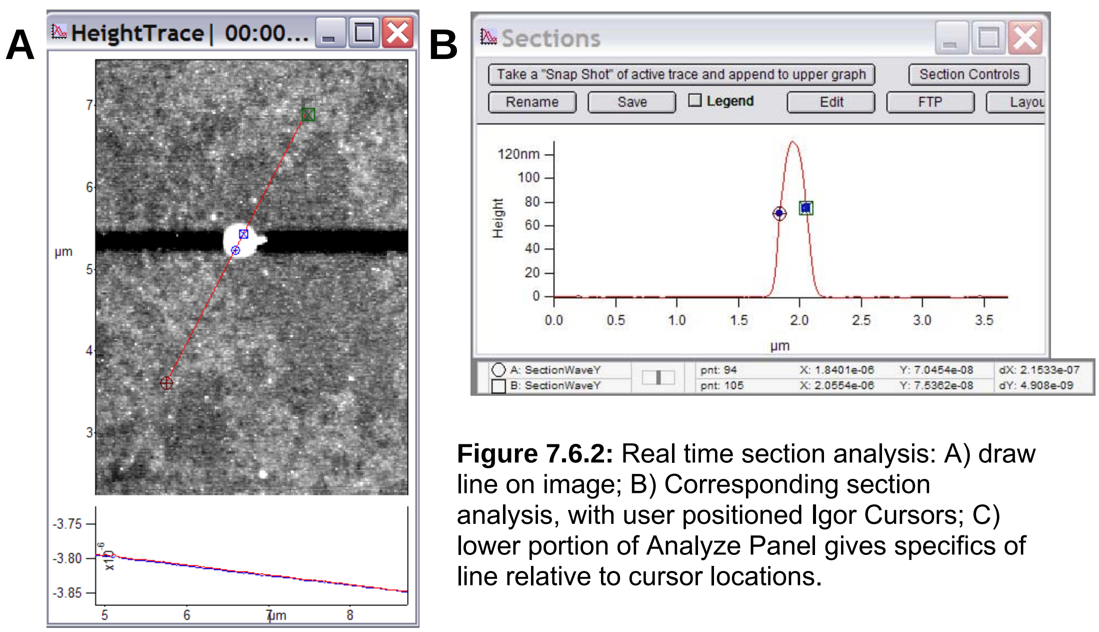


Figure 7.6.2: Real time section analysis: A) draw line on image; B) Corresponding section analysis, with user positioned Igor Cursors; C) lower portion of Analyze Panel gives specifics of line relative to cursor locations.

If the Y axis data values doesn't match the values in the S&D meter, some kind of real time flattening is probably activated, which will offset the data. Switch the real time flattening to 'None' in the Master Channel Panel (Figure 7.1.1A).

▪ If the image happens to possess some tilt, it won't be intuitive to get a proper height from the cursors.

✓ On the section plot, Hold Crtl + Shift key over curve to be 'leveled' (Figure 7.6.3A).

✓ Right or left mouse click over the line scan; a double arrow will appear (Figure 7.6.3A).

✓ Move mouse up or down to make section level. (see Figure 7.6.3B); from here, the Igor Crtl + I cursors can be put on to measure the dY.

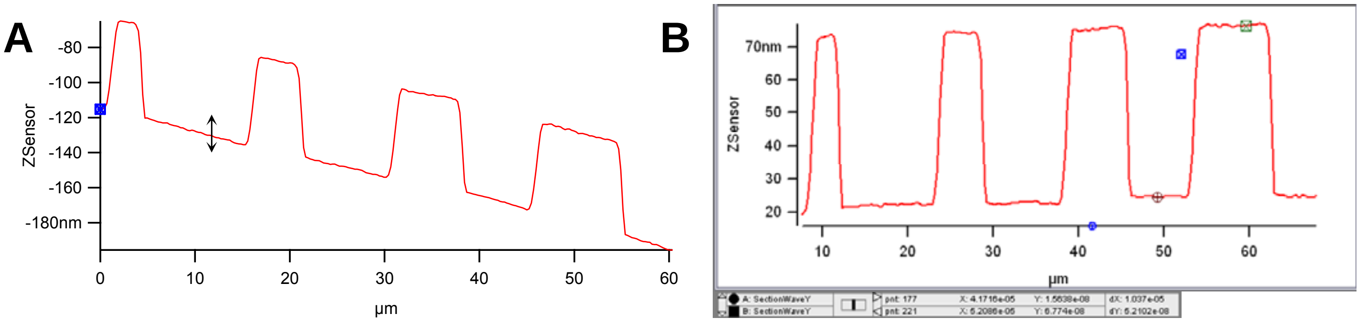


Figure 7.6.3: Real time line section plot leveling to obtain more accurate height differentials. A) crtl + shift + mouse button over section to obtain double arrow cursor; B) move mouse up or down to level section.

✓ Click the 'Export Table' button in the Section Window to get these cursor stats (i.e., X_a, Y_a, Z_a; X_b, Y_b, Z_b; plus differential values and angle values).

7.6B: Adjusting the Angle of the Section Line via three different ways-

❶ Click the 'Clear' button in the Analyze panel and redraw a new line over feature of interest.

❷ Grab one of the end Igor cursors on the section line in the image by left mouse click & hold while dragging cursor to new point (Figure 7.6.4A to B). This will also update the Angle setvar value.

❸ Use the angle check box to manually adjust with the Angle setvar window.

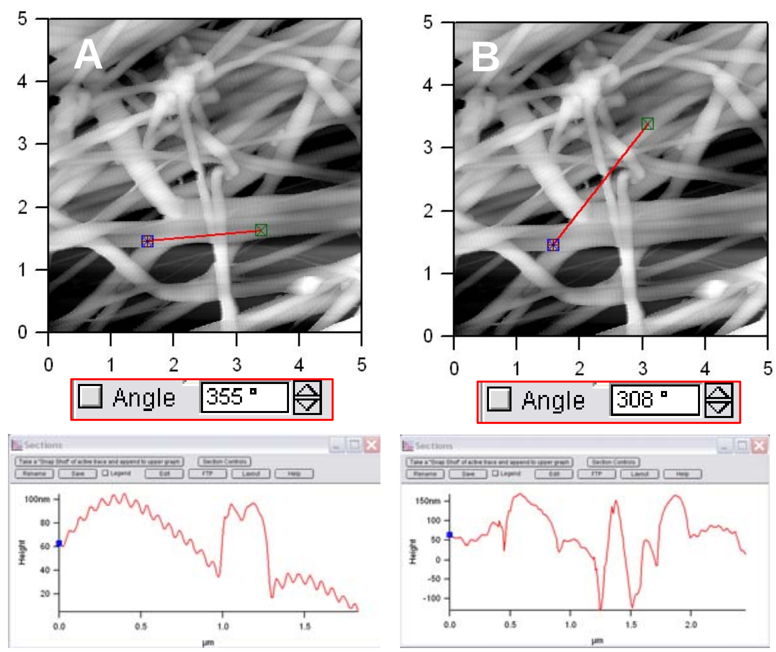


Figure 7.6.4: Adjusting line section angles: A) drag one of the end Igor cursors to a new area; B) result.

▪ To **average multiple scan lines**, enter the desired amount of scan lines to be averaged in the Width setvar window in the *Section* tab of the Analyze panel. Lines will be displayed with solid center indicating center, and dotted lines represent the averaging scan line width (Figure 7.6.5- a 25 line average in a 512 x 512 image resolution).

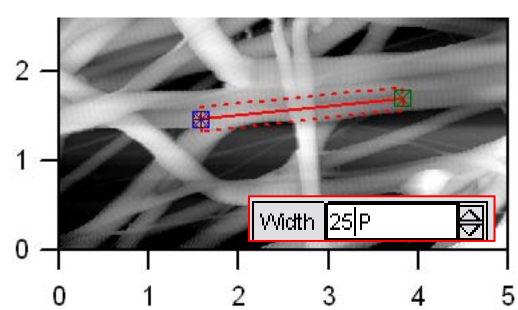


Figure 7.6.5: Averaging multiple scan lines

7.6C: Plotting multiple section lines displayed on one section plot-

✓Create a section line as described above.

✓Click the 'Take a "Snap Snot" of active trace and append to upper graph' Button.

✓Create additional section line.

✓Click the 'Take a "Snap Snot" of active trace and append to upper graph' Button.

▪ Figure 7.6.6 shows an example of this-

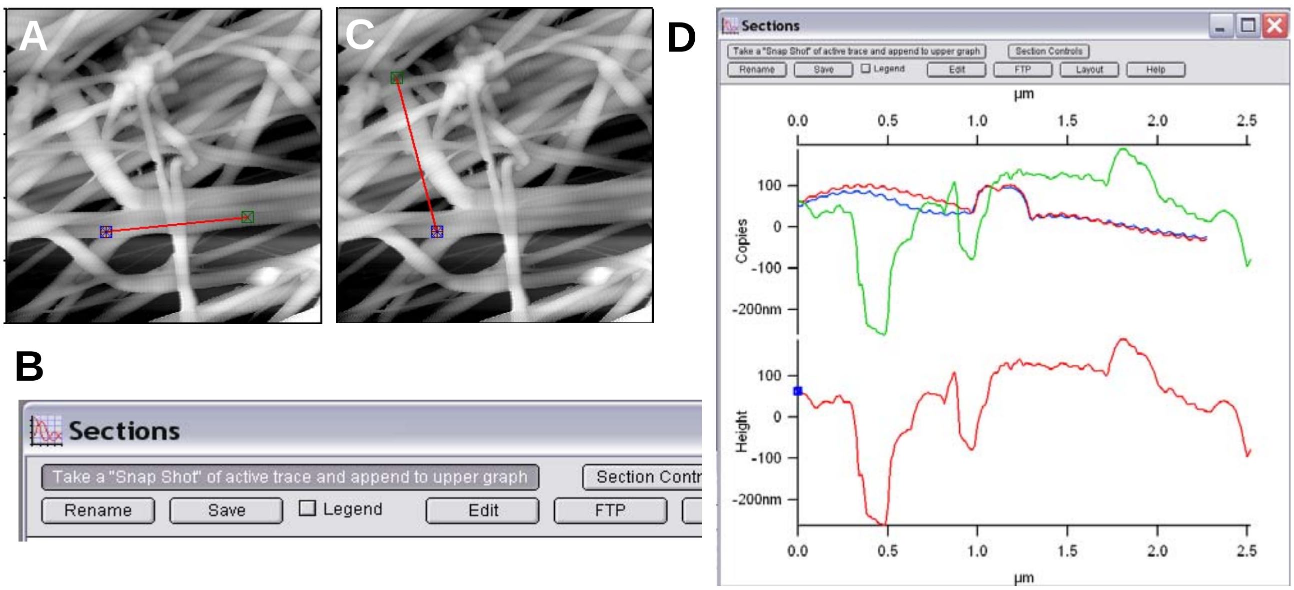


Figure 7.6.6: Plotting multiple section on one section plot: A) create first section plot; B) click "Snap Shot" button in Sections window; C) Create additional Section plot; B) click "Snap Shot" button in Sections window again; D) resulting multiple sections plot(s): red- single line 7.11A; blue: averaged line 7.10; green: single line 7.11C. Sample: Collagen in air.

7.6D: Export the line section XY data:

✓ Click the 'Edit' button in the Section plot- this will bring up a table with a Y value, relative to the points in the Igor Wave (*see Igor 'Getting Started' manual to understand the definition of a wave*), and the points where the blue cursors are- kind of meaningless for use with another spreadsheet.

What the author would then do upon this inquiry is:

✓ Go to Windows → New Table…

✓ Select SectionWaveX & SectionWaveY.

✓ Click 'Do It'- this will create an X,Y delimited table from which numbers/ data can be cut and pasted.
None of the Asylum Research inmates will verbally condone the use of programs other than Igor Pro.

7.7: OTHER STUFF:

7.7A: WINDOWS MANAGER: The windows manager helps find windows when you have a lot of different panels and tables open.

✓Ctrl + 1 opens the window (Figure 7.7.1)- upper left window.

✓Find the panel/ graph or table you are interested in, and double click- this will bring that window forward.

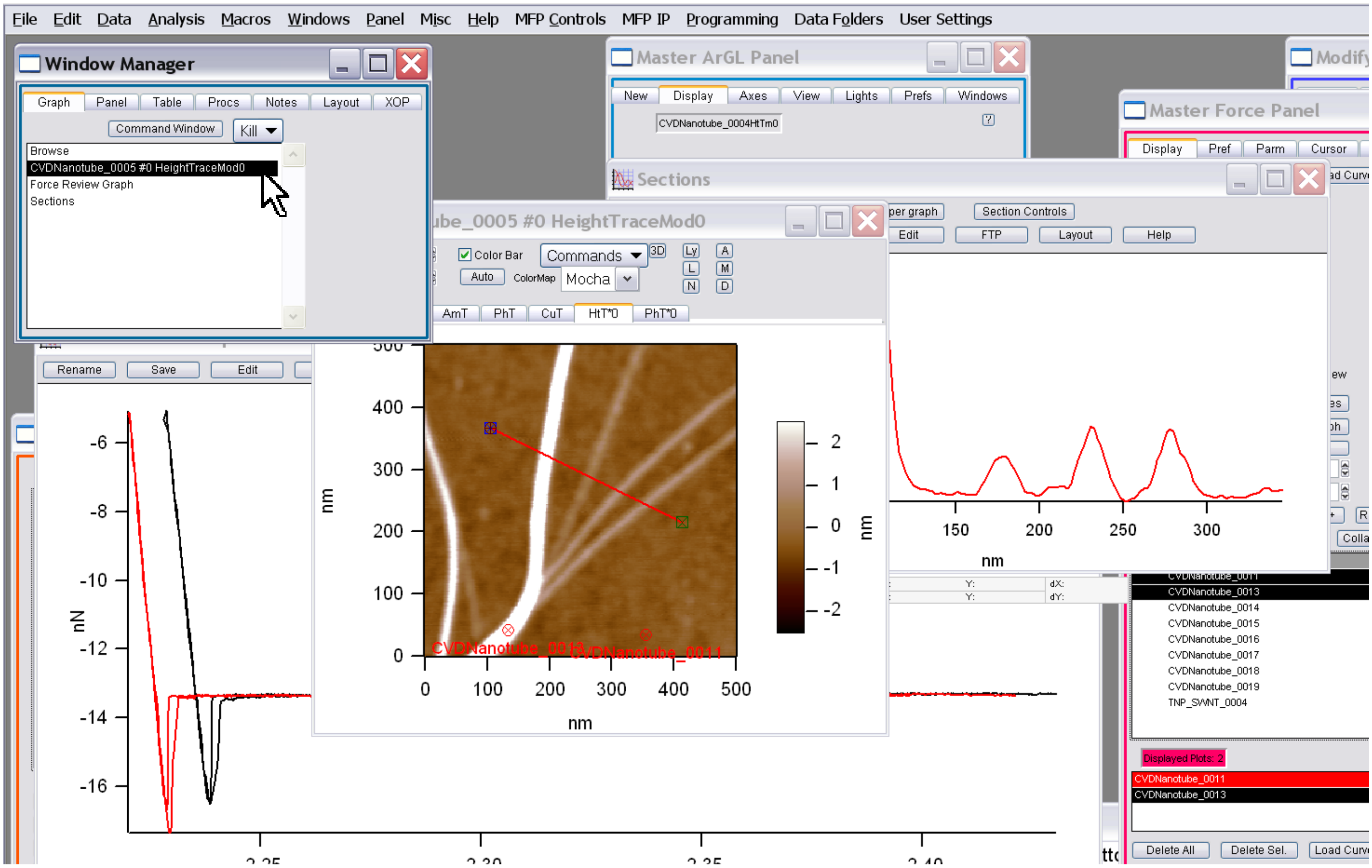


Figure 7.7.1: The Windows Manager panel; hotkey: (Crtl + 1).

7.7B: SAVING IGOR EXPERIMENTS:

▪ Saving the Igor Experiment is a great way to begin right where you may have left off- when you save an experiment, all the panels, graphs, etc. will come up just as it was exited once loaded. Other advantages of saving them is that every command executed is stored in the History window. Recall that Igor is memory based, so many full days of this will make the file large, and ultimately slow the program.

▪ One down side to it is that these .pxp experiments can be pretty big Mbyte wise. If you save pxp's on a regular basis it's a good idea to back these files up, or remove them from the lab PC so it doesn't get bogged down due to a full harddrive.

▪ In the author's experience, the best way to open a saved expt is to have the MFP-3D software open (loaded), then go to File→ Open Experiment and select the saved expt you wish to open. NOTE: it will open if you use the same version (or slightly later) to open the saved experiment with. See Section 14.17 for more on this.

7.7C: Adding Meters to S&D meter:

▪ To view additional signals in the S&D meter:

✓ Click the 'Setup' button,

✓ Change the parameter of interest Status pull down menu From 'Auto' or 'Off', to 'On';

✓Click the 'Done' button.

▪ Figure 7.7.2 shows the phase signal being selected, such that the quantitative value of the Phase signal can be seen during imaging to aid user to whether the tip is experiencing net attractive or repulsive forces as it interacts with the surface (Section 6.1.4).

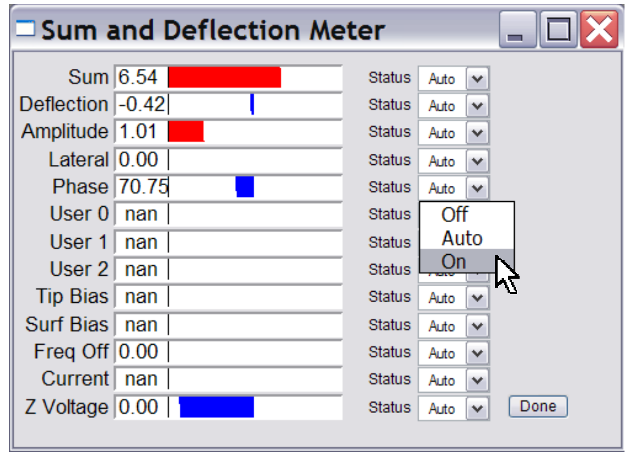


Figure 7.7.2: Turning on additional meters in the S&D meter.

7.7D: Modifying Igor Graphs:

▪ To adjust line thickness (in any Igor Pro window), color or appearance, double click the mouse on the plotted line, and the Modify Trace Appearance dialogue will come up (Figure 7.7.3). Here the thickness, color, line style, and mode can be defined. When complete, click the 'Do It' button.

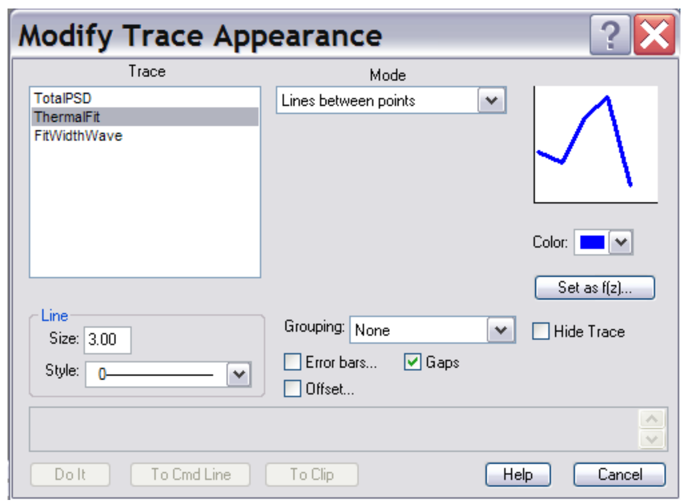


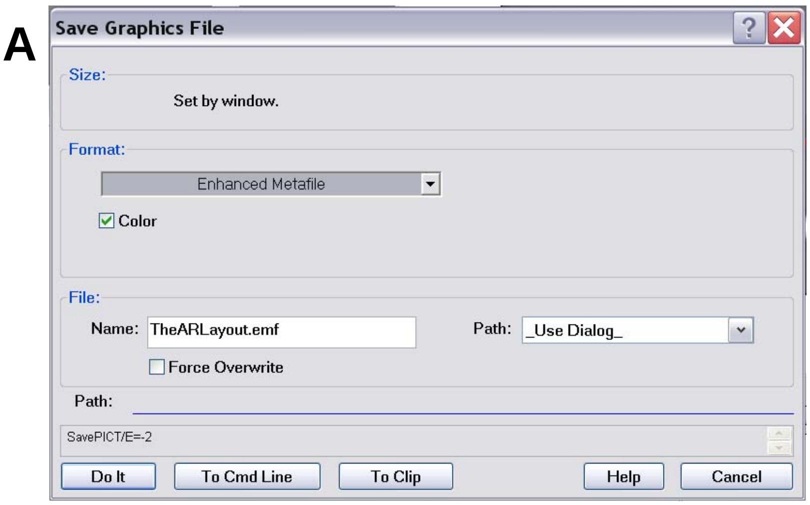
Figure 7.7.3: Modify Trace Apparence dialogue.

7.7E: Igor/ MFP-3D Layouts:

Additionally, the MFP-3D™ software has these items called 'Layouts' , *and they're not kidding when the say they are awesome!*(Figure 7.7.4B).

▪Any data you have worked up in the offline analysis can be dumped into one of these layouts by click the layout button. *(See Section 14.13*).

✓ To save the layout, go to File→ Save Graphics; the Save Graphics File dialogue comes up- here you can determine what file type to save the file as; the name; and the Path (*see Section 14.2*)



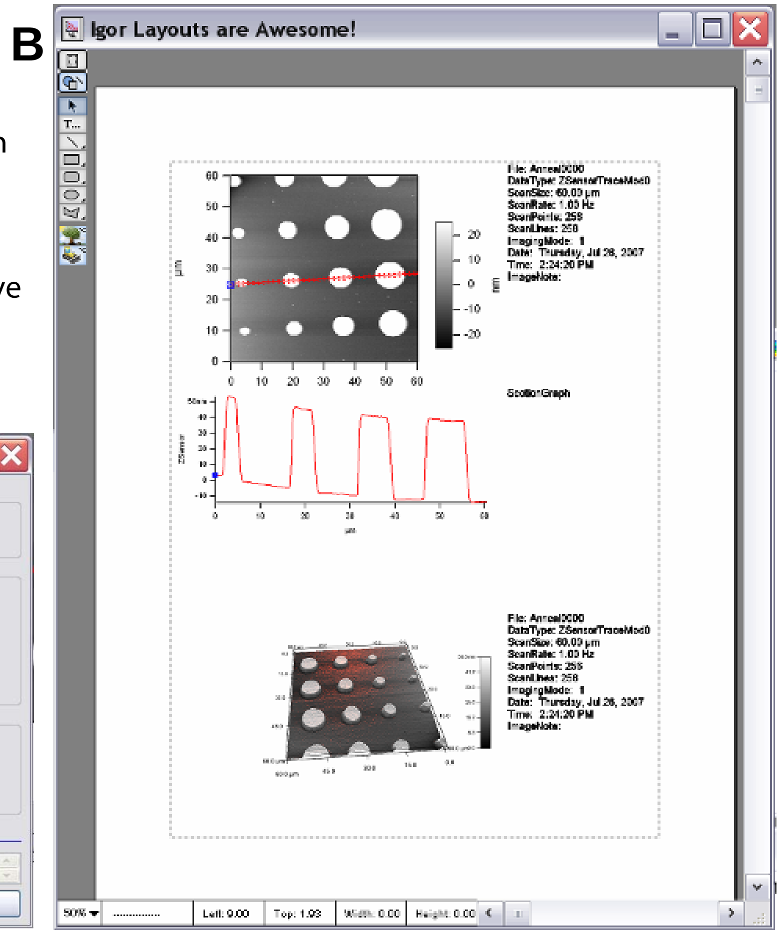


Figure 7.7.4: A) Saving the layout as Graphics file; B) Igor Layout example.

✓ Use the Igor tool box (Ctrl + T; upper left corner to add text or shapes to the Layout.

8. Contact Mode Operation:

Contact mode AFM (also known as constant force mode) is one of the more commonly used imaging modes in AFM. It is often used in imaging of hard materials, in some electrical techniques and to image biological materials- e.g. cells under low set point force and scan rate (because cells are big bags of water, they can be easily oscillated using AC mode on them- contact mode works great, and the Deflection images shows a great deal of detail). The features described in Chapter 7 also apply to this and all imaging modes on the MFP-3D.

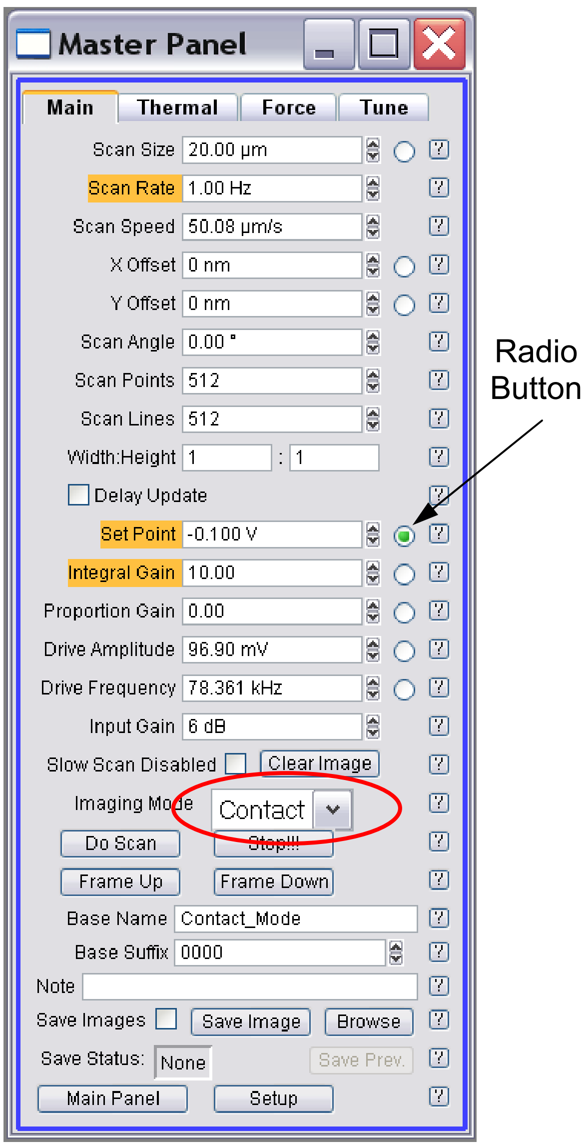


Figure 8.1: Contact mode imaging parameters

8.1: SET UP AND INITIAL PARAMETER SELECTION:

✓In the Main tab of the Master Panel, select 'Contact' from the Imaging Mode pull down menu (Figure 8.1, red ellipse). Parameters highlighted in orange will be mainly used to fine tune imaging parameters when scanning (see below).

✓ **Image channel selection:** Go to the Master Channel panel: At the 'Input' pull down menus choose the following image channels: Height, Z sensor and Deflection (Figure 8.2). If doing Lateral Force Microscopy, choose Lateral in an additional 4th channel.

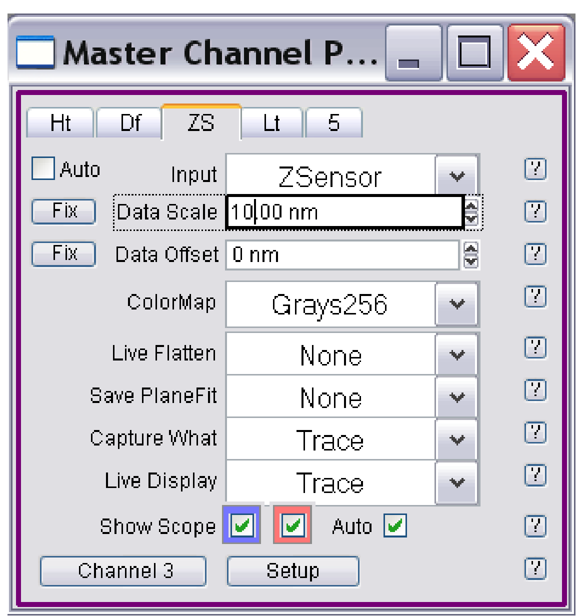


Figure 8.2: For Contact mode imaging, select Height, deflection and Z Sensor channels

If you don't change from the default AC mode image channels of amplitude and phase, these channels won't produce any meaningful data in Contact mode- they must be changed prior to imaging in contact mode.

NOTE: Scan angle should be at 90° for lateral force microscopy (LFM). *This may be obvious, but the author mentions it because some other commercial scopes scan perpendicular to the lever at 0°.* **See Section 8.4 for more on LFM.**

It's a good habit to activate the Z sensor channel when imaging, especially when sample features are larger than a few hundred nanometers- the LVDT sensors are more linear than the piezos, and thus it's a more precise Z measurement.

For more on the Master Channel Tab, see Section 7.1.

✓ With a properly loaded cantilever, adjust the Photodetector (PD) to a slightly negative value. The slight negative free air deflection will place the SLD spot in the middle of the PSD (where the range is more linear) when the tip is engaged.

▪ To conceptually help aid the selection of a Set Point voltage, a qualitative depiction of cantilever deflection *vs.* tip-substrate distance is shown in Figure 8.3. The contact point is when the Set Point deflection voltage is equal to the cantilever Set Point deflection value- this is when the cantilever starts to experience positive deflection due to the applied force from the piezo. Low set point forces will be slightly more positive Set Point voltages than the free air deflection voltage, while larger forces are much more positive than the free air deflection voltage. To know exactly how much force is being applied during imaging or a manipulation, the cantilever spring constant must be determined- *See Chapter 9*.

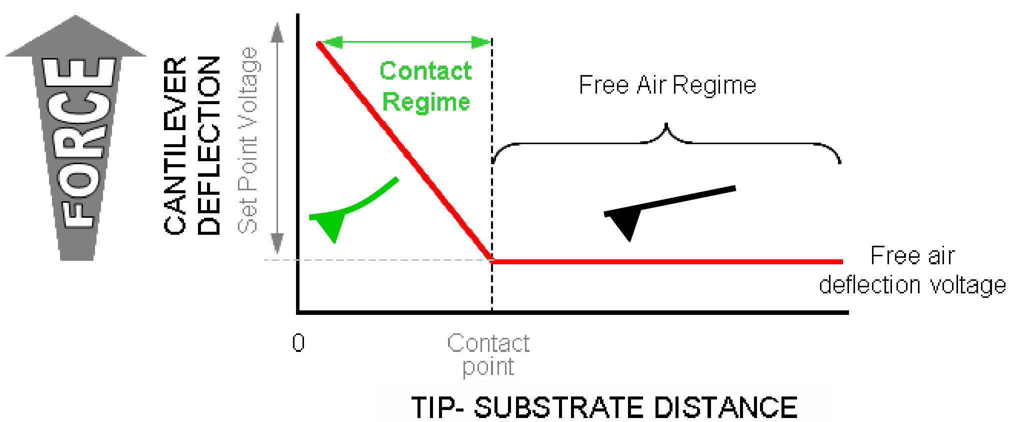


Figure 8.3: Qualitative depiction of Cantilever Deflection *vs.* Tip-Substrate separation.

✓ Choose a Set Point voltage that is more positive than the free air deflection value in the S&D meter. In Figure 8.4, the free air deflection is -0.20 V: therefore a Set Point voltage of -0.19V or more positive is what is needed to engage the tip. Set Point voltages near (just positive of) the free air deflection means the tip will apply low force to the sample (or false engage), while Set Point voltages much more positive than the free air deflection voltage means the tip will apply greater force to the sample.

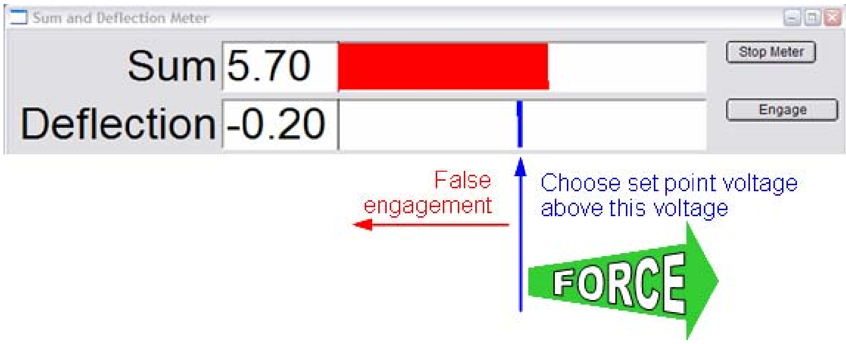


Figure 8.4: Choosing a Set Point voltage in Contact Mode: Set point must be more positive than the free air deflection voltage.

▪ On the *Main* tab (Figure 8.1), adjust the highlighted parameters (dependent on sample):

✓Integral gain (I): 8 to 10: *allows the Integral gain to be responsive enough upon feedback servo activation*.

✓Scan rate: 1 Hz; *for softer samples, choose a slower Scan Rate.*

✓Scan angle, resolution (scan points & lines) and image size is up to you.
Rotating the scan angle results in counter clockwise rotation with positive rotation angle values

▪ At this point you are ready to engage for an imaging-only application (i.e., if you are **not** interested in knowing the cantilever spring constant to know what forces are applied at a given Set Point voltage). See Chapter 9 if you want/need to determine cantilever spring constant.

8.2: TIP ENGAGEMENT:

✓Click 'Engage' in the Sum and Deflection window (Figure 8.4). Notice the Z-piezo voltage maximizing all the way red (150 V), indicating the Z- piezo is fully extended in anticipation of engagement of the feedback servo.

✓ ***Slowly*** turn the thumbwheel counter-clockwise (towards the surface; rate ~ several microns per second) until the deflection voltage value on the S&D meter equals the user defined Set Point, *AND* you here a chime sound (computer speaker volume must be on). The feedback servo is now activated. You will also notice the Z-piezo voltage meter goes from 'railed' in the red (150 V), to some lesser value.

If you have the volume up on the transducer (front of controller, headphones plugged in), you can hear a frequency change as engagement occurs- it will sound like static and will change to a dampened sound when in contact.

GENTLE ENGAGEMENT: Soft engages, similar to those described in Section 6.1.2B, and also can be done in Contact Mode. Choose a Set Point voltage slightly more positive than the free air deflection voltage; you can proceed by clicking the 'Engage' button and wheel the tip down until the feedback servo is engaged.

An even more gentle engagement occurs by wheeling down 10 μm, then click '**Engage**'; if the tip doesn't engage, click '**Withdraw**' button, wheel down another 10 μm, and repeat. *Notice that each graduation on the front thumbwheel is 1 μm.*

✓ Once feedback has been activated, continue to lower the head with the front thumbwheel to ~ 70 V (i.e. no color in Z-Piezo meter). This indicates the piezo is in the middle of its *Z*- range (~ 7.5 μm standard head; ~20 μm extended head).

▪ *At this point the tip is engaged and just sitting on the surface- it does not begin rastering until you tell it to do so. You can now being imaging (see next step) or determine the Spring Constant (see Chapter 9).*

If the Z-piezo voltage is railed all the way blue (-10 V) upon clicking simple engagement, the piezo is fully retracted because it thinks it has crashed. This indicates a false engagement and adjustment must be made to the Set Point voltage to allow extension of the piezo. This often occurs when imaging in fluids/ high humidity, or too low a Set Point voltage (i.e. low force).

Is the acoustic hood hatch still open?-

Probably. Follow steps to preserve the tip's apex while shutting the door:

❶ Use the Hamster to decrease the Set Point voltage to pop the tip off the surface.

❷ Close the hood door.

❸ Increase the Set Point voltage with the Hamster again to hard engage;

❹ Proceed scanning.

8.3: FINE TUNING IMAGING PARAMETERS:

✓Click the 'Do Scan' button on the Main tab. After a brief moment, imaging will begin.

✓Tune the parameters with Set Point force, Integral gain (I) and Scan Rate. Use the arrow clickers (to right of setvar windows) to adjust parameters, opposed to typing values in, *OR- see next bullet…*

▪ *Alternatively*, you can fine-tune the parameters using the '**Hamster**' **wheel** on the front of the controller. Any parameter with a 'radio' button next to it (Figure 8.1) can be changed during a scan when it is activated (looks like black/ green dot in circle) with the 'Hamster'. The Hamster gives "digital control with analog feel". The toggle switch to the left of the Hamster allows you to toggle between radio buttons in the panel. This is a GREAT feature for tuning on the fly, and you don't need to use the mouse. (The Hamster is also functional in the Force panel, described later in the Section 11).

✓ Image quality can be monitored by image resolution and amount of noise in the line traces (located below images).

✓ The Set Point voltage and Integral gain should first be adjusted to achieve good tracking. Figure 8.5 shows an example of imaging a calibration grid, with corresponding parameters from Main tab below. Panel A) shows the initial tracking of the tip upon engagement with a Set Point voltage of –0.4V (free air was ~ -0.45V). B) Shows that with an

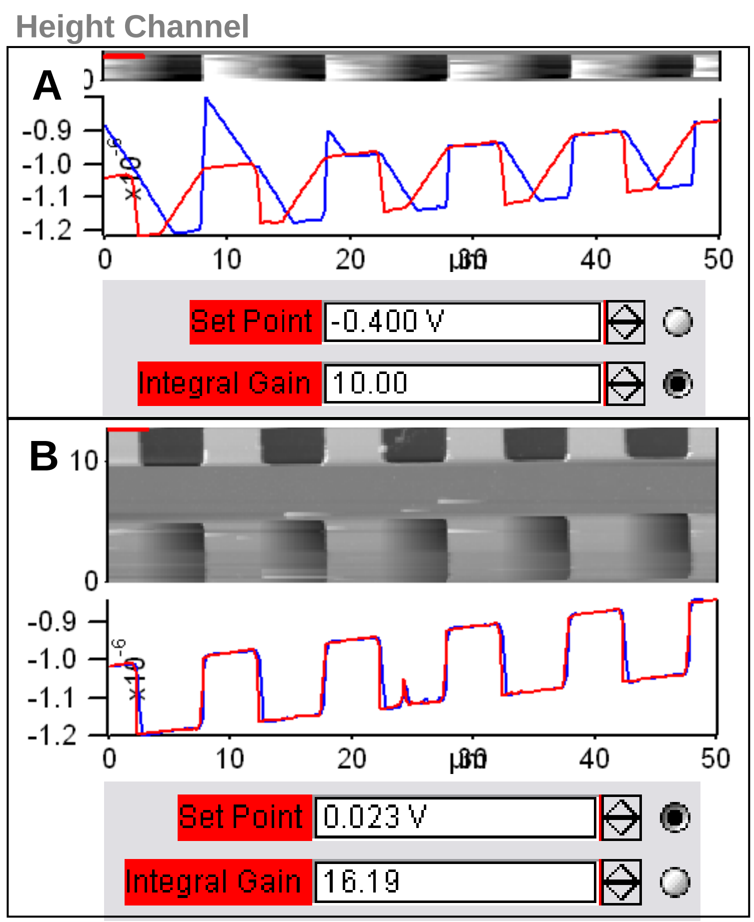
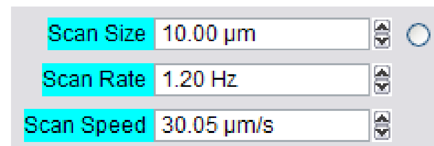


Figure 8.5: Image parameter tuning in Contact Mode of calibration grid.

increase in Set Point force (i.e. more positive Set Point *voltage)* and slight increase in Integral gain, tracking is improved greatly.

Don't be alarmed if you have too crank up the Integral gain when using long floppy cantilevers- the gain is related to the optical lever sensitivity (more gain for less sensitivity).

✓ The second parameters to adjust are the Scan Rate & Scan Angle. Having a vague idea of the orientation of the tip on the cantilever can make scanning at 90º more advantageous than 0º because of the shape of the tip at the end of the cantilever. Some cantilever manufacturers compensate for the slight angle the cantilever is mounted relative to the surface by having the front and back angles of the pyramid at different angles. Typically the



The **Delay Update** ☑ checkbox allows the user to change the parameters during a scan, which will take effect at the end of that scan. During the period before update, the parameters changed will be highlighted in a light blue color. Notice that setvar without radio labels, these highlights also occur because these values only take effect when the frame is finished (e.g. scan rate).

▪ *It's also great for changing the number of scan lines and points on the fly (i.e., image pixel density).*

-Without the Delay Update ☑ checkbox activated, the tip has to be withdrawn to change either the Scan Lines or Scan Points setvar values. The author is superstitious enough, and has had experiences where the tip is somehow changed during this withdraw step.

-However, with the Delay Update ☑ checkbox activated, these parameters can be changed, then click the '**Frame Up**' or '**Frame Down**' buttons to activate this new change- *Notice the tip stays on the surface* (S&D meter- Z piezo voltage). This makes scanning the surface at a lower value of scan points and lines to look for something more efficient (quicker), then increase for higher resolution imaging.

8.4: LATERAL FORCE MICROSCOPY:

Lateral Force Microscopy (LFM) can easily be performed on the MFP-3D.

✓In the Master Channel Panel, confirm that Deflection and Lateral channels are selected. See Section 7.1 & Figure 7.1.1 for more detailed description).

✓In the *Main* tab of the Master Panel, confirm the Scan Angle setvar is set to 90º.

✓Confirm the imaging mode is set to Contact mode.

✓Engage tip; as described in Section 8.2

▪ Figure 8.6 shows an LFM image of a micro-contact printed alkanethiols on polycrystalline gold. The bright areas of the LFM images are terminated with a carboxylic acid, while the dark areas are a methyl terminus.

AR does manufacture a head that has the ability to mechanically zero the SLD spot in the center of the PSD.

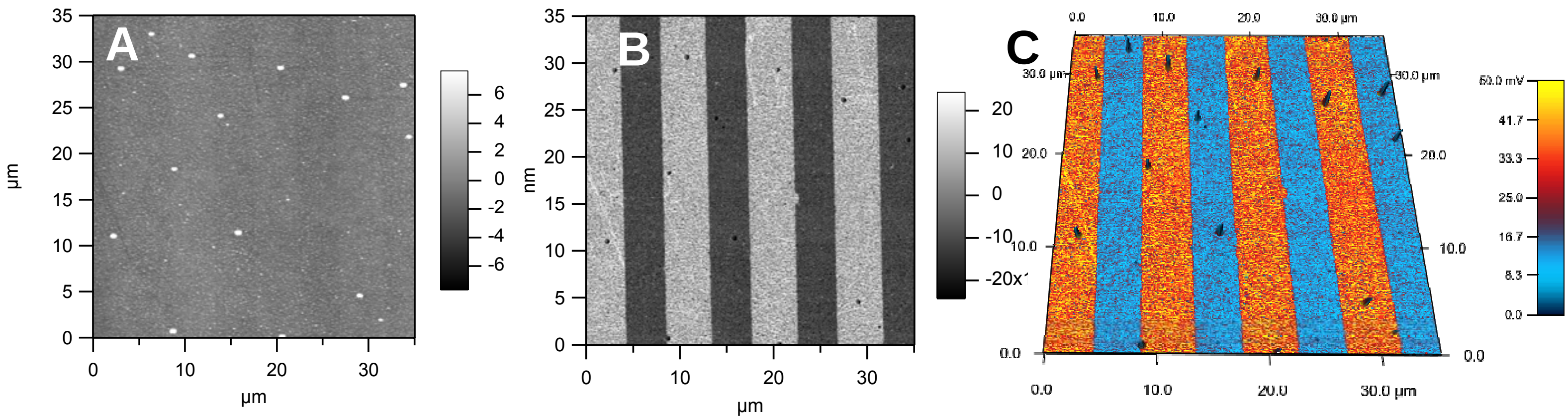


Figure 8.6: A) Contact mode Height image of µCP pattern of –S(CH2)10CH3 and –S(CH2)10COOH; B: LFM image – bright areas of image represent –COOH terminus; C) ARgyle 4D rendering of lateral channel painted onto Height channel.

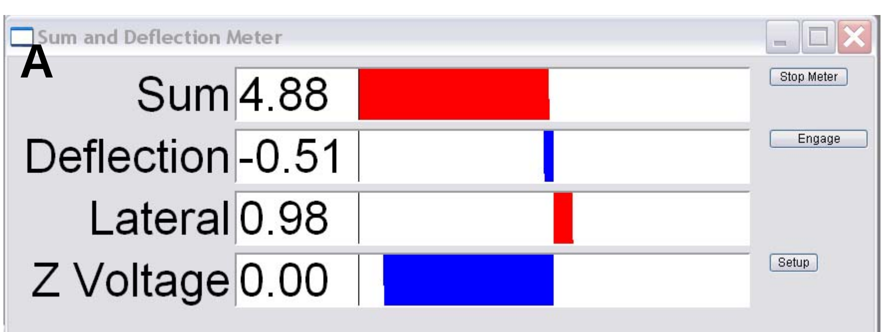
Electronic ZEROING LATERAL SIGNAL:

A standard MFP-3D head does not have a mechanical means to zero the lateral deflection on the PhotoDiode, for reasons not to be discussed herein. There is an electronic way to zero the deflection- it's an igor procedure file (.ipf) called LFM Rocks- contact support for this file.

✓ Load the LFM Rocks ipf- goto File → Open File → Procedure; load LFM rocks from wherever it's saved.

✓ Compile the .ipf by clicking the compile button at the lower left of the .ipf window. Once compiled, this button will no longer be visible. To learn more about compiling Igor procedure files, read the Igor manual.

✓ To zero the lateral signal, go to to Macros→ ZeroLateral; (Figure 8.7B) the lateral signal will electronically be zeroed.



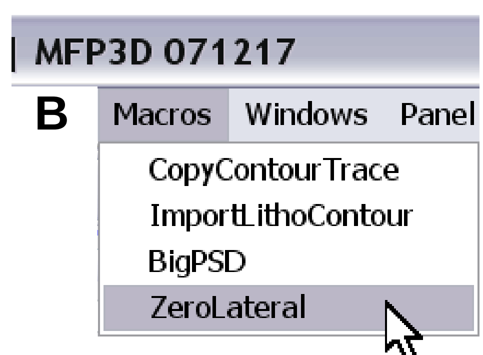
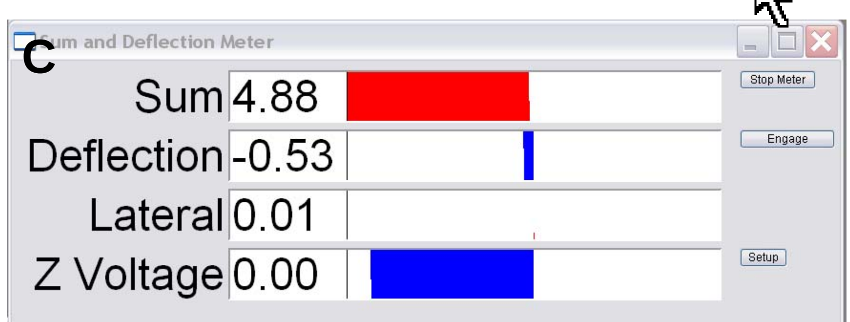


Figure 8.7: Zeroing lateral deflection with LFM Rock ipf: A) typical lateral signal from a given cantilever (see Section 7.7C to view lateral signal): B) with LFM rocks compiled, select ZeroLateral from Macros in menu bar; C) result- electronic zeroing of lateral signal.



ARUP (User Panel) LFM friction loop-

▪ You may have noticed in the AR user panel menu that there is something called LFM Friction Loop. In most cases, this isn't going to be useful to the average or even advanced MFP-3D user- the reason being that unless you have one of the High Bandwidth Heads, and you have the mechanical lateral zeroing head (another option seldom chosen), the BW on the lateral signal is something silly like 50kHz- this friction loop signal is more or less going to be noise.

This user panel was designed by our programmers as an attempt to help a customer (whom has the high BW head with mechanical lateral zeroing) to figure out the lateral InvOLS from the turnaround area at the end of the scan line.

The author mentions it so you don't waste your time with it-

9. Spring Constant Determination

9.1: Thermal Method (for determining Spring Constant)

The determination of spring constant, k, is a quick 3-step procedure:

❶ Determine the 'Virtual Deflection' of the optical path.

❷ Determine the slope of contact region from a force curve to determine the 'inverse optical lever sensitivity' of the cantilever (in nm/V).

❸ Withdraw tip & perform a thermal tune to determine the cantilever's resonant frequency. An algorithm computes the spring constant using the equi-partition theorem.

This protocol can also be found in the ***Spring Constant Tutor:*** *to view it in the MFP-3D™software, go to Programming → Start User Panel → SpringConstantTutor. It reads remarkably similar to this section, just more eloquently. It even has an all in one panel set up that contains everything you need to do to complete the k determination, see Figure 9.9.*

A simplified technical note on spring constant calibration via the thermal method entitled Spring Constant Calibration by Roger Proksch, PhD. can be found at the end of this chapter.

NOTE: If at all possible, it is best to do the spring constant calibration in air due to the higher cantilever Q (relative to the generally low cantilever Q in fluid).

STEP 1: Determine Virtual Deflection in Optical Path:

The virtual deflection is a mechanical coupling of the deflection signal with the Z movement, a result of the mechanical path not being quite perfect, resulting in a slight slope in the force curve. *Although this may only be a few nm's over several microns, if not corrected, it can skew the accuracy in measurement / analysis of the force to be determined.* The exact origin of it is still not fully understood, but it depends on how the light is aligned on the lever as it travels through the piezo range. By performing this step first, it will give you a more precise estimate of the cantilever spring constant.

▪ The virtual deflection correction essentially 'levels' the free air part of the force-distance curve (i.e. constant deflection). This in return aids force curve analysis because the free air baseline will have a constant deflection value until it makes contact or some other force is imparted upon the tip. In many dissociation based force measurements, analysis generally averages some section of the free air to find the zero contact point of the retract portion of the curve; having a baseline with a constant deflection value makes this more precise.

▪ Before performing the first step, put the tip on the surface by engaging in CONTACT MODE on a *clean, (infinitely) hard* surface* (i.e., freshly cleaved mica, clean glass slide, cleaved graphite). Avoid bumpy surfaces such as an evaporated metal film like Au on glass, or dirty sample, because the tip may experience some slipping on the surface if on an asperity.
**An infinitely hard surface will have a slope of unity in a distance vs indentation plot- See Section 11.2.*

A more detailed description of Contact Mode can be found in Section 8, but in brief:

✓Select 'Contact' from the Imaging Mode pull-down menu on the *Main* Tab of the Master panel.

✓Select a Set Point voltage (force): low Set Point voltage (force) is slightly more positive (in volts) than the value of the 'Free Air' Deflection (in Volts) in the S&D meter; higher Set Point voltage (force) are much more positive than the free air deflection voltage value. *Low set point forces avoid truncating the tip's apex. Refer to Figures 8.3. & 8.4.*

✓Integral gain = 7 to 10 is usually sufficient.

✓Click the '**Engage**' button on the S&D meter.

✓Engage tip by slowly turning the thumbwheel (CCW) until the Deflection voltage matches the Set Point voltage; a bell sound will chime if speaker volume available; with continual approach with the thumbwheel, the *z*-voltage value goes from railed (150 V, *red*), to some other lower voltage value; adjust this down to around 70 V (no color in z-voltage meter).

NOTE: if you need to engage as gently as possible, it's best to do a false engagement in AC mode first, increase the Set Point force until the tip hard engages at the minimum possible force; then switch over to Contact mode once engaged. See Section 6.1.2 for this gentle engagement procedure.

✓ Click on the *Force* Tab of the Master Panel (Figure 9.1): This panel is set up so the user can define the specific distance the piezo travels during the force-distance cycle (called 'Force Distance').

For a more detailed description of the Force Tab, see Section 11 (Force Spectroscopy)-

▪ The white bar on the left side of the window represents the entire Z range of the *z*- piezo in the AFM head. The red bar represents the 'Force distance'- a user defined approach/ retract cycle distance that the *Z*-piezo moves during the force-distance curve acquisition. As you change the value of the force distance, notice that the size of the red bar's length changes. This can also be activated with the Hamster wheel, or by clicking on the red bar and dragging the mouse.

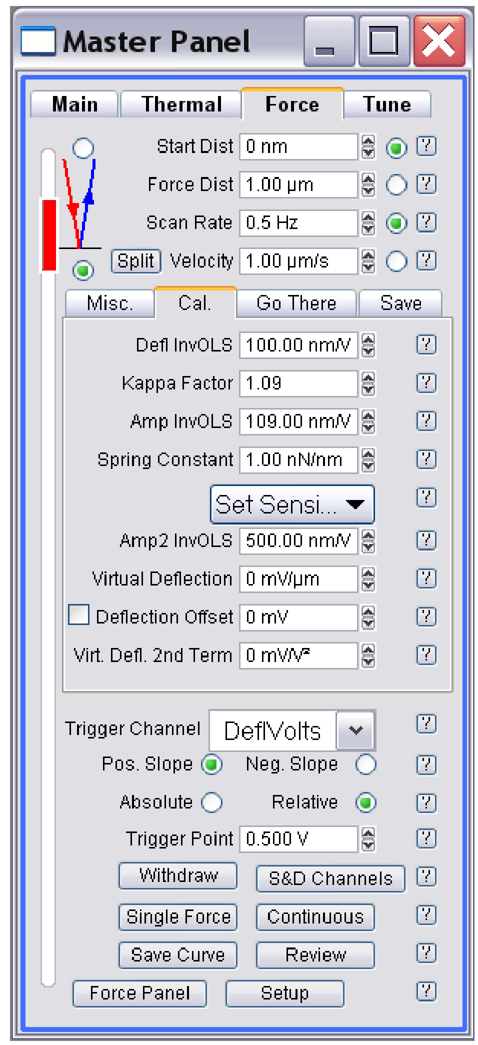


Figure 9.1: Force tab of the master panel with calibration sub-tab shown.

***Initial Parameter adjustment**- set parameters similar to Figure 9.1.*

✓Activate the radio button for 'red' mode (force distance bar is red)- *this means the tip starts the approach/ retract cycle off the surface .*

✓Slide the red force distance bar all the way to the top of the white vertical Z range bar.

✓Choose a 'Force' distance (0.5 to 1 μm).

✓Velocity of 1 μm/s.

✓Activate the 'Trigger' from the Trigger channel pull-down menu- choose the 'DeflVolts' channel.

▪ The ***trigger point*** value is the amount of positive deflection (or force, or Z distance) the tip experiences in contact with the surface before the piezo switches direction for the retraction part of the curve.

✓Activate the 'relative' trigger radio button.

✓Enter a 'trigger' point value of 0.4 to 0.6 V. *Most other parameters in Figure 9.1 are not important at this point.*

✓Click 'Single Force' button; Notice the Z piezo voltage extends the tip to approach the surface, reverse direction when it finds the surface, then retracts and eventually stops at a voltage value that correlates with the user defined Force Distance.

▪ A Force – distance curve should look similar to one in Figure 9.2A; Notice *the very long approach (red) curve-this is because the piezo was traveling toward the surface waiting to reach a force equal to the trigger point selected.* This long free air is what's needed for Virtual deflection correction so the linear region of it can be fit.

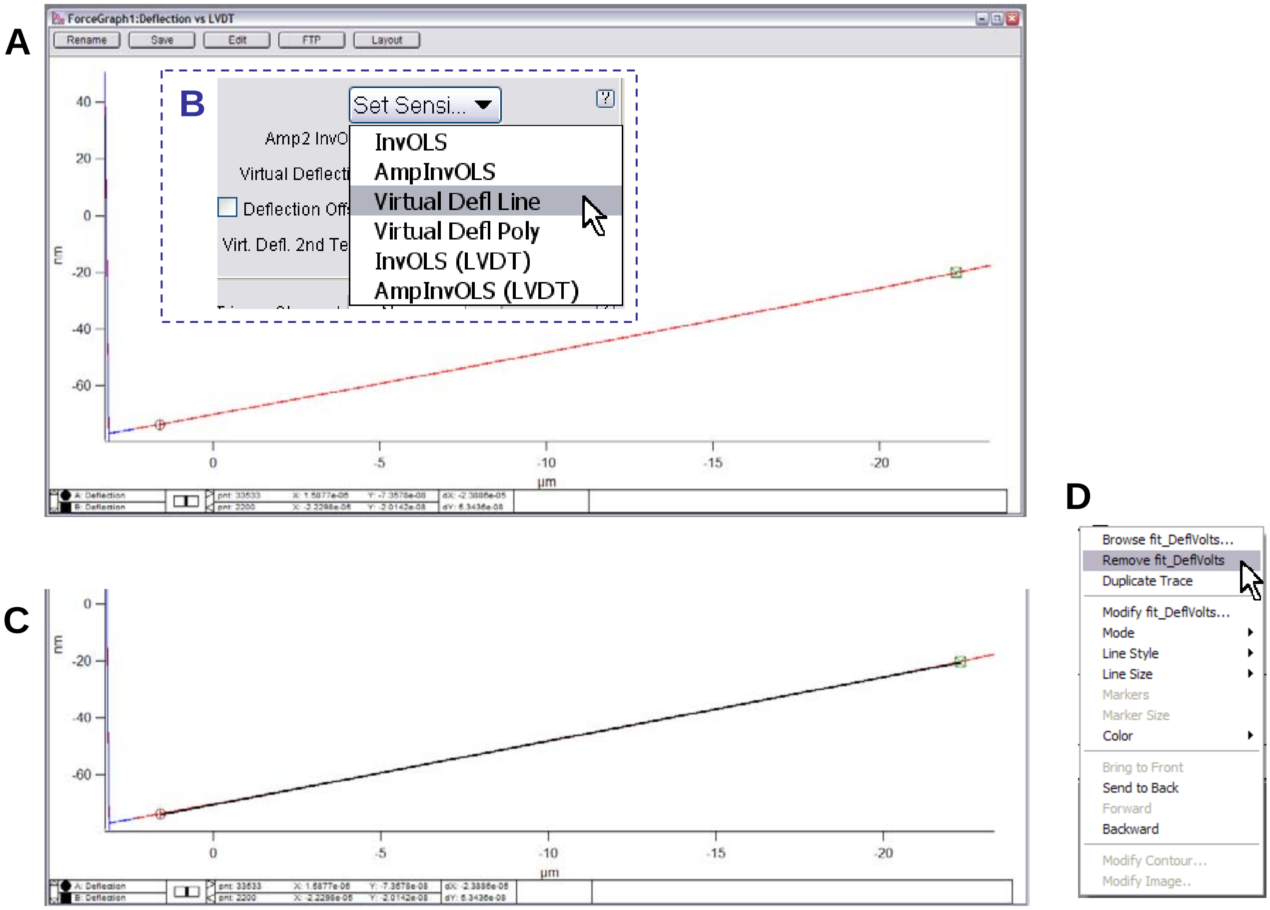


Figure 9.2: Calibrating the virtual deflection: A) initial force curve has long approach curve (red); place the Igor cursors on linear region of free air part of the approach curve; B) select Virtual Defl from Set Sens pull down in Cal sub-tab of Force tab in master panel; C) resulting linear curve fit; D) removing fit trace from force curve

✓ With the force plot window active, hit Ctrl + i which brings up a panel on the bottom of the plot that has Igor cursors (A:⊗, B:☒; with their respective *X*-, *Y*-, dX & dY values). *The author calls these Igor Cursors because they can be called up on any Igor Pro plot.*

✓Place the Igor cursors on linear part of free air approach (red) curve (Figure 9.2A). The object of this exercise is to fit a large Z distance (several microns) to make the virtual calibration more accurate.

✓Make sure both cursors are on the same (approach/ retract) trace by using the keyboard arrow keys to assure they both move in the same direction. If they move away from (or towards each) other, they are not on the same trace.

✓ Go to 'Set Sens' pull-down menu in Cal Sub-Tab in the Force tab (Figure 9.2B, inset)- select 'Virtual Defl Line'.

✓ The portion of the curve between the Igor cursors gets fit, represented with black line (Figure 9.2C); Also notice the virtual deflection value has been updated in the calibration sub tab.

✓ To remove this line fit, place the mouse cursor (+) on the black fit line, right click; choose 'Remove fit_DeflVotls' (Figure 9.2D). Removal of this curve allows you to perform another force curve for the next step; if you don't remove the fit, then the updated virtual deflection parameter will have the next curve off scale and you won't see the new curve in the plot window.
Alternatively, just close the Force graph window- a new one will open open then next curve acquistion and will be properly rescaled.

▪ The virtual deflection has now been corrected so the free air now looks 'level' in subsequent force curves (i.e., has constant deflection), relative to the signal to noise ratio of the curve.

ALTERNATIVELY, you can calibrate the virtual deflection with the tip far off the surface such that the full range of the piezo is taken into account.
See images on thumbdrive that do this.

If you are using a entended Z range head (*older*- 28 µm or *current*- 40 µm), this part of the curve may not appear linear; in this case you should select 'Virtual Defl Poly' instead to account for the curvature of the approach curve. *An example of this can be seen in Figure 9.11 at end of chapter.*

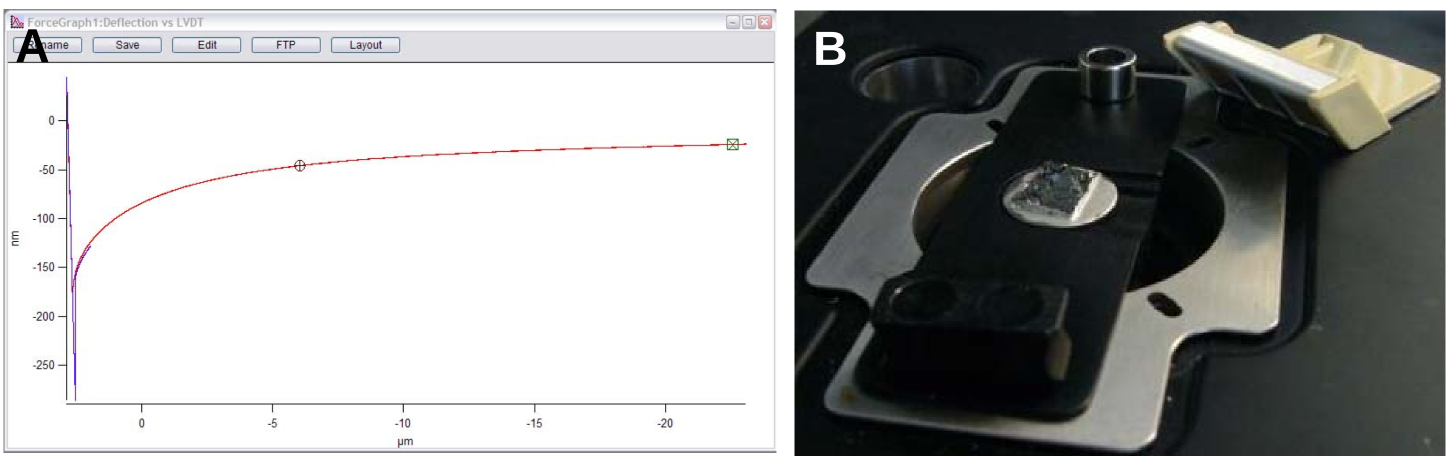


Figure 9.3: trying to eliminate charge by removing any glass components in sample mounting. Here, the sample (graphite) is fixed to sample puck via silver paint, and placed on an AR magnetic sample holder.

SURFACE CHARGE CAN ALSO AFFECT YOUR FORCE CURVE AQUISTION-
Occasionally, the flat substrate chosen can be subject to surface charges, causing some crazy non-linear deflections in the free air and near point of contact when using very low spring constant cantilevers. The author has found this to be true of 0.03 N/m and below levers using clean glass, or mica glued to a glass slide. For example, glass is notorious for having a surface charge, and mica's negative charge can cause a thicker than normal inherent water layer to accumulate. Figure 9.3 shows an example of surface charging affecting the force distance curve: notice the non -linearity in the approach and retract parts of the curve- this is caused by the long range forces of the surface charge pulling the tip towards the surface- makes it tough to get a proper virtual deflection curve fit. To solve this, it's a good idea to have a atomically flat material that doesn't possess strong surface charge- Figure 9.3B shows a piece of graphite adhered to a magnetic sample puck on an AR magnetic puck holder. Sometimes you can't escape the surface charge though…

STEP 2: Determine the cantilever InvOLS:

▪ The objective of the second step is to measure the slope of the contact region (called the 'inverse optical lever sensitivity'; or *InvOLS*), a parameter necessary for the algorithm to determine the spring constant. Figure 9.4 gives a sense of what the optical lever sensitivity is, given in units of V/nm; the software inverts it for the algorithm. Notice this is a similar curve as in Figure 8.3.

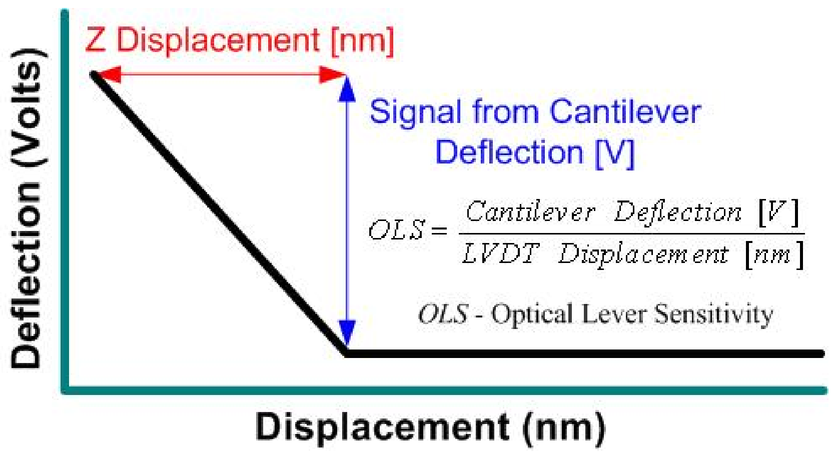


Figure 9.4: qualitative description of calculating the Optical Lever Sensitivity

✓Click the 'Single Force' button again. This time the force distance will be equal in both approach and retract cycle because the instrument now knows where the surface is. *Alternatively, you can do this step on the contact region of the initial curve (Figure 9.2) by expanding on the contact regime area after correcting the virtual deflection.*

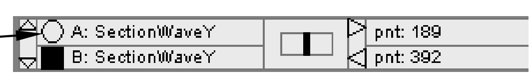
▪ You should have a force curve that looks similar to the one in Figure 9.5A. *Notice the free air part of this curve has constant deflection value.* It's time to determine the InvOLS (*inverse* optical lever sensitivity) of the cantilever from the slope of the contact (repulsive) region of the force-distance curve.

✓ Expand on the contact region of the curve (Figure 9.5B).

✓Place the Igor cursors on the either extension or retract part of the contact regime; use arrow keys to confirm cursors are on same curve (Figure 9.5B)- they will move in the same direction if they are. If not, correct this.

Deactivate one of the cursors on the curve by going into the lower margin of that Igor window and clicking on the open circle/square. When it's deactivated, it will turn black allowing you to finely position the other cursors on the curve. When done, reactivate and check that both cursors are on same trace.

De-activated Igor cursor



▪ In the *Force* tab, click on 'Deflection' in the 'Set Sens' pull-down menu (Figure 9.6C). The 'Defl InvOLS' (deflection inverse optical lever sensitivity) value will automatically be updated in the Force tab, and connects the two cursors (⊗,☒) in the deflection *vs.* LVDT plot with a thick black fit line.

It's a good idea to record this InvOLS in your notebook. (Amp InvOLS is Defl InvOLS times the Kappa factor).

A

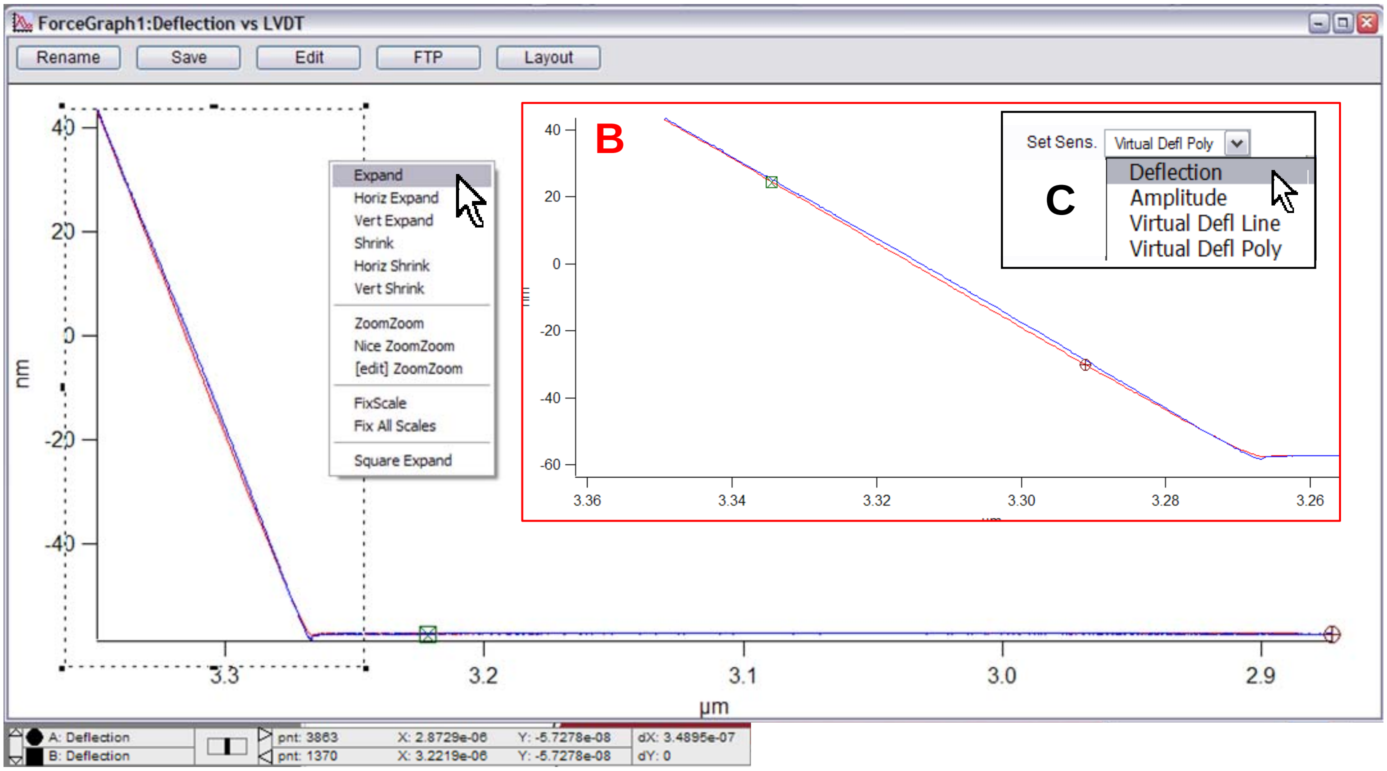
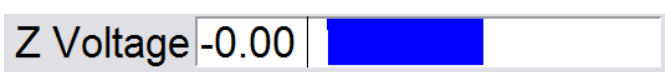


Figure 9.5: Determining the cantilever's InvOLS : A) Force curve on a hard surface; B) expand on contact region, place Igor cursors on retract curve; C) select 'Deflection' from SetSens pull down menu in Calibration sub-tab in the Force tab.

▪ *Amplitude should be selected if doing force distance curve in AC mode.*

NOTE: If planning to work in fluid after determining the InvOLS in air, you must re calibrate the InvOLS of the lever to be accurate- it will change slightly due to the fluid medium.

✓ Disengage the tip by clicking the 'Stop!!!' button on the bottom of the 'Main' tab of the master panel, *OR* by clicking the 'Stop' button to the right of the deflection meter on the Sum and Deflection Meter window, *OR* by clicking the 'Withdraw' button in the *Force* tab. All do the same thing: the S&D meter should show a piezo voltage of 0.0 V in its 'withdrawn' position.



'Averaging' InvOLS: For more precise measurements of the InvOLS (because they can be slightly different from curve to curve), statistical representation can easily be performed using the MFP-3D.

✓ In the *Force* Tab of the Master Panel, click the 'Set Up' button towards the bottom of the panel-

✓ Check the 'Limit Cont. to:' Show? ☑ checkbox; this will allow a user defined number of force curves to be sequentially acquired at the same XY location, without clicking the 'Single Force' button repeatedly.

✓ Click the 'Looks Good' button to clean up the panel.

✓ Enter a value for how many force curves to be acquired in one spot. For the example shown in Figure 9.7, the author chose 100.

✓ Click the 'Continuous' button to acquire the force curves.

Once the curves have been collected- (also described in Chapter 11).

✓ In the Master Force Panel, load the curves that you want to average the DC InvOLS on. Take note of the suffices of these curves because they will have to be designated in the Analysis tab (Figure 9.6B).

✓ Confirm that only the 'Ext' ☑ checkbox is activated, indicating that you only want to look at the extension part of the force curve.

✓ Plot the Force cursors as LVDT (X) versus Deflection Volts- typically that is what is done to calibrate the spring constant since the force constant of the cantilever isn't known (Figure 9.6A).

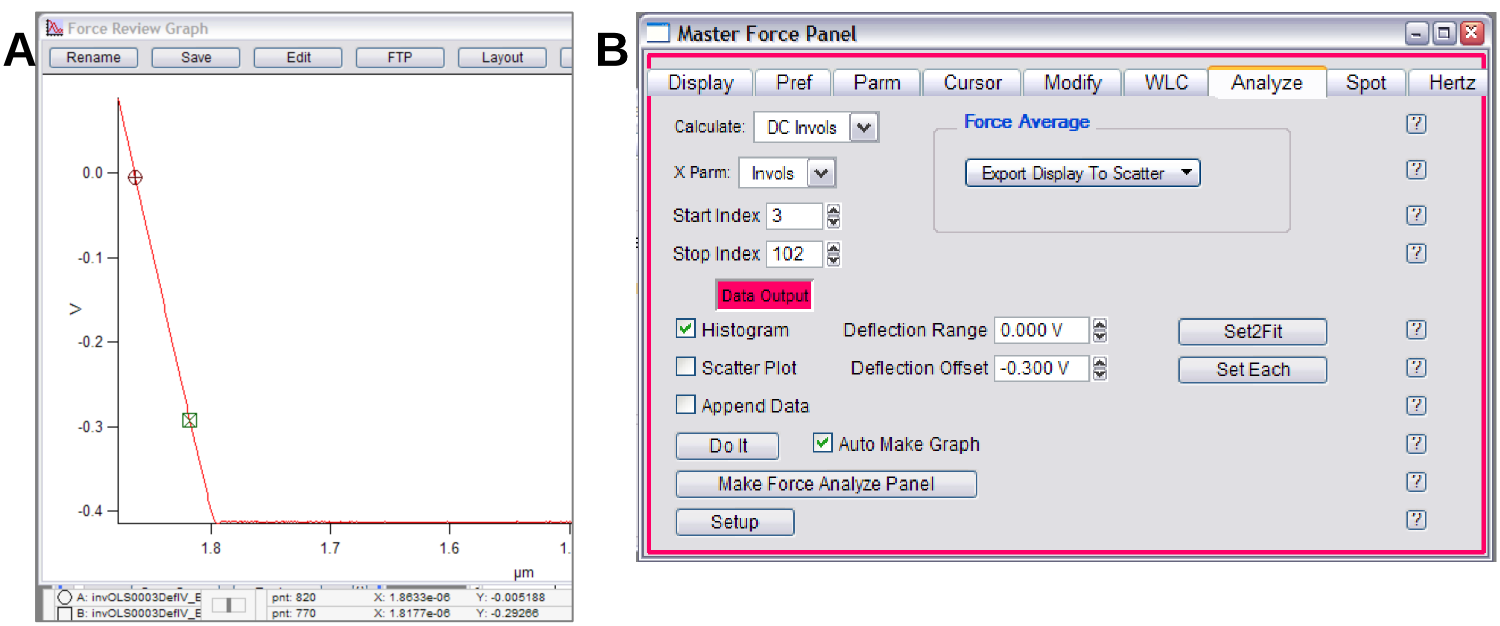


Figure 9.6: Averaging InvOLS to display as histogram distribution: A) determine Deflection range on extension part of one of the curves to be averaged; B) enter these range values into the deflection range and Offset setvars in the Analysis tab of the Master Force Panel. Set up Analysis tab similar to above for averaging InvOLS.

✓ Install the Igor Crtl + i curves on the contact regime part of the curve. Take note of the Y axis values- these will also be used in the Analysis tab to designate the 'Deflection Range' during the averaging (Figure 9.6A).

✓ Go to the Analysis tab of the Master Force Channel.

✓ Under the 'Calculate' pull-down, select DC InvOLS (i.e., InvOLS in contact mode, select AC InvOLS if you are doing Dynamic Force Spectroscopy).

✓ Under the 'X Parm' pull-down, select 'InvOLS' (InvOLS is what you want the Histogram to have on its X axis).

✓ Enter the appropriate suffix range in the 'Start' and 'Stop' Indices.

✓ Under the 'Deflection Range' setvar, enter the value of the Igor cursor closer to the trigger point (Figure 9.6A).

✓ Under the 'Deflection Offest' setvar, enter the value of the other Igor cursor (Figure 9.6A).

✓ Check the 'Histogram' ☑ Check box-

✓ Click the 'Do It' button- a histogram should be generated that will show a distribution of InvOLS values (Figure 9.7).

▪ In Figure 9.7, a Gaussian fit was applied, although other fit types are available in the 'Fit Type' pull-down. Notice the Mean and Width of the histogram fit is display, and that the Bin size can be changed using the slider bar.

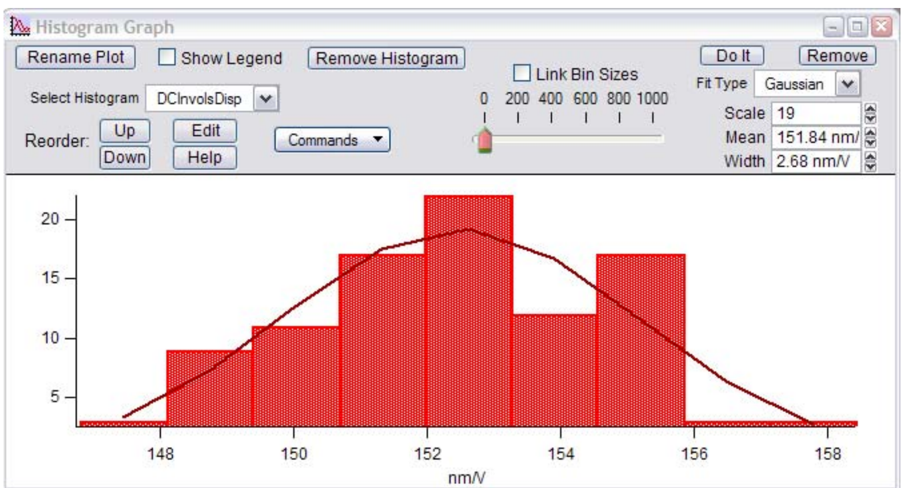


Figure 9.7: Histogram of InvOLS values. The fit type pull down allows different fitting functions to be applied- for an example, a Gaussian fit was applied (*not that the histogram looks Gaussian*).

STEP 3: Perform a Thermal tune:

✓Click on the *Thermal* tab on the Master Panel (Figure 9.8). *To be very precise, retract head back a hundred microns or so (with the thumbwheel) to eliminate the possibility of the cantilever experiencing any long range forces.*

✓Adjust the deflection (PD) on the AFM head so it reads ~0V deflection in the S&D meter, if needed.

✓Click the 'Do Thermal' button at bottom of panel. This detects the natural resonant frequency of the cantilever by doing an iterative series of frequency sweeps and averaging them. A real-time amplitude *vs.* frequency (called a power spectrum) plot is generated (similar to Figure 9.9A). Let it collect many cycles (the more you collect, the more it filters out noise).

▪ *How fast the Thermal tune proceeds is dependent on the resolution value: 3 is very good, but slow; 7 is efficient but produces a noisier plot.*

▪ For a thermal power spectrum in fluid, see Figure 9.10A at the end of this section.

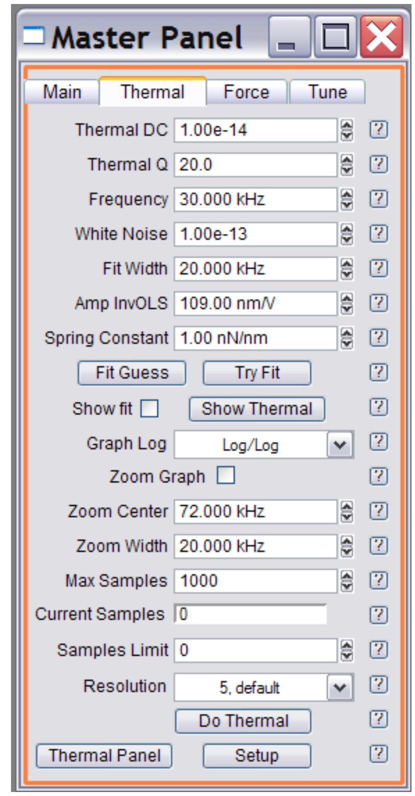


Figure 9.8: Thermal Tab.

✓ Hit 'Stop Thermal' to cease sampling when plot looks like it has definition enough to fit a curve to it. On the power spectrum plot (Figure 9.9A), expand the first large peak (red dash line). Do this by holding down the mouse button and creating rectangle around peak, then right or left clicking the mouse button; select 'Expand' to give a plot similar to Figure 9.9C.

✓ Hit Ctrl + i to get the Igor cursors (⊗,☒ at bottom); place one on the peak of the first resonant peak. (Figure 9.9C).

✓Type the value from the cursor (X value, which is in Hz) into 'Zoom Center' parameter (in kHz) on the thermal tab (Figure 9.9C). If working in fluid, the peaks are much broader (see Figure 9.10A), and their resonant frequencies shift to about ½ to $\frac{1}{3}$ the 'in air' values.

In Igor, Ctrl + A will get you back to full spectrum in any expanded Igor window / image. For example, this would get you from Figure 9.9C to 9.9A.

✓Click on the 'Show Fit ☑' checkbox in the *Thermal* tab.

✓Click the 'Fit Guess' button (this brings up a blue Gaussian shaped curve very near to the thermal resonant peak); (Figure 9.9D).

✓Click on the 'Show Fit ☑' checkbox in the *Thermal* tab.

✓Click the 'Fit Guess' button (this brings up a blue Gaussian shaped curve very near to the thermal resonant peak); (Figure 9.9D).

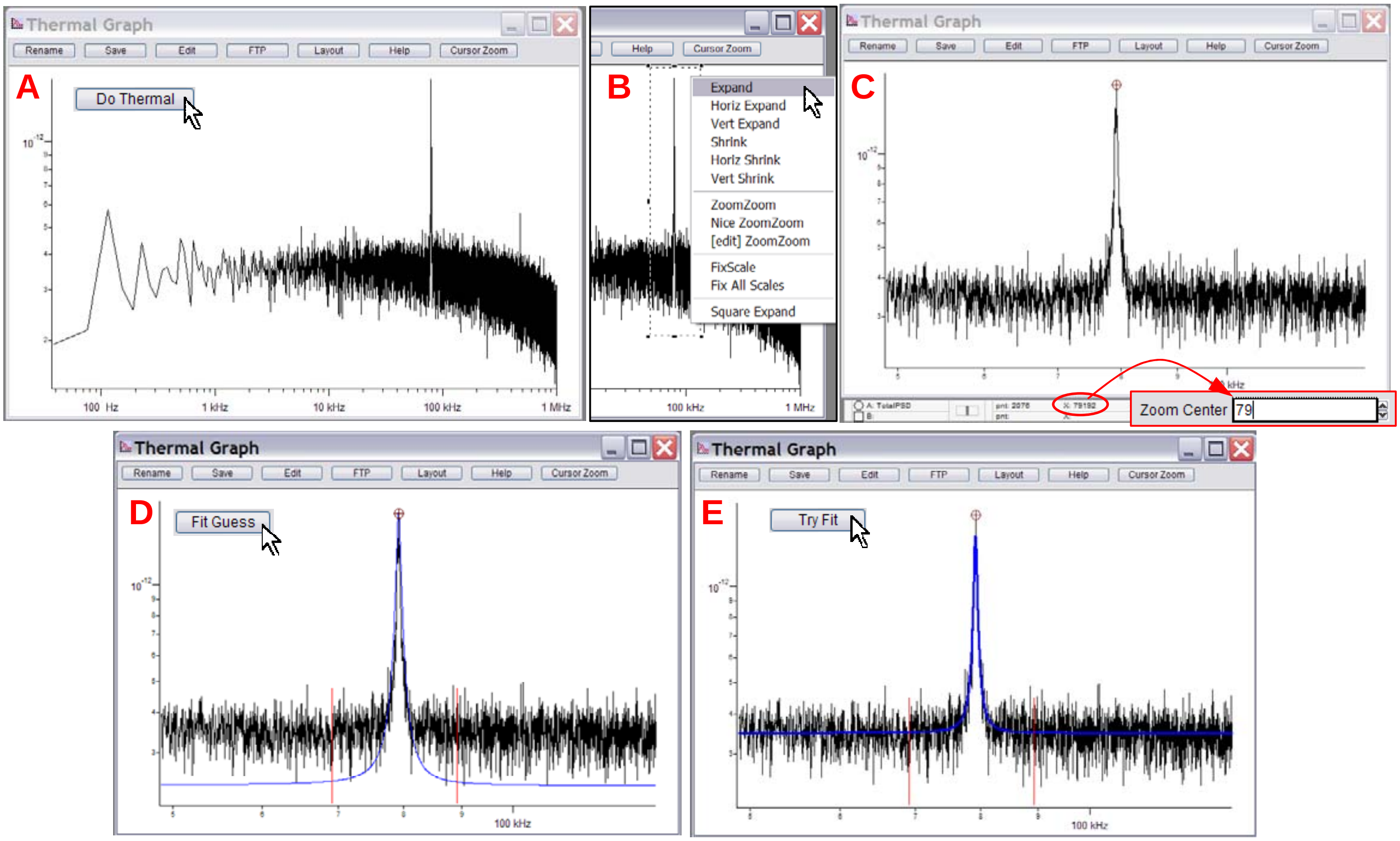


Figure 9.9: The thermal tune: A) Power spectrum after clicking 'Do Thermal' button in Thermal Tab ;B) Expanding on fundamental resonant peak; C) Zoomed peak, bring up Igor cursors, transfer X value (in Hz) to Zoom center parameter in thermal tab (in kHZ); D) After clicking 'Fit Guess' button; E) After clicking 'Try Fit' button, completing spring constant determination.

✓Click on the 'Try Fit' button- a blue curve will fit to this resonant peak (Figure 9.9B). The software automatically calculates the spring constant and updates the resonant frequency, seen in Thermal and Main tab. (Figure 9.9E).

▪ At this point the software has determined the cantilever's spring constant by the equi-partition method. This is very useful when performing force distance curves, or needing to know how much force the Set Point is being applied by the tip. This value will be store in any saved data parameter file.

9.2: Misc. Operations:

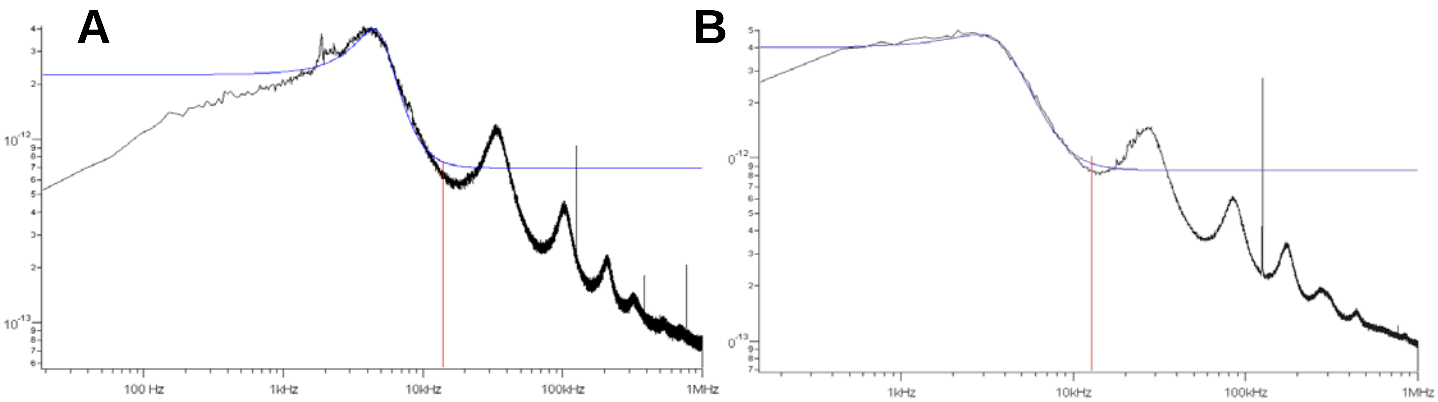


Figure 9.10: Thermal tunes of contact mode cantilevers *IN FLUID*. A) Typical plot of a 0.03N/m triangle cantilever. B) One that has fundamental peak dampened (*see note below*): Program has difficulty fitting to dampened fundamental peak.

▪ Sometimes when using a floppy cantilever *IN FLUID*, the author has found that there is a dampening effect that causes difficulty computing this peak position (see Figure 9.7B below). There are ways around this: 1) take the frequency spectrum before engaging/ taking force curve; perform a try fit once you have the InvOLS value (there will be some greater error in *k* determination with this approach). 2) Disengage the tip and manually retract of the tip a turn or two of the thumbwheel (20 μm to 40 μm) before taking the frequency spectrum. This seems to work much better, with a low number of iterations.

As mentioned in Step 1, an MFP-3D™ equipped with an extended head will have an initial force curve with a non- linear free air approach. Figure 9.11 below gives an example of this, as seen with a 28 µm extended head. This is when 'Virtual Defl Poly' would be selected for this calibration.

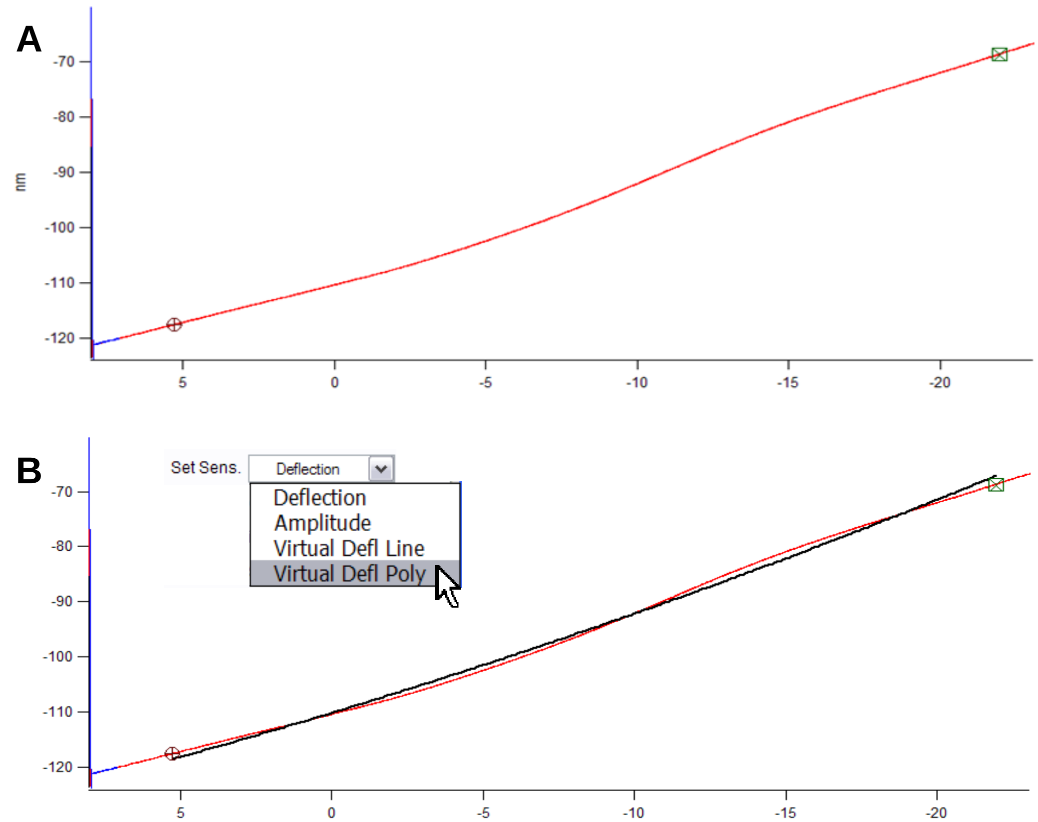


Figure 9.11: Virtual Deflection found in extended heads. A) Force distance curve after first force curve (as described in Step 1); B) after free air portion of approach curve is fit with polynomial.

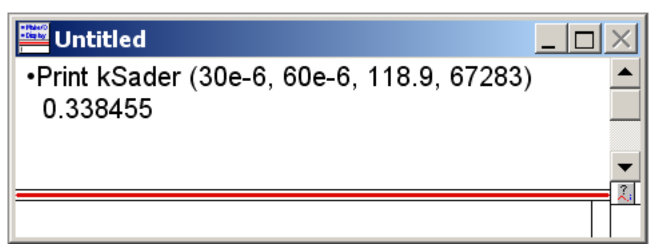
9.3: Sader method:

The MFP-3D software also has the option of using the Sader method to determine the spring constant. This technique uses the dimensions of the cantilever (in meters), the Q and frequency (Hz) to back out a k value (N/m).

✓ In the command line type: Print kSader(w,l,Q,f)

Where w is the width, l is the length, Q is the q from the thermal tune and f is the frequency.

Here's an example-
Print kSader (30e-6, 60e-6, 118.9, 67283)
⇨ *k*=0.338N/m



For more on spring constant determination, the following references may be helpful-

J. E. Sader, J. W. M. Chon, and P. Mulvaney, Rev. Sci. Instrum. 199, *70,* 3967
J. P. Cleveland et al., Rev. Sci. Instrum. 1993 *64, 403*

9.4: Spring Constant Tutor-

As mentioned on page 9.1, the Spring Constant Tutor allows all k determination steps to be performed in a single software panel.
✓Go to Programming → Start User Panel → SpringConstantTutor;

▪ This panel is set up where the progression of determining the spring constant goes from top to bottom. This is an example of building a User Panel (*for more on this, see Section 14.14*).

① (Although not described above) Virtual deflection is intended (with Spring Constant Tutor) to be determined when the tip doesn't engage the surface- the entire range of the piezo is fit.

✓ Engage the tip- notice the active Z Piezo voltage meter.

② Determine the InvOLS using a trigger channel and trigger point. Notice the Deflection voltage is displayed b/c this Force curve acquisition is done in Contact mode.

③ Thermal tune: first withdraw the tip, then click 'Do Thermal'; bring up Igor cursors to fit the fundamental resonant peak to complete the spring constant determination.

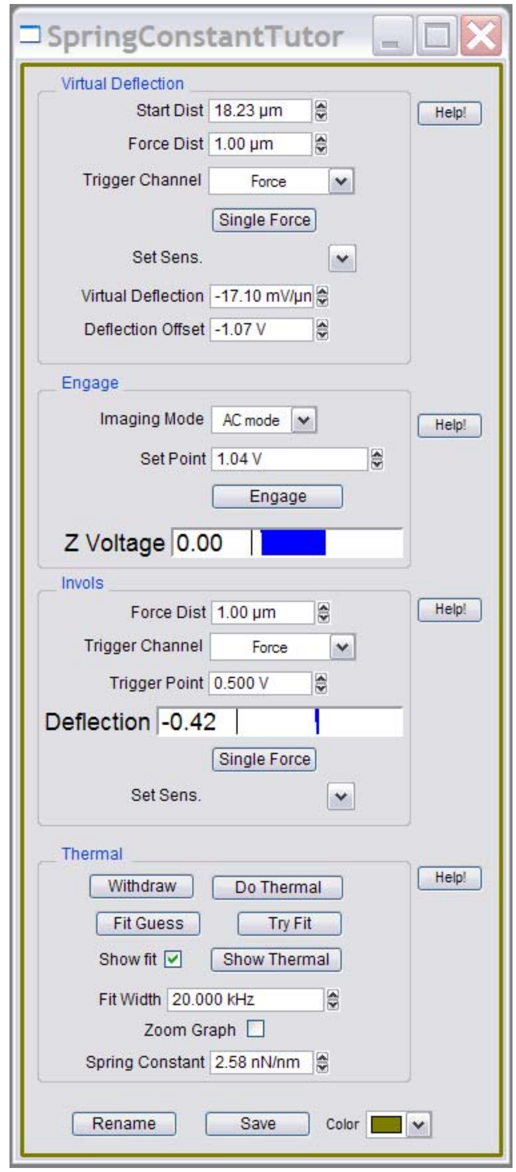


Figure 9.9: Spring Constant Tutor window allows all three steps of the spring constant determination to be performed in one single panel.

Reference Spring Cantilever Calibration Technique with the MFP-3D™ AFM

Roger Proksch, Ph.D., Asylum Research

In this calibration method, the spring constant of the unknown spring is calibrated by pressing it against a very stiff surface and then against a reference spring of known and lesser compliance. The spring constant of the cantilever under test is then calculated using

$$k_{unknown} = k_{std}\left(\frac{InvOLS_{std}}{InvOLS_{unknown}} - 1\right).$$

In the above equation, $InvOLS_{unknown}$ is the inverse Optical lever Sensitivity (with units of nm/Volt) for the cantilever under test measured on a very stiff surface and $InvOLS_{std}$ is the same quantity measured on a compliant surface with spring constant k_{std}.

Typically, we expect uncertainties in this method of 20% or greater. Sources of error include positioning the tip of the cantilever under test at the proper position on the reference lever or spring and surface contamination of the either the test, reference spring or very stiff surface. We are currently involved in a project to compare this calibration method with others and will publish the results once they have been analyzed. For additional information, please see the paper "Finite optical spot size and position corrections in thermal spring calibration", R. Proksch, et al, *Nanotechnology* **15,** 1344-1350.

References

A. Torii et al., *Meas. Sci. and Tech.* **7**, 179 (1996).

To extend this a little further- (Courtesy of Mike Falvo, UNC-CH physics)
To first order, the cantilever can be treated as a simple spring:

The force of the spring is simply F=-*k*x where x is the displacement from equilibrium.
The potential energy of the spring is U= ½ kx^2

The equipartition theorem decrees that every degree of freedom has ½ kBT thermal energy. So the spring (on average as it vibrates) has ½ k_BT energy which will show up as an average max deflection. This is what you measure doing thermal tuning (given InvOLS has been taken such that the spectrum is calibrated).

So the equipartition theorem says **$\frac{1}{2} k\langle x^2\rangle = \frac{1}{2} k_BT$**

Where x is the time averaged deflection determined from thermal tune; T is temperature and k_B is boltzman's constant

Solving for *k* gives :

$$k = k_BT/\langle x^2\rangle$$

10. MicroAngelo™

Lithography and Manipulation software

The MFP-3D™ closed loop system makes it ideal for accurate and complex scanning probe based lithography, and nanometer to micron scale tip manipulations. Typically, an area of the substrate is first imaged non-destructively (in AC mode or gentle contact) to determine what the 'real estate' looks like; then mouse strokes representing the tip's path(s) can be added to the image using the MicroAngelo™ software. Images can be imported and scaled appropriately, set point voltage (or tip bias) ranges defined for the execution, and the scripting performed.

These instructions assume that the MFP-3D™ is set up with desired tip and substrate, and is engaged.

10.1: Introduction to Software Panel:

▪ In the main menu bar of the MFP-3D™ software, open the lithography panel by choosing MFP controls ⇨ Litho Panel. The panel consists of four tabs including MicroAngelo™, Groups, Step & Velocity. See Figure 10.1 below.

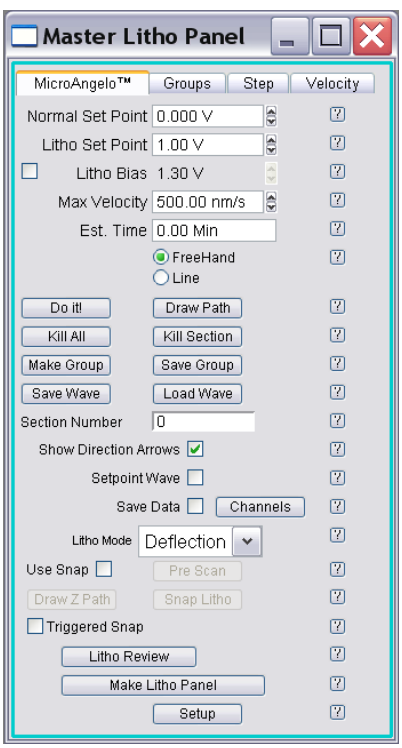


Figure 10.1: MicroAngelo tab of the Litho Panel.

▪ The **MicroAngelo™** tab allows the user to draw lines on the image representing tip paths, determine the set point during the manipulation and the set point between paths (kind of like a retraction set point); make groups of paths; define constant velocity during the path; determine mode (contact or AC); apply constant tip bias (if performing an oxidative or reductive manipulation; other features of this window will be described later in this procedure text.

▪ The **Groups** tab allows the user to import an image, rescale a saved path (or group of paths) that you have previously drawn, or select between saved groups in memory. There are slider bars that allow X& Y rescaling preserving the aspect ratio; offsetting the group in X and/or Y; and group rotation.

▪ The **Step** tab allows the user to make arrays of points by defining number of points, spacing between points, dwell times (& ranges), and tip voltages (& ranges, if applicable).

▪ The **Velocity** tab varies the tip velocity along the path based on the pixel color on an imported image.

The help menus are excellent for the MicroAngelo™ software. To access them, just click on the question marks to the right of every parameter or button in the software panels.

✓ Image the substrate to determine an area suitable for lithography or the manipulation. Depending on your substrate material or application, it may also be a good idea to have the spring constant determined prior to the manipulation, so the user is aware of the amount of force applied at a given Set Point voltage.

10.2: Drawing lines:

10.2.1: Basic operation:

MicroAngelo™ allows the user to draw linear or free hand lines representing tip paths on an image window. Multiple lines can be grouped together, offset, rotated and rescaled if necessary.

✓In the **MicroAngelo™** tab, activate the FreeHand or Line radio button depending on what kind of line you want to draw.

✓ Click on the 'Draw Path' button (see Figure 10.1); Notice the button now reads 'Stop Draw', the free hand and line selections become faint because they can not be switched between each other, and a cross hair cursor (+) will be present when the mouse is on one of the image channel windows.

✓Draw a path on image window with mouse/cursor; if drawing more than one path (with the line tool or free hand tool), left click mouse button at each segment; double click mouse button when finished drawing line segment.

▪ Paths can be grouped together: If you want to save the pattern for future use, Click 'Make Group', then 'Save Group', name it in the dialogue that shows up. Notice this name will now appear in the list in the Groups tab.

▪ If you want to switch between the FreeHand & Line tools, click 'Stop Draw', switch to the other; then click 'Draw Path' again.

▪ Notice the drawn line turns from red to blue once clicking 'Stop Draw'.

✓For scratching based lithography or manipulations, switch the imaging mode to Contact in that Main tab imaging mode pull down menu of the Master panel, or in the 'Litho Mode' pull down in the Litho Panel.

✓Select a 'Normal Set Point' that will not damage the surface while the tip is moving between paths. In contact mode, a deflection value that is the same as the free air deflection (or more negative than that value) won't damage the surface between litho paths.

✓Determine/ select a 'Litho Set Point' value that will apply enough force to perform the desired lithography result (Scratch, oxidative/reductive, diffusion based direct write, etc.), or have enough force to perform the manipulation (without riding over what is intended to be pushed).

✓ Click 'Do It!' to execute the litho event. Notice this button now reads 'Stop Litho'.

▪ The tip will be withdrawn when completed with the lithography/ manipulation execution.

▪ The 'Show Directions Arrow' checkbox ☑ will show the direction the path will traverse, and will become apparent on the image. To adjust the number of arrows on the path pattern, double click on the path and a dialogue comes up allowing you to reduce or increase the number of arrows, similar to how plots are customized in Igor.

▪ Figure 10.2 shows a simple set of lines drawn with MicroAngelo™: one was by free hand, one by line. The two paths were grouped by clicking the 'Make Group', then 'Save Group' buttons- a dialogue came up asking what the group is to be named. The Free Air deflection (from S&D meter) was -0.57V, so I defined the 'Normal Set Point' as -0.5V, while choosing the Litho Set Point as 0.5 V. The velocity was 600 nm/s.

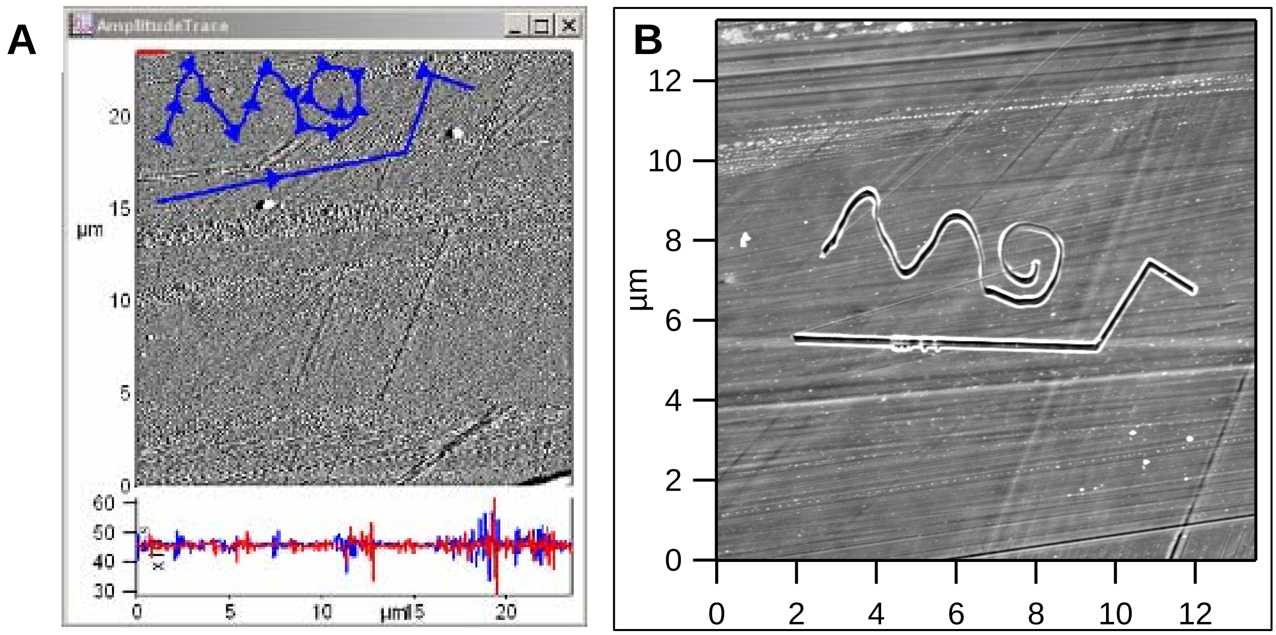


Figure 10.2: A) line and free hand paths drawn & grouped, also showing 'direction arrows'; B) AC mode image of resulting pattern on polycarbonate.

Similarly, this same approach can be applied for sample manipulation via the tip.

▪ If you rescale the image/ scan area to view results, you will probably have to kill the section by clicking 'Kill All' button.

10.2.2: ☑Using the Setpoint Wave checkbox in MicroAngelo™ tab to *systematically vary the Setpoint*

▪ When this is checked, a new dialogue is presented that allows Set Point voltage ranges to be applied along an individual line/ path, in either a linear or staircase waveform. *Note that is not able to distinguish between objects in a group*.

▪An example of scratch lithography using the varied Set Point Wave is demonstrated in Figure 10.4.

✓After imaging the surface in AC mode, the path was drawn by clicking the 'Draw Path' button; then 'Stop Draw' button was clicked (results in Figure 10.3A).

✓ Check the 'Set Point Wave' checkbox ☑, which brings up the window similar to the one shown in Figure 10.3B below. The control parameters at the top of this window allow the user to define a start and ending Set Point voltage, and whether that ramp is stepped linearly, or as a step function.

▪ In the example below, the Set Point range was from 0 V to 7 V with a linear ramp wave. The 'Voltage Step' setvar value is insignificant when using linear waveform ramps- it only applies when using step wave forms.

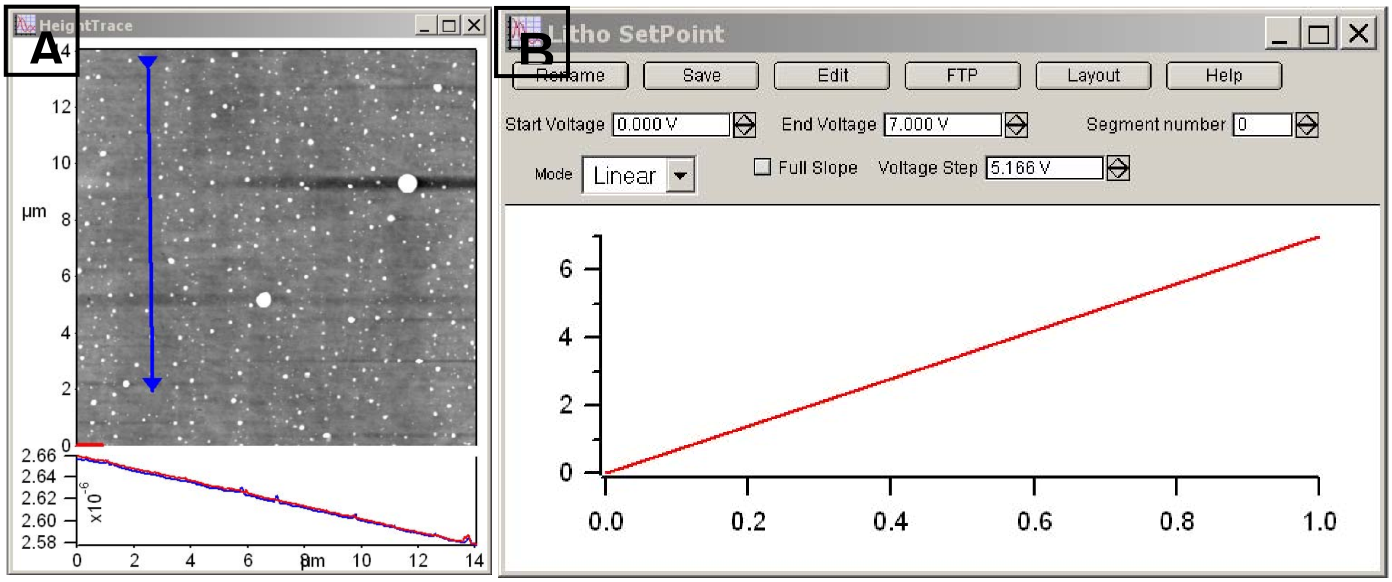


Figure 10.3: A) path drawn on acquired image; B) defining linear ramp after checking the Setpoint Wave box on the MicroAngelo™ tab

✓ Click the 'Do It!' Button to execute the MicroAngelo™ event.

▪Figure 10.4 shows the results of this ramping Set Point (applied force). As expected, with increasing Set Point voltage/ force, the tip plows further into the polycarbonate surface as it traverses the defined path, seen as a feature increasing in width.

▪ The path was defined such the tip would experience increasing torsion as the pattern was traversed, to demonstrate what is described in the next section.

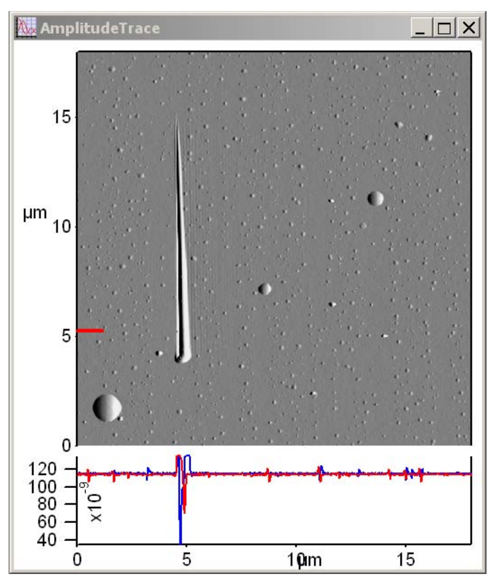


Figure 10.4: Results of Setpoint Wave

10.2.3: ☑ Save Data checkbox allows the deflection, and lateral signals during the event to be viewed. See Figure 10.5B for an example of this.

▪To view the deflection, lateral and time signals during the manipulation event, click the 'Save Data' checkbox **BEFORE** the event (*before* clicking the 'Do It!' Button).

✓ After the event, click the 'Litho Review' button, which will bring up a panel similar to the one seen in Figure 10.5A (below). This panel shows a list of the litho events that were performed in Igor's memory.

✓ Choose the event you want to see the saved data for during the event.

✓ Choose X & Y wave desired from the respective pull down menus.

✓ Click 'Make Graph' button to view the data; a plot similar to the one seen in Figure 10.5B is displayed.

▪ In this case, the lateral data is shown, representing the torsion on the cantilever during the lithographic event shown in Figure 10.4 (above).

Note that the lateral signal during the manipulation is limited by the bandwidth of the ADC used for the lateral signal (~100 kHz). The bandwidth on the deflection signal uses the Fast ADC at 2.5 MHz.

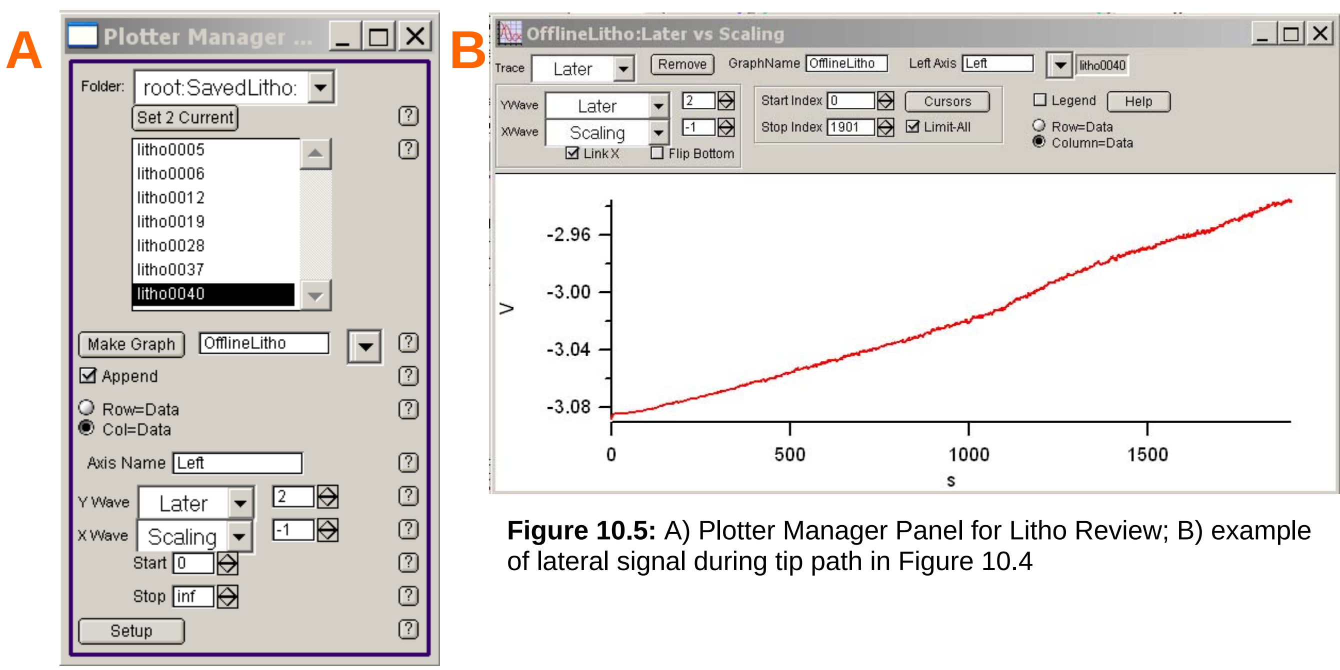


Figure 10.5: A) Plotter Manager Panel for Litho Review; B) example of lateral signal during tip path in Figure 10.4

☑ Use Snap checkbox & 'Prescan' Button- this litho mode is not understood by the author yet. Read the help menu if you want to use it.

10.3: Importing Images as Patterns:

Importing an image to use as a group in MicroAngelo™ is a very straightforward task. Many image files (tiffs, jpeg, Bitmap, etc.), as well as GDS (graphics design system) CAD drawing files can be imported.

✓Open the **Groups** tab (Figure 10.6)

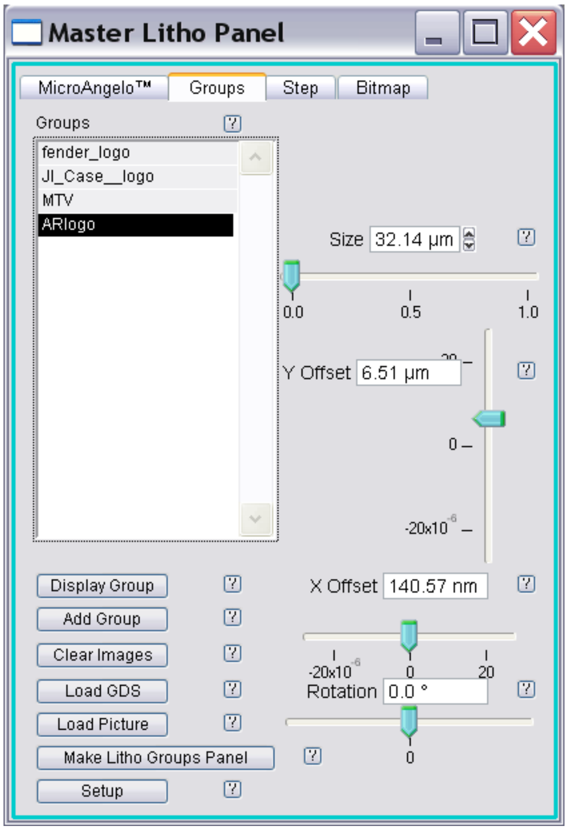


Figure 10.6: *Groups* tab.

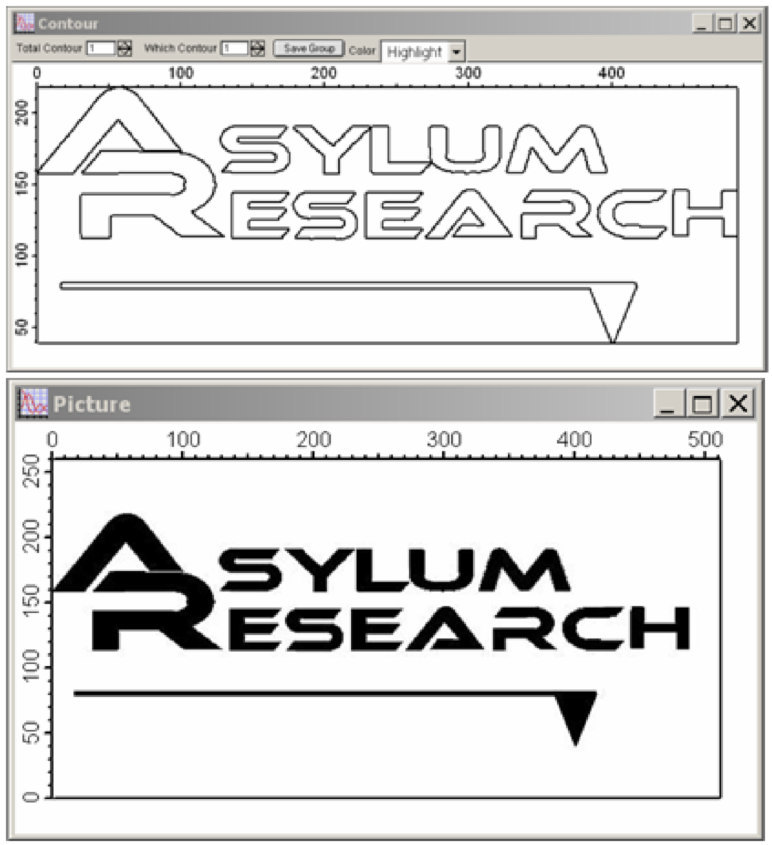


Figure 10.7: Contour & Picture windows.

✓Click on the 'Load Image' button; a browse dialogue comes up letting you select the stored file

▪ The image loads and Igor immediately makes a 'Contour' of the image. Figure 10.7 shows the results of loading the AR logo.

▪ The contour image defines the edges of the imported image as the path the tip will traverse during the manipulation. Notice at the top of the Contour window, there are some parameters you can adjust: 'Total contour' has something to do with edge effects- its kinds of like a threshold in the mask function in the modify panel; 'Which contour' chooses which contours will be included in the group ultimately used as the path; the Color is a pull down menu that will color the different contours. These parameters are discussed further towards the end of this section. For now, this simple image will be contour value '1'. Contour effects are more pronounced in imported images that are grayscale or have color. It's best to try it on your own…

✓ Click the 'Save Group' button to add this group to the list. I happened to call this group 'ARlogo', as seen in Figure 10.6.

✓ Highlight the group of your choice (in this case, the AR logo).

✓ Click the 'Display Group' button- this will display the group (colored red) in the image window (see Figure 10.8 A).

✓ Position the group where you want it in the image area using the X & Y offset slider controls, along with rotation and size slider controls.

✓ Click the 'Add Group' button, which will make the group pattern blue in the image window (Figure 10.8 B).

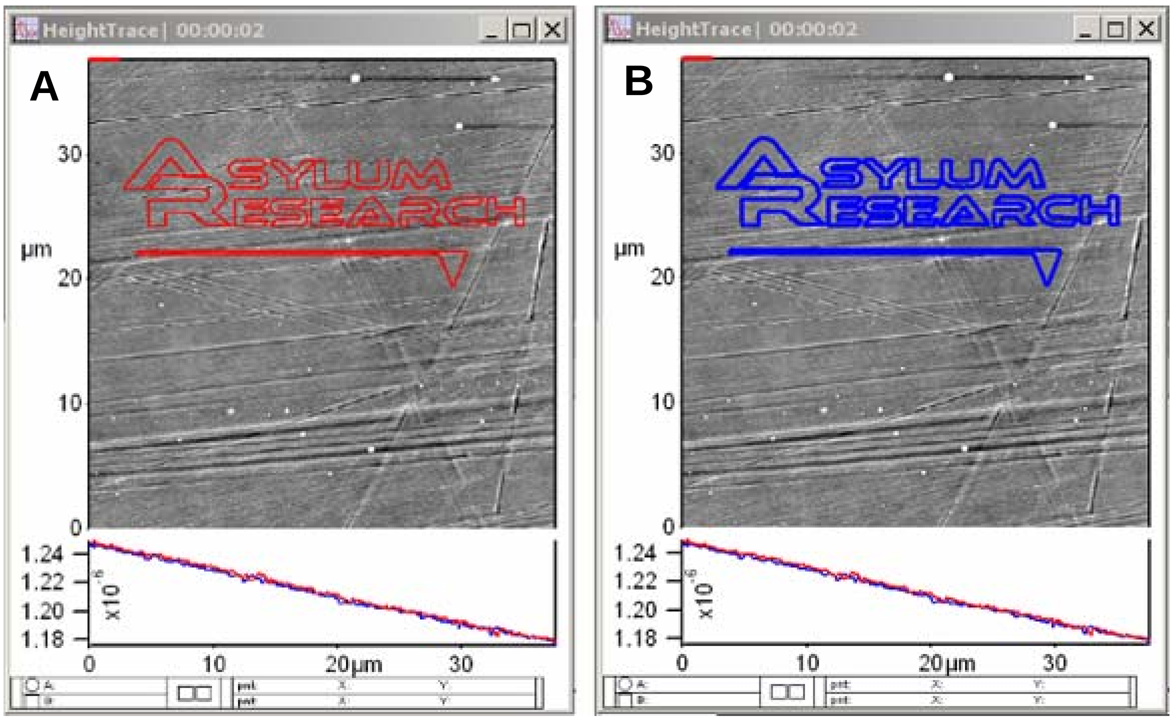


Figure 10.8: A) group displayed, positioned to users preference; B) group after Add Group button activated

✓ *Go back to the MicroAngelo™ tab.*

✓ Define the 'Normal' & 'Litho Setpoint' voltages, and tip velocity. Choose a 'Normal Set Point' voltage value that doesn't damage the surface between paths; Choose a 'Litho set point' value that will induce enough force to scratch the surface; Choose a tip velocity that will be at a rate sufficient enough to do what is intended.

If the manipulation requires an oxidative or reductive potential, click the 'Litho Bias' checkbox, and define an appropriate bias to the tip.

✓ Make sure 'Contact' is selected from the pull-down menu (if that is the desired mode); Incidentally, if you change the imaging mode in the Main tab of the Master panel, it will also be updated here as well.

☑ the 'Show Directions Arrow' checkbox will show the direction the path will traverse, and will become apparent on the image. To adjust the number of arrows on the path pattern, double click on the path and a dialogue comes up allowing you to reduce or increase the number of arrows, similar to how plots are customized in Igor.

✓ When all parameters are defined, click the 'Do It!' Button;
▪ During the manipulation, A red dot that represents the location of the tip, based on the values from the X,Y LVDTs is shown on the screen. You'll also notice the 'Draw Path' button now shows what section of the group path is traversing. If you click this button during the litho procedure, it won't do anything.

▪ At the end of the patterning, the tip will be withdrawn.

▪ In the pattern example shown in Figure 10.8 above, an AC 160 Si cantilever was used to scratch a polycarbonate surface. The resulting scratch can be seen in Figure 10.9 (right):

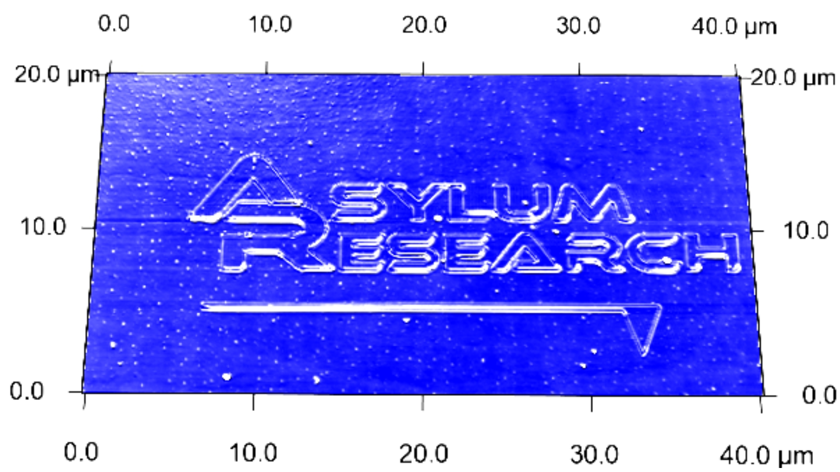


Figure 10.9: AC mode image of resulting patterned from an imported image scratched into a polycarbonate image

10.4: Creating Array Patterns: *The Step Tab*.

▪ Arrays can be created using the **Steps** tab (see Figure 10.10 below); this tab can have the tip dwell at each subsequent array point for a user defined time, or apply a different force. Both functions can be systematically increased, or custom values can be manually entered.

Creating simple arrays-

✓ Choose number of points desired in the array with 'X Count' and 'Y Count' setvar inputs.

✓ Choose the distance desired between points in the array, in both X & Y dimensions using 'X Step' & 'Y Step' setvar inputs.

The 'Time Start' setvar value is the amount of time the tip will be in contact with the surface (dwell) at each point in the array. If it is desired to have the tip have the same dwell at each point, enter 0 s in the 'Time Step' value input.

✓ Click 'Do It' button to fabricate the array pattern. Notice the S&D meter deflection values reaching the defined Set Point during the indents.

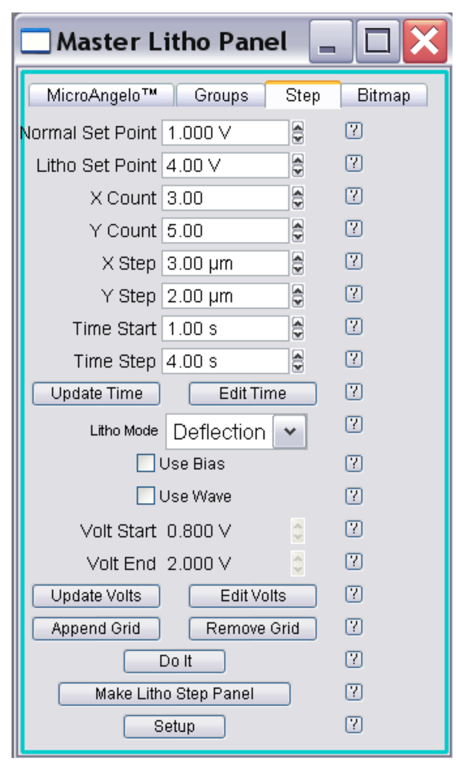


Figure 10.10: *Step* tab

▪ Point 1 in the array start with the lower left, move to the right, then moves to left array in next row.

The array will be centered in image window; the XY offsets in the group tab doesn't move the array to a user defined area in the image (like it does when using a pattern).

▪ In the example shown in Figure 10.11, a 3 x 5 array was created using the parameter values shown in Figure 10.10. Large forces were applied using a rather dull tip, resulting in the poor quality image below.

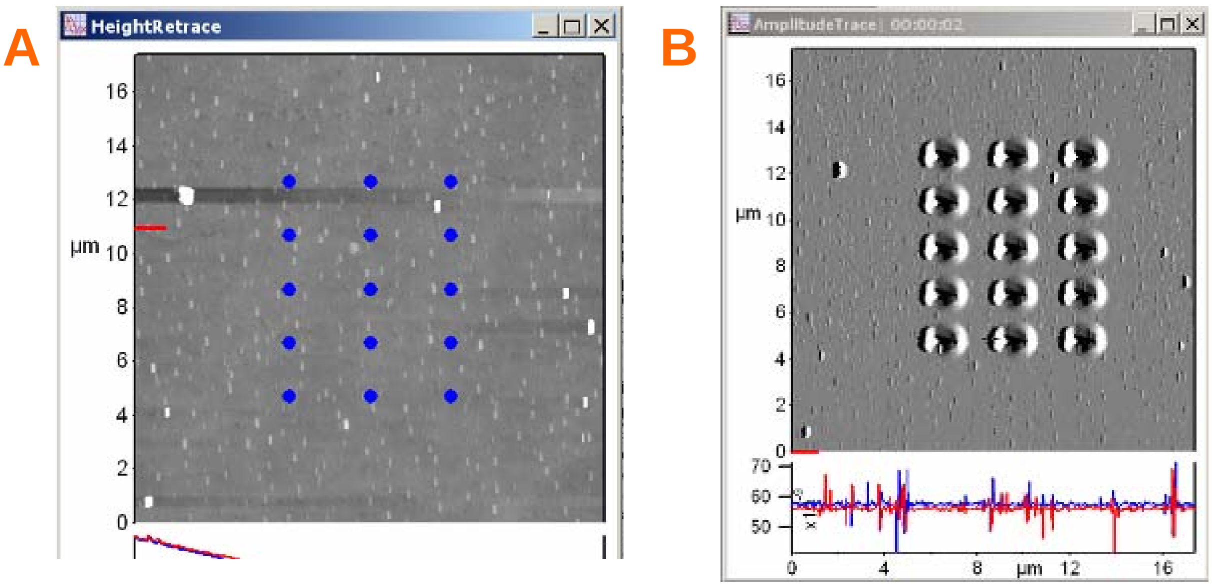


Figure 10.11: A) array pattern created using the steps tab; B) resulting indent pattern array on polycarbonate.

10.4B: Varying tip dwell time at each array point:

▪ The amount of time the tip stays in contact with the surface at each array point can be independently or systematically varied using the Steps tab.

To systematically vary the dwell time at each subsequent point in the array-

✓ Define a 'Start Time' value which represents the amount of time the tip dwells at point 1.

✓ Define a 'Time Step' value: this is the amount of additional time spent at each subsequent point. For example, if you want the tip to spend 3s longer at each subsequent point, enter 3s in 'Time Step'; (Start Time being 1s). Point 1 (lower left of the array) would spend 1s; point two would spend 4s; point three, 7s, etc. *Make sure to click the* 'Update Time' *button to ensure this change takes effect.*

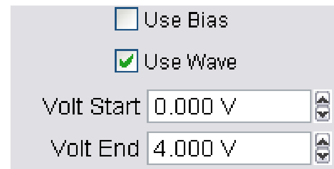
▪ The individual time values at each point can be viewed in spreadsheet form by clicking the Edit Time button.

To independently vary the dwell time at each subsequent point in the array-

▪ Alternatively, maybe a more custom variation is required- in this case, click the 'Edit Time' button. This will bring up a spreadsheet that allows the user to manually change the time at each respective point.

10.4C: Varying Tip Set Point Voltage at each array point:

The amount of applied force the tip imparts to the surface the surface at each array point can be independently or systematically varied using the *Steps* tab. This can be helpful when doing a series of indents at different applied forces in an array.



☑ Check the checkbox above the 'Volt Start' setvar input (this is actually called 'Use Voltage Wave); this will disregard the 'Litho Setpoint' input value in the MicroAngelo™ tab.

To systematically vary the applied force at each subsequent point in the array-

✓ Define a starting Set Point voltage using 'Volt Start' setvar input.

✓ Define an end Set Point voltage using 'Volt End' setvar input.

✓ Click 'Update Volts' button to activate this change.

▪ The individual Set Point setvar values at each point can be viewed in spreadsheet form by clicking the 'Edit Volts' button.

✓ Click the 'Do It' button; *the deflection at each array point can be monitored in the S&D meter.*

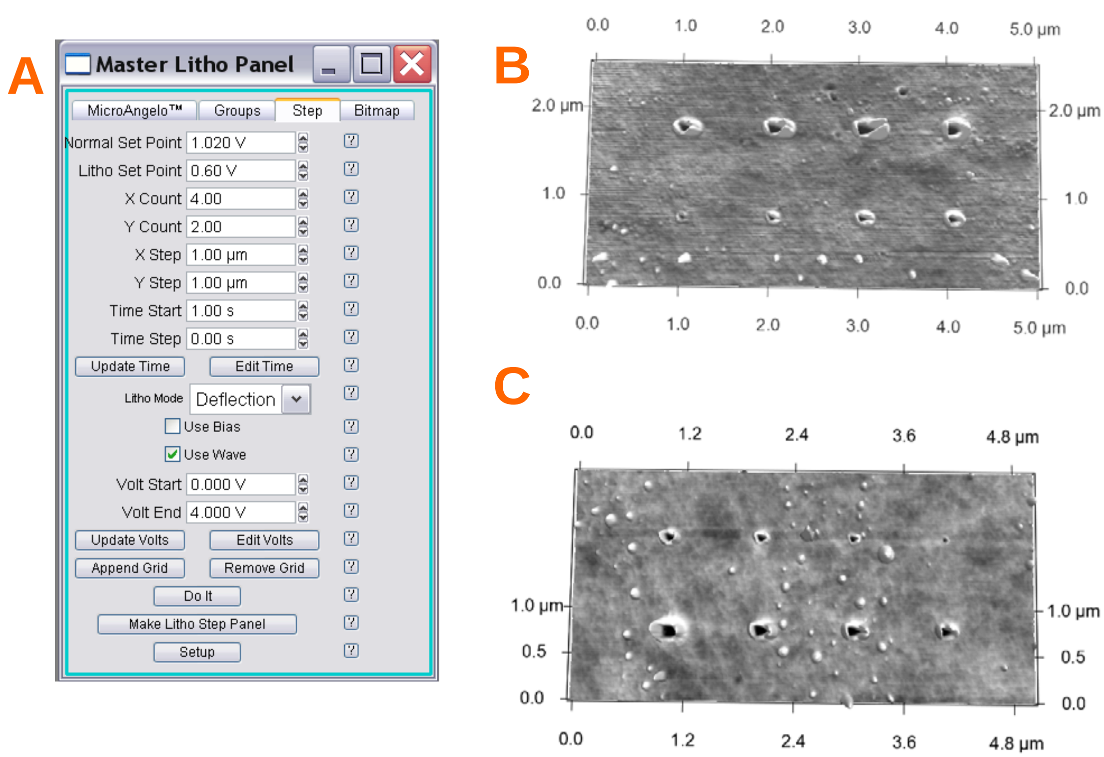


Figure 10.12: A) parameters used to make a 2 x 4 array with constant dwell at each point, while varying: B) the applied force from 0 V to 4V; C) varying the Setpoint (applied force) from 4V to 0V. Recall point 1 begins in the lower left of the array.

▪ In the example above, two 2 x 4 arrays were produced by varying the Set Point voltage from 0 V to 4 V (Figure 10.12B); and varied the Setpoint from 4 V to 0V (Figure 10.12C). In both examples, the tip dwell time was constant at each point (Tine Start = 1s; Time Step = 0s).

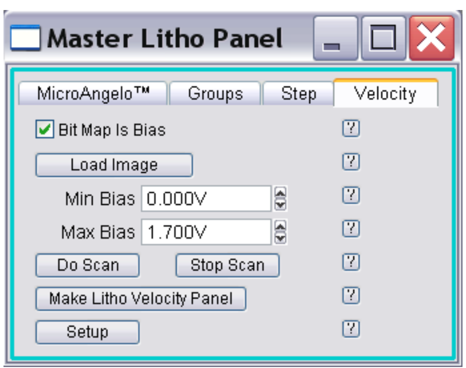
To independently vary the applied force at each subsequent point in the array-
▪ Alternatively, maybe a more custom Set Point variation is required- in this case, click the 'Edit Volts' button. This will bring up a spreadsheet that you can go into and manually change the Set Point at each respective point.

Although the author hasn't tried this yet, when the 'Litho Bias' check box is check in the MicroAngelo™ tab, perhaps using Start and End Volts value inputs allow arrays to be produced with oxidative or reductive tip potentials.

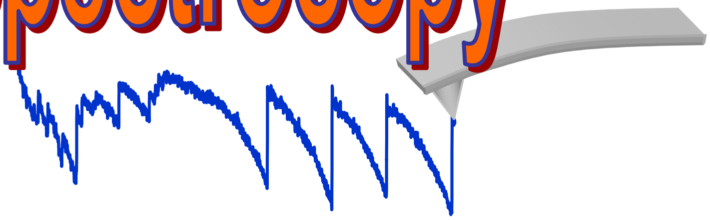
10.5: The Velocity Tab

Using the Velocity tab- It is best to read the help menu on what this is for. Although I haven't tried to do this yet, I am under the impression that it is used to draw images that have shading in them. An image is loaded, the colors are converted to gray scale- dark grays are patterned using the 'Min velocity' while the bright grays are patterned at the 'Max Velocity'. All grays in between are patterned at a velocity determined by the grayscale value and the velocity range entered into the value inputs.

I suspect this feature is good for diffusion dependent direct write scanning probe lithography techniques, among others.



11. Basic Force Spectrocopy



11.1: Introduction to the Force Spectroscopy

In brief, Force spectroscopy measures the deflection (displacement) of the cantilever (with known spring constant) in the Z direction with the intent that an adhesion dissociation event between the tip and substrate will be measured, *OR* the compliance of a material under the 'tip' as it pushes into it. the process usually begins with the tip approaching the surface with some 'free air' (non-contact) deflection value, make contact to the sample for some period/ load until it reaches a user defined cantilever deflection (force), to then reverse direction for the retract cycle *(although there are some exceptions).*

There are two major classifications that most force spectroscopy experiments measure:

❶ A pulling event in which the tip interacts with the surface, and some adhesion dissociation event between the tip and surface is measured on the retract cycle (Figure 11.1.1A).

❷ The tip pushes into the (material on the) surface to measure compliance (Figure 11.1.1B).

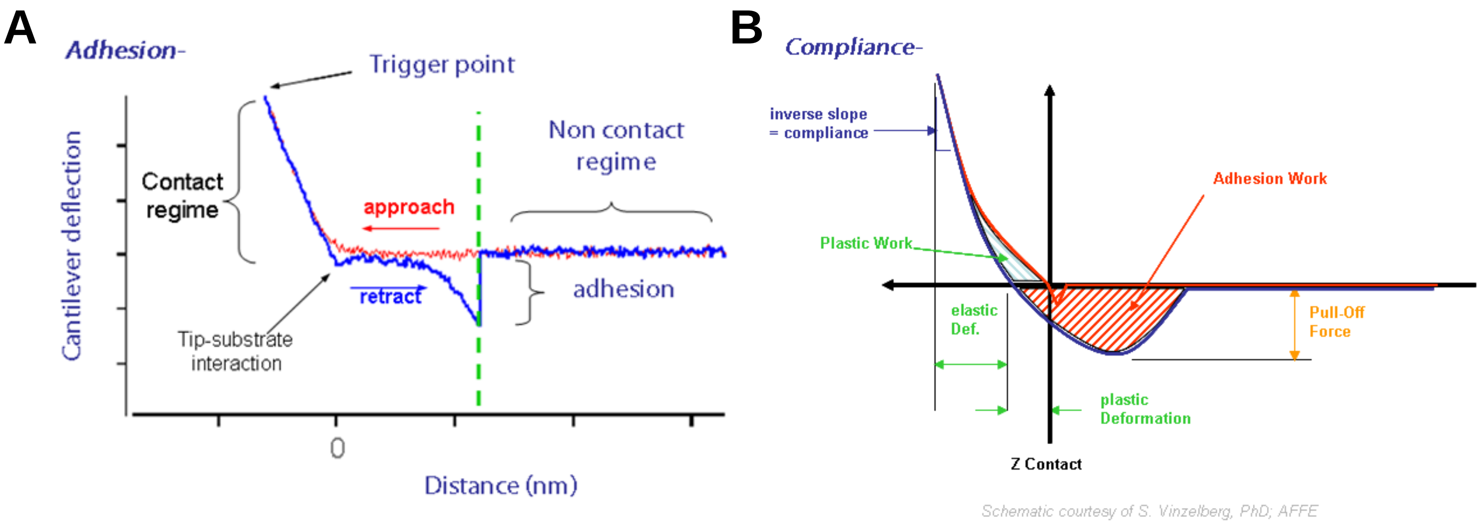


Figure 11.1.1: Two classifications of force spectroscopy measurements: A) Pulling can yield adhesion forces; B) Pushing can yield compliance forces.

Historically, the MFP-3D head was designed specifically to measure forces more accurately than traditional tube based scanner designs: specifically, the entire optical detection assembly moves together, so there is no change in the SLD reflective spot location on the back of the cantilever, allowing the (inverse) optical lever sensitivity (InvOLS) to remain constant across the entire X,Y or Z range of the piezo actuators. There are other more subtle contributors to the accuracy of the measurement relative to other commercial AFMs.

This chapter was designed to help describe, and in some cases reveal, the versatility of the force spectroscopy capabilities with the MFP-3D software and some empirical considerations. It was also the most challenging chapter for the author due to this versatility.

Cantilever selection is very important in force spectroscopy: if too stiff, then small forces may not be recognized; if too floppy, perhaps not enough force can be applied during compliance measurements, or tip get stuck to the surface via adhesion or surface charges. Other considerations are the cantilever material regarding how well it will work in air/ fluid, or the ability to do chemical cross-linking to it.

There are some excellent force spectroscopy review papers in the literature; although the author is likely missing many, a list of some useful ones are:

▪Butt, H.-J., Cappella, B., Kappl, M., 'Force measurements with the atomic force microscope: Technique, interpretation and applications', Surface Science Reports, **2005** *59* 1-152

▪Cappella, B., Dieter, G., 'Force-Distance Curves by AFM', Surface Science Reports, **1999** *34* 1-104

▪Heinz, W., Hoh, J. H., Journal of Chemical Education, **2005** *82*(5) 695-703.

▪Heinz, W., Hoh, J. H., Nanotechnology, **1999** *17* 143-150.

▪Dupres, V., Verbelen, C., Dufrêne, Y. F., 'Probing molecular recognition sites on biosurfaces using AFM', Biomaterials **2007** *28* 2393–2402.

▪Reif, M., Grubmüller, H., 'Force Spectroscopy of Single Biomolecules', ChemPhysChem **2002** *3* 255-261

11. 2: The Force Panel and Force Curve Acquisition:

11.2.1: The Force tab of the Master Panel:

The *Force* tab of the Master Panel is where most the basic force spectroscopy software control occurs- *the subtabs in the center of the panel will be described below.*

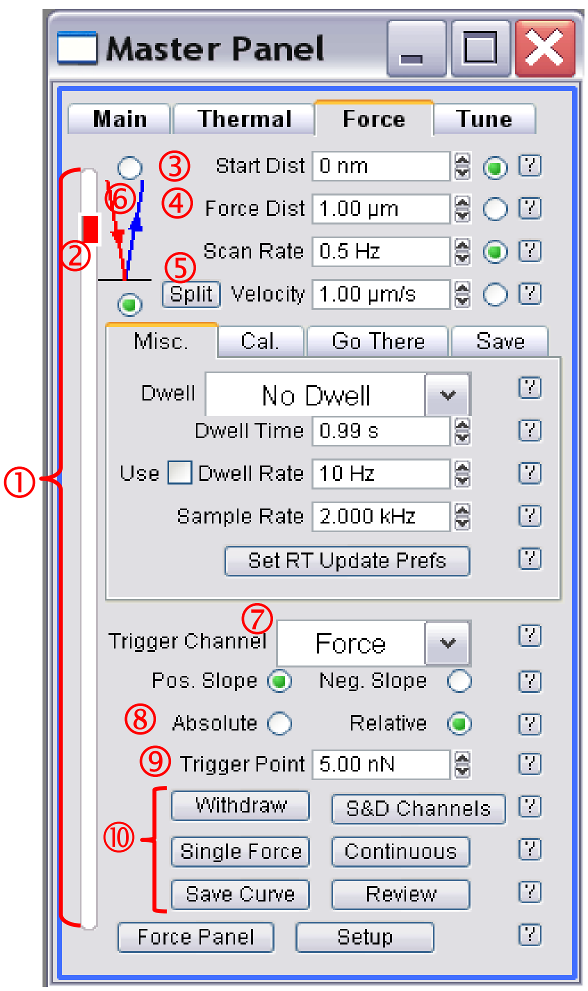


Figure 11.2.1.1: Force tab of the Master Panel.

▪ *The Z piezo operates in OPEN LOOP by default. See Section 11.2.8 to activate closed loop for constant velocity pulls or constant loading.*

① The white bar along the left of the panel represents the entire travel distance of the Z piezo; the top of the white bar represents the piezo as fully retracted; the bottom represents the piezo fully extended.

②The red bar within it represents the 'Force Distance' of the force distance cycle, relative to the entire travel of the Z piezo (*see below*).

③ **Start Distance**- this setvar is the point at which the piezo begins its Z/ force distance cycle (i.e., the starting voltage applied to the piezo is another way to think about it). The red bar's upper position in the white slider bar qualitatively depicts this position. The red bar can be moved with the mouse cursor two different ways:

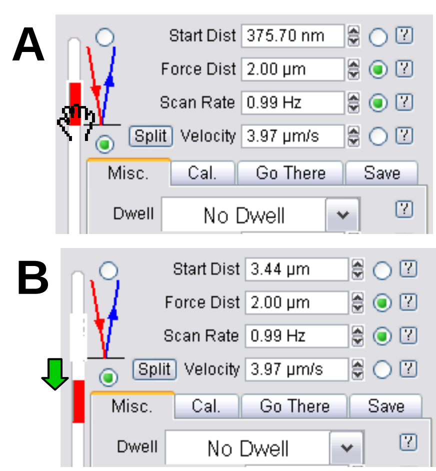


Figure 11.2.1.2: Moving 'Start Distance' with mouse: A) left click mouse in red bar until hand icon appears; B) drag cursor to move red bar.

❶ Place cursor somewhere in red region; left mouse click until hand icon appears, drag red bar throughout the Z piezo range (white slider bar);

❷ Left click mouse in the white area to roughly scroll the red cursor up or down.

▪ the Hamster wheel (on front of MFP controller) can also execute this movement in real time when the setvar radio button is activated.

④ **Force Distance**- this setvar is how long the force-distance cycle will be, *provided a trigger point isn't arrived at prior to that*. Notice that as this setvar value is changed, the length of the red bar changes (i.e., the length of the red bar qualitatively depicts the force distance relative to the entire length of the Z piezo's distance (white slider bar)). *The length and position of the red bar can be manipulated with the mouse by holding the left button down over it, then moving it (crudely depicted in Figure 11.2.1.3).*

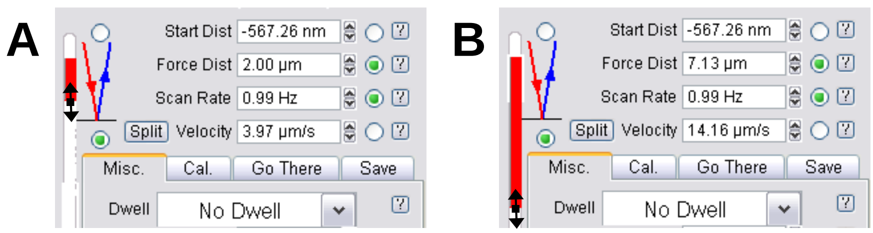
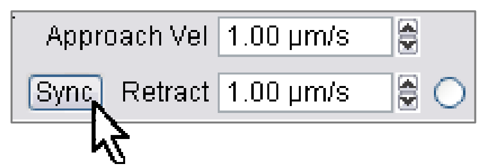


Figure 11.2.1.3: Changing 'Force Distance' with mouse cursor: A) place cursor at edge of red bar; hold left mouse button down until double arrow appears; B) drag cursor to desired force distance value (see setvar).

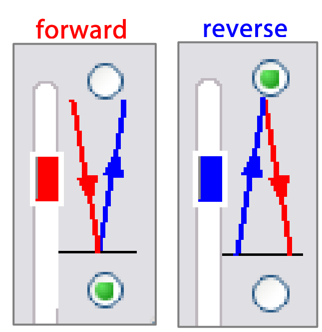


⑤ **Scan Rate/ Velocity**- This is the estimated PIEZO velocity (NOT the tip velocity); changing the velocity, or scan rate, affects the other.

'Sync' button: Split / Synch Approach/ Retract velocities can be made different in 'Split' mode; ***for experiments that require constant velocities, turn on the closed loop in the Z axis*** (*see Section 11.2.8*).

See Section 11.2.9 to select velocities that are not subject to hydrodynamic effects.

⑥ **Forward/ Reverse pulls** *(Red mode/ Blue modes)*- these radio labels change whether the tip starts on or above the surface :



Forward pull (Red Mode): tip starts cycle in free air (non-contact) and approaches surface; then retracts; this is the default mode upon MFP3D software start up. This is the most commonly used mode for force spectroscopy experiments.

Reverse pull (Blue mode) tip starts cycle at surface and travels (retracts) to free air (non-contact), then returns to surface; This mode is good for 'Fishing' , possibly Force Clamp (*see Section 11.4.1*).

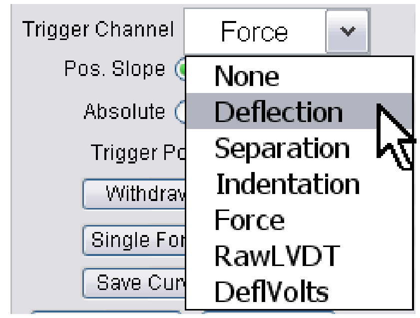


Figure 11.2.1.4: Selecting a trigger channel.

⑦ **Trigger Channel**- this pull-down menu allows the user to define what units to measure for 'triggering'- *essentially a user defined Force that the tip applies before switching direction* (Figure 11.2.1.4).
▪ *None*- no trigger applied- the piezo will go through its force distance cycle regardless of either not making contact with surface (Figure 11.2.7.3), or grinding tip into the surface.
▪ *Deflection*- monitors the deflection (nm) on the PSD- best used if the spring constant has been calibrated (See Chapter 9).
▪ *Separation*- This is Deflection subtracted from the LVDT signal; puts contact regime on left side; makes the contact regime have a slope of infinity if the cantilever is pushing against an infinitely hard surface.
▪ *Indentation*- This is Deflection subtracted from the LVDT signal; puts contact regime on right side; puts increasing indentation depth to right of start. (Force on Y axis)
▪ *Force*- will monitor Force applied- value only accurate if the spring constant has been determined (See Chapter 9).
▪ *RawLVDT*- monitors the distance on the Z axis Closed loop sensor
▪ *DeflVolts*- Monitors the amount of volts on the PSD- best used as trigger if the InvOLS has not been calibrated.

⑧ **Relative/ Absolute Trigger**- this triggering choice with applies the user defined trigger value (relative); or takes the user defined trigger plus what ever the free air deflection is converted to the trigger channel dimension/ unit (absolute). *For examples of triggering, see Section 11.2.*

Slope- this has to do with whether the force curve trigger is for a contact mode or AC mode (sometimes called Dynamic) force curve. Most force curves are acquired in contact mode, and would require a positive slope. However, in Dynamic Force spectroscopy, the AC mode curves are plotted as amplitude, (or phase) *vs.* distance, often requiring a 'Negative' slope. See Section 11.4.6 for more on Dynamic (AC) Force Spectroscopy.
NOTE: Dynamic force spectroscope also refers to studying the loading rate by applying the same load to a material at different velocities.

⑨ **Trigger Point**- this setvar defines trigger point/ load values. *The 'Trigger point' is a user defined value detected on the PSD that tells the piezo to switch directions even if the force (deflection) curve hasn't achieved the completed Force distance (deflection) value.*

See section 11.2.8 for a more complete description on trigger points

⑩ *BUTTONS-*
'Withdraw'- withdraws the tip; puts Z piezo voltage at 0V (i.e., almost fully retracted).

'S&D channels'- brings up the 'Force Channel Panel'; see Section 11.2.6 / Figure 11.2.6.1.

'Single Force'- activates a single force cycle (Crtl+3 also does this); downward toggle on black MFP3D controller face plates- *there is no ARC2 controller equivalent analog action*.

'Continuous'- initiates force cycles to be continuously executed, until told to stop.
▪ The amount of continuous curves can be limited by clicking the to 'Setup' button.

✓ Activate the 'Limit Contin. To' ☑ checkbox. *Stands for limit continuous to.*
✓ Click the 'Looks Good' button.
✓ Enter the amount of curves needed to collect continuously in the Limit to Cont setvar.
✓ Do some force spectroscopy.

'Save Curve'- if the curve looks like it's something you want saved after the fact, and you don't have all curves being saved in the *Save* subtab, click this button.

'Review'- loads force curves for review/ analysis by bringing up the Force Display panel *(see Section 11.5.1).*

There are four subtabs in the Force tab of the Master Panel to preserve some panel real estate in the software, described in Sections 11.2.2- 11.2.5-

11.2.2: The *Misc.* subtab-

This sub-tab controls two things-

❶ DWELL PERIODS & SAMPLING- The MFP-3D software allows the user to define a period of 'Dwell' where the tip stops moving (or maintains some load) in the middle or end of a scan whilst continuing to collect data at some rate. This feature is great for data acquisition where perhaps a chemical reaction timescale needs to be expanded to accommodate some receptor-ligand event, or to assess some creep /compression event while applying/ maintaining a load to the sample with the 'tip'.

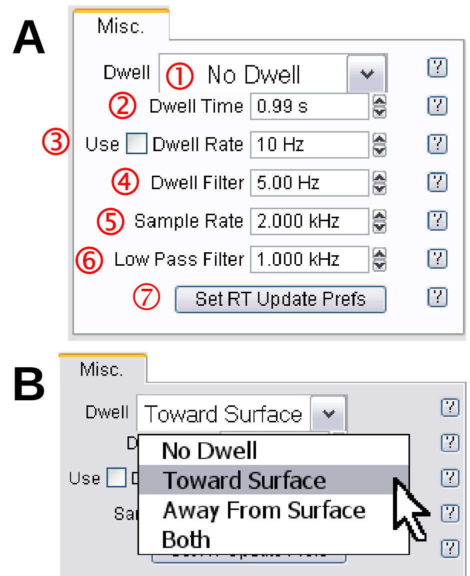
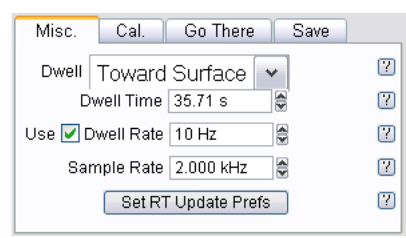


Figure 11.2.2.1 *Misc*. tab allows user to define temporal and low pass filter settings for Dwell and force curve collection.

▪ For now there is no feedback on during the dwell for non-triggered force curves. For triggered force curves there is either a Z LVDT -> Z Piezo feedback loop running, or a Defl -> Z Piezo feedback loop running during a dwell.



①**Dwell Pull-down menu**- activate Dwell function here. (Figure 11.2.2.1B).

None- no Dwell period is applied

Toward Surface-this is a Dwell period applied when the trigger point is reached; Bandwidth options during this segment can be choosen (*see below*)

Away from Surface-This occurs for reverse pull (see blue mode); a dwell is applied after the tip pulls away from the surface, before it approaches it again.

Both- this applies a dwell when tips retracts from surface, then again as tip approaches surface again.

②**Dwell Time**- this defines the length of Dwell. Click the 'Use' checkbox to increase Dwell rates- maximum dwell time is 500s (8.3 minutes).

③**Dwell Rate**- This is the rate at which data is collected during a dwell- i.e., maybe the experiment calls for more or less points to be collected during this time. It must be activated with the Use ☑ checkbox to the left of the setvar.

④ **Dwell Filter**- this is a low pass filter that can clip from 1 Hz to 1kHz; often not a default parameter- must be activated in the setup menu of the panel.

▪ An example of a force distance curve (plotted as deflection vs. time) with a multi second dwell setting is seen in Figure 11.2.2.2.

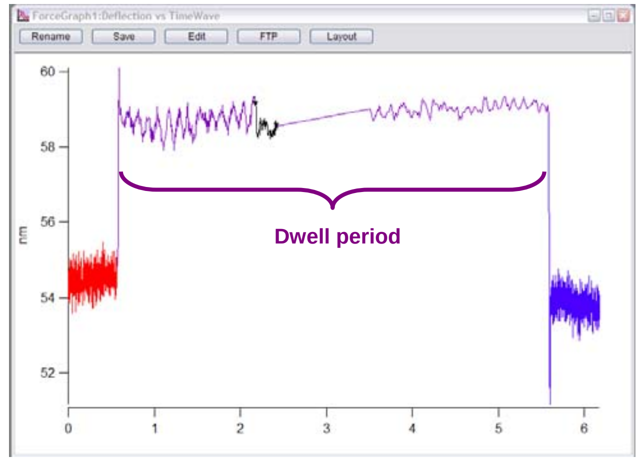
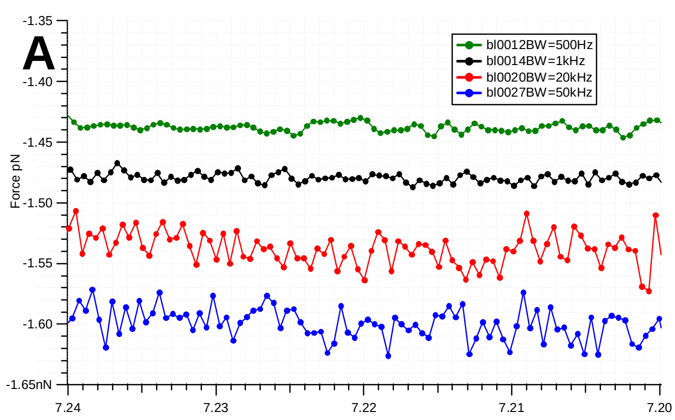


Figure 11.2.2.2: Example of a force distance curve (plotted as cantilever deflection vs. time, with a multi second Dwell towards surface.

❷SAMPLING/ BANDWIDTH OPTIONS- The band width (BW) options for the curve collection. Note, to view the BW options (in most version of MFP-3D software), they must be activated to be shown using the 'Setup' button.

⑤ **Sample Rate**-this is essentially how many points per second that is collected during the force curve acquisition (See Figure 11.2.2.3A for example).

⑥ **Low Pass Filter**- this effectively changes the low pass filter on the signal so you can adjust the amount of signal noise; the lower the frequency, the more data that gets clipped. Use this filter with caution- if small forces must be measured, too small of a filter setting (i.e., < 1kHz) could clip the event of interest. (Figure 11.2.2.3A).



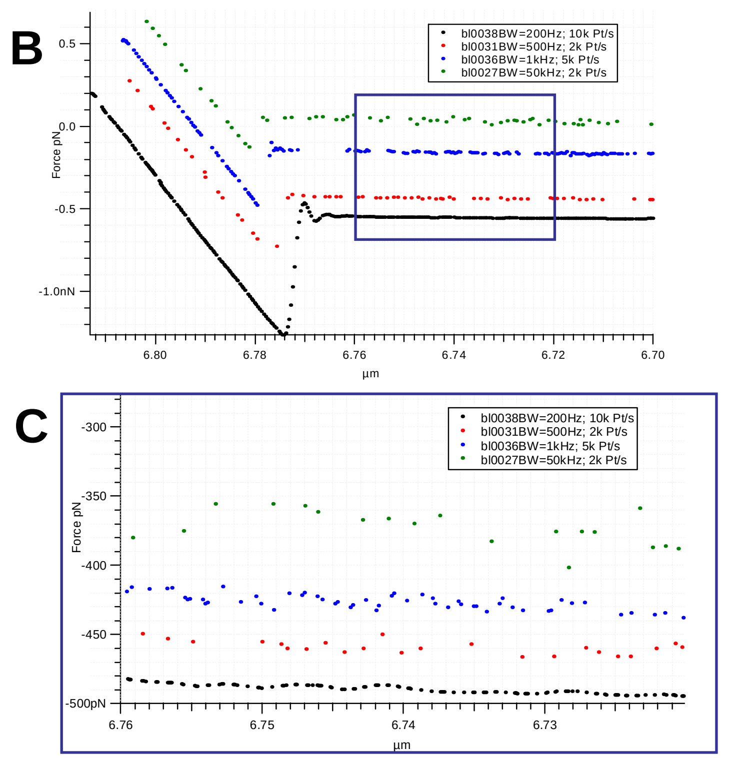


Figure 11.2.2.3: Examples of filter settings for force curve acquisition: A) various data streams from a free air portion of a force curve collected at 2kHz (i.e., 2,000 points/sec) with different low pass filter settings- Notice higher filter settings are more noisy; B) Examples of force curves collected at various sample Rates and low pass filter settings: C) zoom of boxed area in B) too illustrate sampling point density.

▪ The author typically sets the sample rate to at least twice the *Nyquist Rate*, but often as much as 4x's just to collect the data with the notion of more points are better because one never knows about the analysis fitting requirements.

▪ Examples of varying Sample Rate and Low Pass Filter values can be seen in Figure 11.2.2.3B,C: this was acquired with an Olympus Biolever in water on a mica surface, but is shown to demonstrate using the Sample Rate and Low Pass Filter setvar settings to get the effect needed.

▪An example of using high sample rates and lower LPF settings can be seen in Figure 11.2.2.4, where all curves were collected at a sampling rate of 10kHz, while the various (lower) LPF settings were employed. Notice that at lower LPF settings, the data may be clipped too much for certain applications were deflection resolution with distance is important.

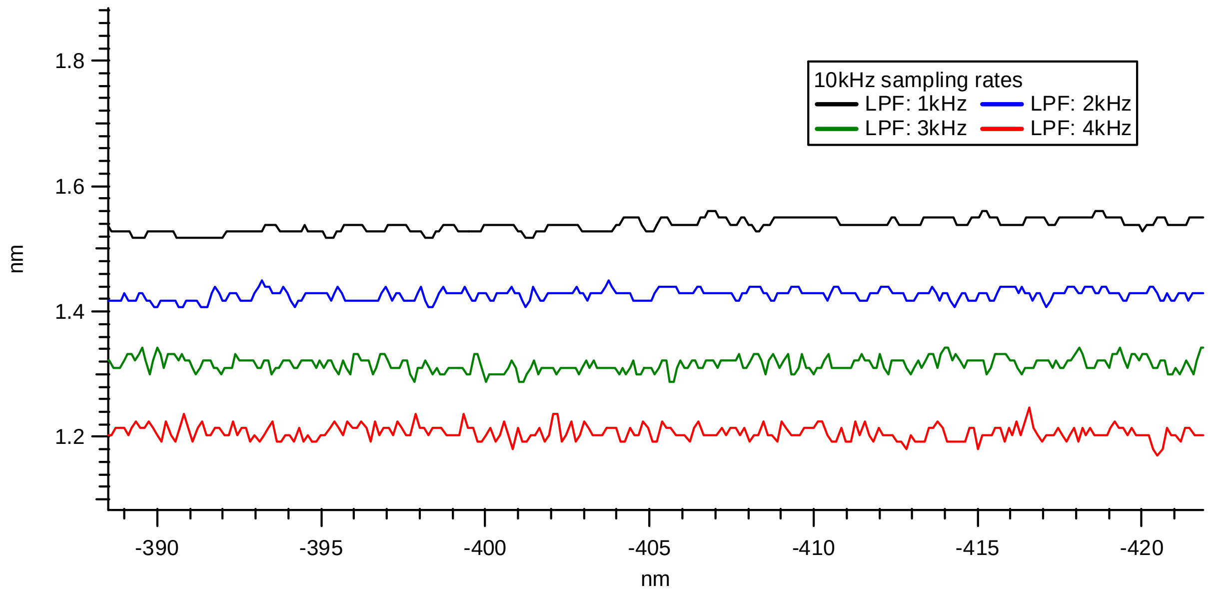


Figure 11.2.2.4: Four force curves collected at 10kHz sampling rate with different low pass filter (LPF) band width settings. Notice at low LPF BW, the data is clipped sufficiently such that data fitting might not occur at desired point values (in Y).

⑦ 'Set RT Update Prefs' button- this brings up the UiMenuPanel (Figure 11.2.2.5) allowing the user to choose how the Force graph gets updated:

Never- force plots updates *after* **the acquisition is complete.**

Auto- will update with some time constraints within the acquisition parameters.

Always- force plot is updated as its being collected; this will be evident by a black line that appears within the data stream in the force curve; See Figure 11.2.2.2 an some example of this.

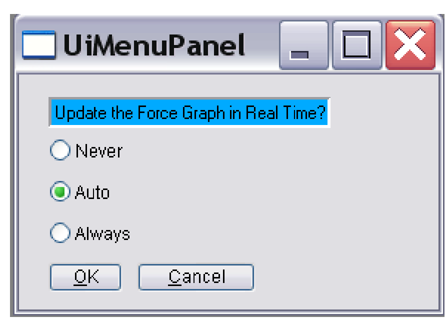


Figure 11.2.2.5: Real time updating of force plots using the UiMenuPanel.

11.2.3: The *Cal.* subtab-

▪ This sub-tab is where the calibrations are executed and displayed for spring constant determination (see Chapter 9 for a complete description of that protocol).

Defl InvOLS- deflection inverse optical lever sensitivity- the slope of the contact regime (of a force distance curve on an infiniety hard surface) acquired in CONTACT mode, in which the units are nm/V (i.e., amount of detected distance of cantilever deflection per votlage on the photodetector).

Kappa Factor- coefficient between Defl InvOLS and AmpInvOLS; value is usually 1.09. The author forgets the meaning of it, and seemingly the author of the help menu has as well.

Amp InvOLS- Amplitude inverse optical lever sensitivity- slope of the contact regime (of a force distance curve on an infiniety hard surface) acquired in *AC mode*, in which the units are aslo nm/V. Slightly different because its an AC signal on the photodetector.

Spring Constant- the calibrated spring constant will be displayed here. See Section 8 for protocol for determining spring constant *k* via the thermal or Sader methods.

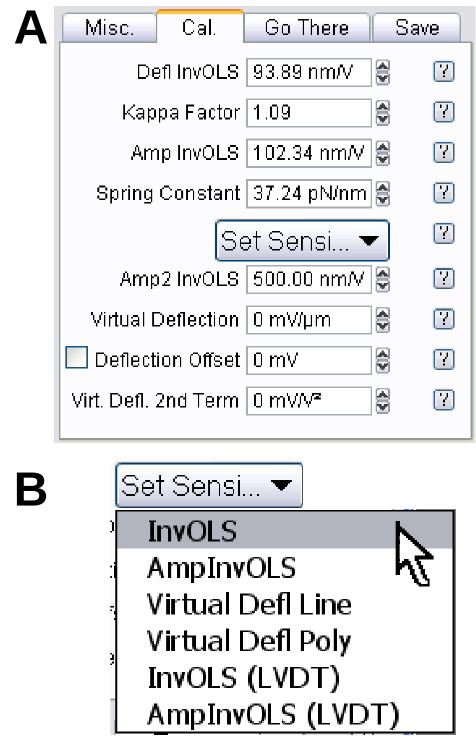


Figure 11.2.3.1: A)The 'Cal' sub Tab of the Force tab; B) Set Sensitivity pull-down options.

Set Sensitivity pull-down menu- this is where to select what is being calibrated (Figure 11.2.3.1).
InvOLS refers to Deflection InvOLS-
AmpinvOLS- see description above.
Virtual Defl Line- fits the virtual deflection to a linear line
Virtual Deflection Poly- fits the virtual deflection to a 2nd order polynomial- *use for Extended Z heads*;
InvOLS (LVDT)- used data from LVDT closed loop sensor channel as the *y*-axis data
AmpInvOLS (LVDT)- used data from LVDT closed loop sensor channel as the *y*-axis data

Amp2 InvOLS- Amp InvOLS for second frequency in DualAC™.

Virtual Deflection- this is how to calibrate the slight abberant deflection that occurs in the optical detection system using a very long force distance cycle- the free air often has several nm worth of deflection over the entire 15 µms of Z travel. It must be calibrated to 'level' the free air part of the curve to allow for more accurate analysis/ determination of contact point; it's updated upon calibration.

Deflection Offset ☑ checkbox- this will *electronically* set the Deflection voltage in the S&D meter to 0V; The offset the same magnitude is made to whatever the free air delfection is, this will electronically zero the deflection, and then an absolute trigger works like a relative trigger (*see Section 11.2.7 for more on Absolute and Relative triggers*).

▪ *For example*, if there was a -0.88V free air deflection in the S&D meter, typing 0.88V into the deflection offset setvar, and clicking the checkbox to activate it would electronically set the free air delfelction to 0.0V.

Virtual Defl. 2nd Term- this is for extended Z range heads that need a polynomial to fit the Virtual Deflection (i.e. using the Virtual Defl Poly fitting function described above) ; value is updated upon calibration.

For a more complete protocol description of spring constant calibration, see Chapter 9.

11.2.4: The *GoThere* subtab-

▪ The *Go There* subtab of *Force* tab in the Master panel allows the user to designate points on a previously acquired image for subsequent force curves. This is very useful when you have imaged the surface and want to immediately acquire force curves at specific points/features on your image. The MFP-3D's closed loop sensor accuracy makes this possible.

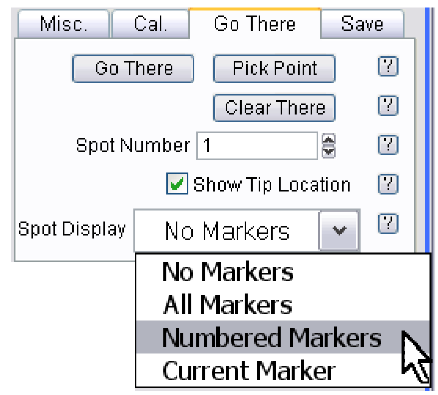


Figure 11.2.4.1: The Go There subtab for Point and Click force curve acquisition.

Once an area has been imaged for the (immediate) subsequent user defined force distance-curve (or I(V) curves) to be acquired, this tab is used for defining the location placement.

Procedure:

✓ Acquire image in AC or Contact mode.

✓ If the image was acquired in AC mode, but the desired force curve acquisition is to be in contact mode (i.e., not dynamic force spectroscopy), withdraw the tip, and change the imaging mode to 'Contact' from the imaging mode pull down menu in the Main tab of the Master panel.

✓ Click the 'Show Tip Location' ☑ checkbox- this will bring the location of the tip within the XY scan area, displayed as a red dot (by default).

✓ In the 'Spot Display' pull-down menu, select 'Numbered Markers'(Figure 11.2.4.1).- this will correlate the 'Spot Number' with the defined cursor(s) on the image area (Figure 11.2.4.2B).

✓ Click the 'Pick Point' button; this will bring a ⊕ cursor (on the all the open image channels) usually designated as '0', and will be in the center of the image (Figure 11.2.4.2A).

✓ Using the left mouse button, drag the ⊕ cursor to desired position on one of the image channels.

✓ Click on the 'That's It!' button; which locks the point at that spot, meanwhile the Spot number index is advanced by one in anticipation of the next point location being chosen. Notice the user defined location/ spot is marked on the image with an 'X' and also labeled with the spot number (Figure 11.2.4.2B).

▪ Also, notice the Spot Number base suffix will increase by one, expecting another point to be picked.

✓Repeat as necessary/ desired.

✓ When ready to acquire the force curves, use the setvar arrow keys (or type) cursor location number to acquire force curves at into the 'Spot Number' setvar.

✓ Click the 'Go There' button. Notice the red dot designating the tip position will move to that point (Figure 11.2.4.2B), generally at a rate the scan rate is set to in the *Main* tab of the Master Panel.

✓ Click the 'Single Force' button , 'Continuous' button, Crtl +3, or downward function selector toggle (Figure 11.4.1.1) on the controller to acquire force curve(s).

▪ Figure 11.2.4.2 shows an example of designating some user defined points on a DualAC™ second amplitude image of some water based paint (see Dual AC Section 6.1.5). In A), the ⊕ cursor was moved from image center to designate first defined location (as seen in Figure 11.2.4.2B); In B) three different points are selected at different contrasted image locations- Notice tip is at point three because of red dot marker.

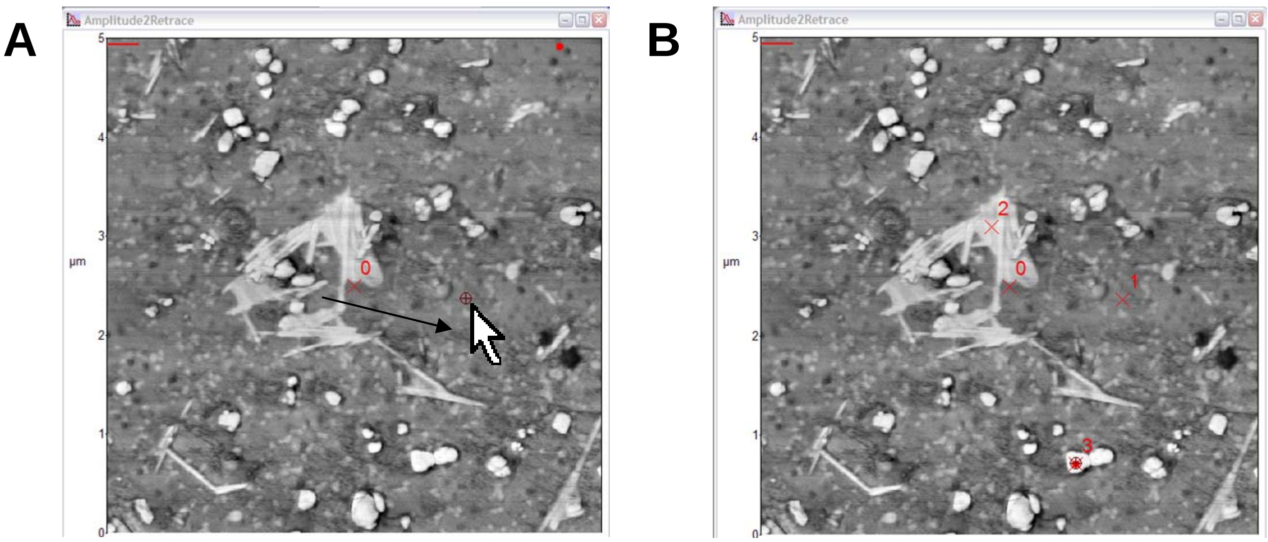
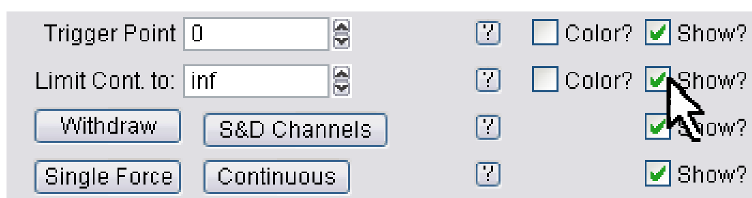


Figure 11.2.4.2: Designating 'Point and Click' force curve locations on a previously acquired image; A) drag the ⊕ cursor from image center to desired location; B) many locations selected; tip located at position 3, indicated by red dot (•). *Data shown is a DualAC™ amplitude2 image of water based latex paint in air.*

To acquire multiple curves at a given point, click the '**Continuous**' button, and designate the 'Limit to Cont' setvar to a desired value (*see Section 11.2.1*).

▪ Recall the '**Limit to Cont.**' setvar isn't shown in the default MFP-3D software, so it must be activated by clicking the 'Setup' button. If the setvar is set to 'inf' it means it will collecte curves continually (infinitely) until told to stop.



NOTE: Every time a new file name is chosen, the numbered markers will refresh and you have to pick points over again. Click the '**Clear There**' button to reset the file name.

The locations of these force curves can be labeled on the previous acquired image (if captured)- see Section 11.5.2H (In Analysis).

▪ If you continue to image in another location or scan size, while having the 'Show Tip Location' checkbox ☑ activated, Igor will have trouble scaling the image. Difficult to describe, but obvious when its happening- just deactivate in the *Go There* sub-tab of the *Force* tab (See Figure 11.5.2H3).

The 'Go There' functions also work for the electrical characterizations techniques.

11.2.5: The *Save* subtab:

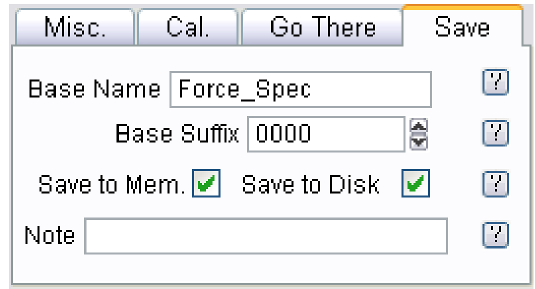


Figure 11.2.5.1: The Save subtab.

▪ The Save sub-tab is where force curves get saved (Figure 11.2.5.1). Use the same 17 character filename as with saving images; the filename can't start with a number or Igor lets you know its displeasure with your desire to attempt this.

▪ As curves are acquired, the base suffix will increase by 1.

NOTE: When the 'Save to Mem' (memory) checkbox☑ is selected, Igor can really get bogged down. The author prefers to save everything to disk- also makes the analysis easier because all the individual curves are spearate files.

▪ *To learn how to save each force curves as an .ibw IF the experiment was only saved to memory, See Section 11.5.2E*

11.2.6: Force Channel Panel:

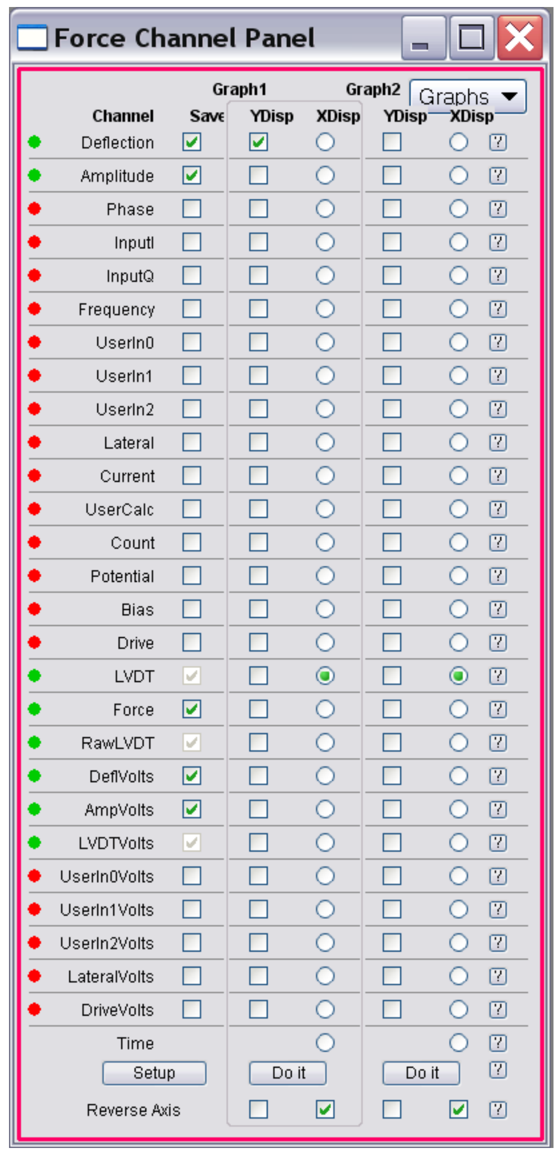


Figure 11.2.6.1: Force Channel Panel: activate axis dimensions here.

This panel assigns the data channels displayed and saved in Force Plots.

The Green / Blue / Red circular dots to the left of the channels describe the settings of those channels.

● Red = Channel is not on (no activation checkmarks)

● Blue = Channel is ONLY displayed (i.e., YDisp checked for a given Channel, but Save not checked)

● Green = Channel is saved (does not care if channel is displayed or not).

▪ Up to 5 channels can be plotted simultaneously.

The default force channel panel should look like Figure 11.2.6.1.

This panel can be a little confusing initially:

▪The 'Channel' column gives all the possible data channels- *note that depending on the experiment, some will give no real signal.*

▪ The 'Save' column is what data is always saved - the channels checked here are what can be called up in the Force Display Panel in the Analysis section of this software (i.e., there is a pull-down menu that you can select the Y axis for- see Figure 11.5.2A.1).

▪ The 'Graphs' pull-down allows up to 5 channels to be plotted simultaneously. Depending on the number of graphs selected in the pull-down menu, that many X&Y columns will be displayed.

▪Each Graph column (1,2,…5) allows selection of two dimensions to plot the desired curve/ signal in. On each plot, multiple Y-axis dimensions can be plotted vs. a single X-axis.

✓ To display time as the X axis, activate the Time radio button towards the bottom of the Force Channel Panel. Figure 11.2.6.2 shows an example of a deflection (Y) vs. Time (X) plot assigned via the Force Display Panel. The inset shows the X axis radio label selected to assign time as the X axis; Deflection would be checked in the Column tab.

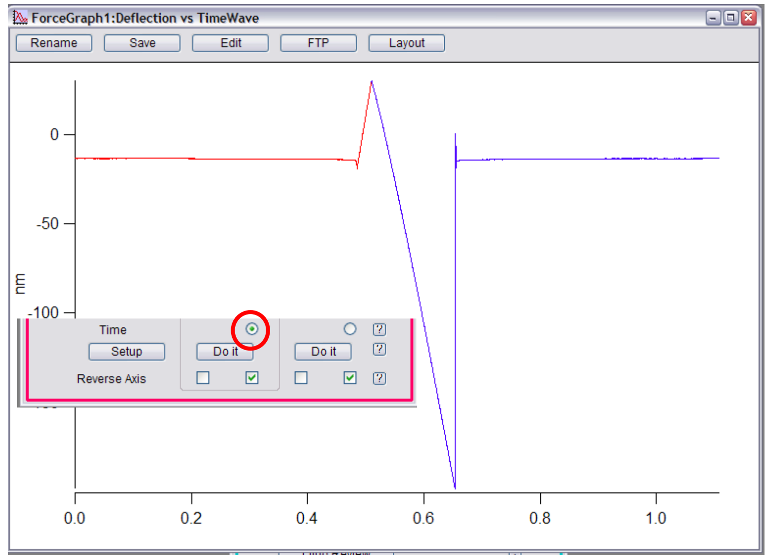


Figure 11.2.6.2: A Time vs. Deflection plot

Some notes on the channels-lifted from the help menu.
'Raw' is the LVDT signal from the Z sensor (LVDT is the type of sensor- linear variable differential transformer). This is noisy, and for most things in the offline, you would probably rather work with something with a little less noise. LVDT (in the context of the force plot software) is the fitted form of that.
How it works in your version of the software:
Each section of the force plot is broken down (extend, retract, dwell) and fit individually.
-fits any **ramps** to a 7th order polynomial, that fit is used as the LVDT signal.
-fits any **dwells** to a 5th order polynomial, that fit is used as the LVDT signal

11.2.7: Force Triggering:

The term 'trigger' can mean a lot of different things to a lot of different folks-

▪ **Trigger points**: Here, *'trigger point'* is a user defined value detected on the PSD that tells the piezo to switch directions even if the force (deflection) curve hasn't achieved the completed Force distance (deflection) value.

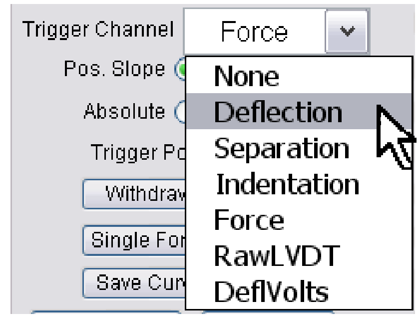


Figure 11.2.7.1: Selecting a trigger channel.

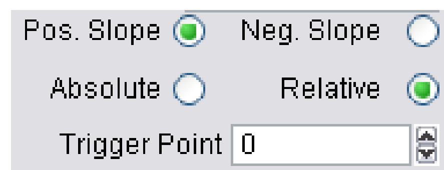
✓Activate the desired trigger point channel by choosing what channel you want it to trigger on (RECALL Figure 11.2.1.3); (for example, I like to use the 'deflection' channel which will be in nm; alternatively, your can choose DeflVolts, which will be in Volts, or Force in Newtons).

✓Type in your desired trigger value.

Determine slope sign- the default is Positive slope, used in contact mode applications

▪**Positive Slope**- software expects the trigger channel will increase before reaching the trigger point.

▪**Negative Slope**- the trigger channel will decrease before it hits the trigger point. For Contact mode applications, the trigger channel is already < the trigger point, it assumes that the trigger point has already been reached, and so it triggers at the start. For amplitude triggers you probably want a negative slope (as the amplitude decreases once you hit the surface).



✓Select what kind of trigger you want; *the difference between the two are-*

▪ ***Absolute*** trigger is the default trigger type: This takes the defined trigger (setvar) value and adds it to what ever the free air *deflection* happens to be (both values read as voltages). What results is a Force curve with larger deflections plotted then you might expect (see Figure 11.2.7.2B description). *(absolute is (1) in parameter file list)*

▪ ***Relative*** trigger is more sensitive and should be used for more accurate force distance curve acquisition, unless you electronically offset the Deflection- *see Section 11.2.3,* . The author uses relative trigger because it triggers at whatever the defined setvar trigger value is. Note, very low trigger values will show inherent noise floor of cantilever/ system. *(relative trigger is (0) in parameter file list)*

the Author usually picks 'relative'…

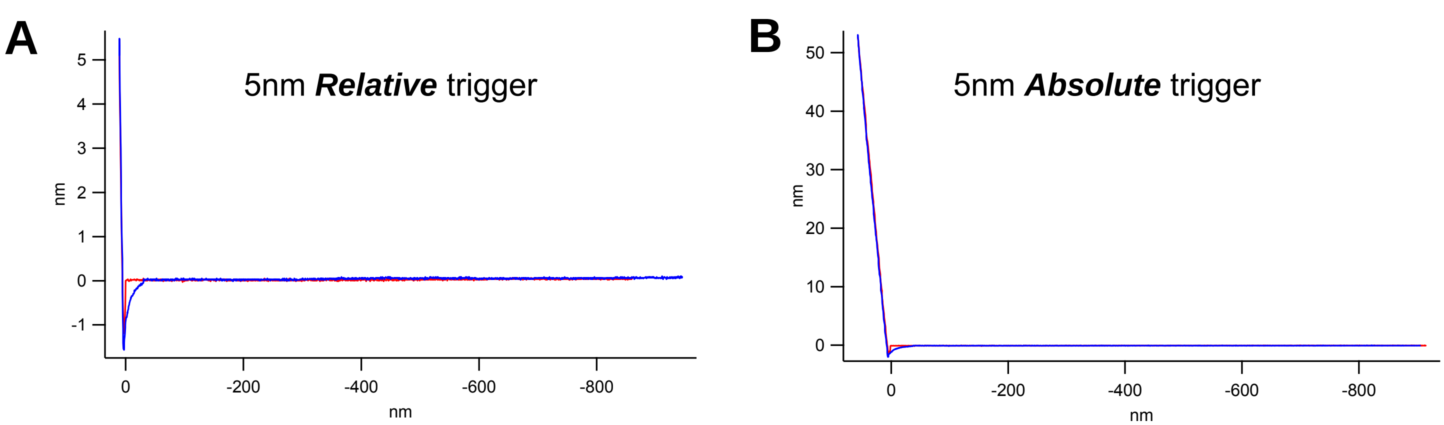


Figure 11.2.7.2: Relative vs. Absolute trigger: A) 5 nm relative trigger (notice 5nm deflection on Y axis); B) 5 nm Absolute trigger with -0.88V deflection which adds an additional 47.74 nm of deflection to trigger point (InvOLS was 54.27nm/V for this AC160 Si lever), giving about 52nm of deflection before trigger.

▪Figure 11.2.7.2 shows the difference between Relative trigger and Absolute: In panel A), a 5nm relative deflection trigger was acquired, while panel B) shows a ~53 nm Absolute trigger because the Deflection voltage was -0.88V- which converts to an additional 47.74 nm (InvOLS = 54.27nm/V for that lever). Both example curves were collected with Deflection as the trigger channel.

▪ Some notes for choosing 'None' as the trigger point - a couple things can happen depending on what the Start Distance is-

❶If the start distance/ Force distance combination doesn't allow the tip to engage on the surface during its cycle, a noisy looking curve like the one in Figure 11.2.7.3 will be seen. Although it looks noisy due to Igor's autoscaling, the noise is essentially the noise floor of the cantilever (about a1nm peak to peak for this lever and it's environment), and looks big just because Igor scales it that way. In addition, the history window notifies you that the curve 'Never triggered'.

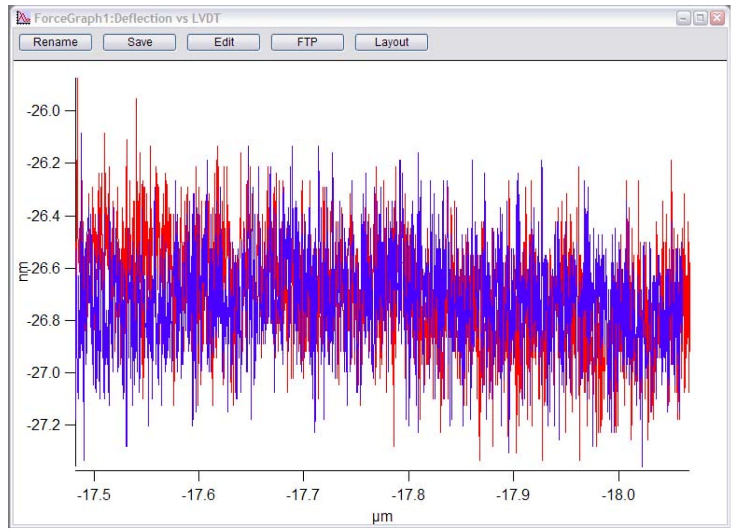


Figure 11.2.7.3: No trigger reached- curve represents noise floor of cantilever.

❷If the Start Distance/ Force Distance combination allows the tip to engage in the surface, it will mash the tip into the surface until the end of the force distance cycle has been reached…*could be bad for the tip apex.*

Relative triggers-

Figure 11.2.7.4 shows two examples of user defined deflection triggers (the cantilever *k* had been determined); Panel A) shows a ~1 nm relative trigger- small triggers are good when using functionalized tips to protect the tip apex or chemistry (i.e., functionalization that someone may have spent time on) to be ground off the tip from excessive force; Panel B) has a relative deflection trigger of 15nm.

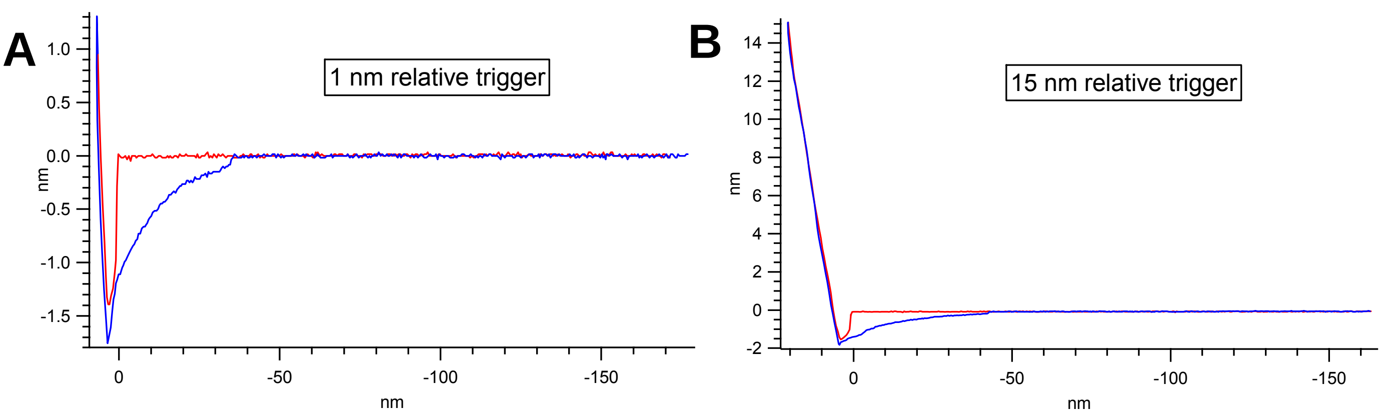


Figure 11.2.7.4: Two examples of low force relative Deflection triggers: A) a ~1 nm relative trigger- notice contact regime of force curve is minimal- best used for functionalized tips in which some sort of dissociation event is being measured; B) increasing the relative trigger to a few nm's worth of deflection. *Note there is some weird adhesion to the surface.*

▪ With a calibrated spring constant, choosing force as the trigger parameters allows forces to be dialed in, which is may be more intuitive than using cantilever deflections.

SURFACE CHARGE CAN ALSO AFFECT FORCE CURVE AQUISTION-

Occasionally, the substrate chosen can be subject to surface charges, causing some crazy non-linear deflections in the free air and near point of contact when using very low spring constant cantilevers. [The author has found this to be true ~0.03 N/m (and below) levers using clean glass, or mica glued to a glass slide. Glass is notorious for having a surface charge.] Figure 11.2.7.5A shows an example of surface charging affecting the force distance curve: notice the non-linearity in the approach and retract parts of the curve, caused by the long range forces of the surface charge pulling the tip towards the surface, or an inherently deep water layer. Either way, it's tough to get a proper virtual deflection curve fit. To solve this, it's a good idea to have an atomically flat material that doesn't possess strong surface charge- Figure 11.2.7.5B shows a piece of graphite adhered to a magnetic sample puck on an AR magnetic puck holder, along with the Static Masters to help dissipate charges. All this said, sometimes you can't get rid of the charge. Good luck-

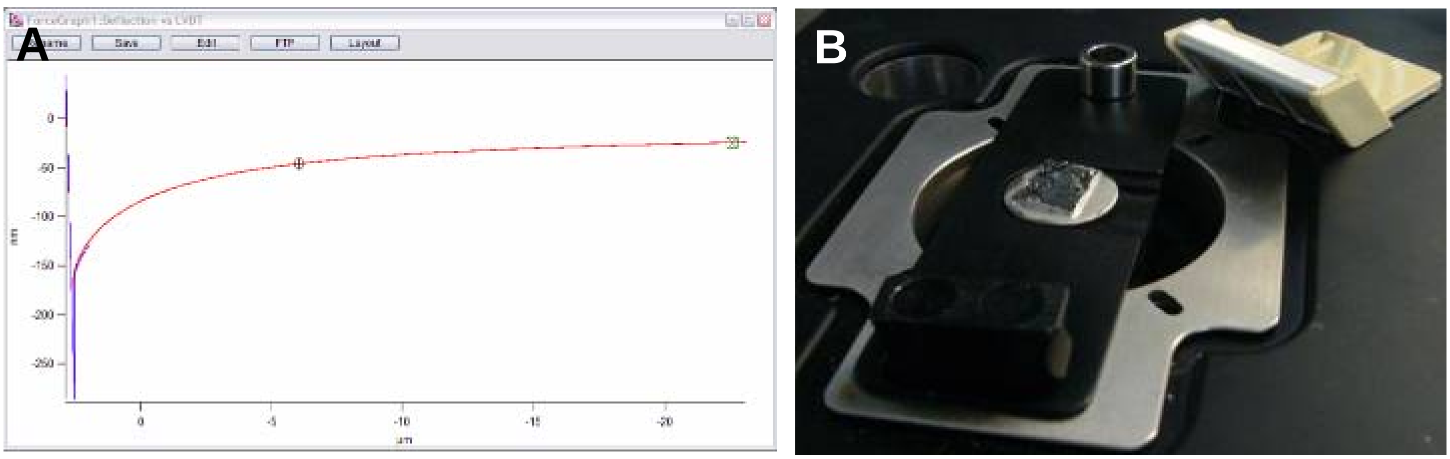
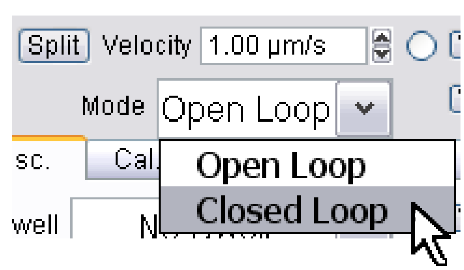


Figure 11.2.7.5: Surface charge can affect cantilever deflection in force-distance curves: A) attractive forces pulling cantilever towards surface in approach; B) Eliminating charge by removing any glass components in sample mounting. Here, the sample (graphite) is fixed to sample puck via silver paint, and placed on an AR magnetic sample holder, and the use of a Static Master.

11.2.8: Closed Loop (Z) Activation:



To turn on the closed loop in force measurements that call for constant velocity or consistent loading. Note that is will have more noise associated with it than in open loop.

✓ Go to the setup menu to activate it;

✓ In the mode pull-down menu, select Closed loop

▪Closed loop is also activated when activating the Indenter ☑ checkbox in the Indenter Panel

Force curves can be monitored with the headphones. This is great for a variety of reasons: You can have the headphone volume up to listen/monitor to the force-distance cycles while you work on something else across the room. Large (non-specific) multiple events curves can be heard through a sort of 'ripping' sound when working with bio systems; or you can hear if the tip comes off the surface by a constant static noise (as opposed to static, dampen, static sound of a regular force-distance cycle).

11.2.9: Empirical considerations:

Software Parameters:

Force Distance- choose a force distance that is large enough to have some of the 'Free Air' (non contact) portion in the curve, such that the contact point can be properly determined during the analysis. Using force distances that are too long (i.e., mostly all free air in the curves) are just wasting time, unless they are specifically required (perhaps heterogeneous surface topographies might require this in an adhesion or compliance force map), *OR* it is desired to have many sampled points from the 'free air' region for determination of a contact point in the analysis.

Trigger points- these are very important, especially when using functionalized tips- applying too much force with a high trigger point value can cause the tip to be ground into the substrate, seriously compromising the chemistry on the tip.

A caveat to small triggers is if the analysis calls for averaging the InvOLS throughout the force data set, a small trigger won't give enough points such that the InvOLS appears to shift more throughout the data set.

Loading rate: Determining the loading rate is important for publication (depending on the intended audience). Acquiring the force curves at the same spot with different velocities will suffice- the force values generally increase with velocity. Using different cantilevers over the course of the experiment will require some velocity (or force distance) matching so the different cantilevers can be compared properly in the analysis.

Sampling points- having a sufficient amount of data points can be beneficial such that a small force event can be detected in the analysis portion of your experiment (which the author suspects is 90% of the force spectroscopy experiment). It's good to look at a few curves in the offline to confirm enough points are present. A safe bet is to choose about four times to 10 times the Nyquist rate while keeping the low pass filter (LPF) around 1kHz to 1.5 kHz maximum. Both the sampling rate and LPF setting can depend on the material. *See Figure 11. 2.2.3.*

InvOLS / *k* changes: before and after an experiment- sometimes you can get different InvOLS values numbers before and after an experiment- this can happen, although it's not really intuitive when you think about it. *This can also manifest itself to some degree in a wider than expected force distribution results.*
▪There could be slight spot movement (maybe some knobs were brushed at somepoint
▪Sometimes the tip just lands on some spurious local asperity which contributes enough to the deviation.
▪Floppy cantilevers can be dampened by the surface even when the tip is retracted several microns- this in turn compromises the thermal peak fit on the final step of the k determination. Wheel that tip several hundred microns up (if you can), and the peak should be sharper (Example seen in Figure 9.10B).

There are some ways around this though-
▪Average all the InvOLS values throughout the experiment (preferably if you have a several 10's of nanometers of deflection trigger to get a decent InvOLS fit), *OR* a series of FCs before and after to be combined and averaged after the experiment – This averaged value will have to be entered into the Parm setvar in the Force Review Panel (see Section 11.5.2C) .

NOTE: This approach does depend what is being done: softer material won't be especially suitable for this approach because they are not infinitely hard surfaces to get accurate InvOLS values. One way to get around this, you can calculate the InvOLS (or average InvOLS) on an infinitely hard surface with the same tip and then plug that into the spring constant setvar in the Force Review Panel (see Section 11.5.2C).

Other questions to consider:

- *Will a dwell, (aka- load hold) be needed?*
- *What kind of data will you need for your analysis (low triggers, long free air deflections, etc.)?*
- *Do you need long force distances because the material is so compliant relative to the chosen cantilever*
- *Do I need to move at every new point (use GO there tab- Ch11).*
- *Are more or less sampling points needed; how about the bandwidth that the LP filter is clipping? (Ch11- force tab stuff).*

Experimental:

Cantilever selection- choosing a cantilever that is appropriate for the measurement is also important. Trying to measure small forces with a stiff lever might not be sensitive enough. Conversely, with too floppy a lever, the sample may be less compliant than the cantilever deflects with the applied load. (See Figure 11.4.3.X)

Sample Prep- it's important to have the substrate affixed very well- AVOID two sided tape- it can introduce a lot of creep into the experiment, but more importantly, if working in fluid it can come detached- ultimately causing the sample to 'float'. When this occurs, there can be some hysteresis in the contact regime of the curve because the cantilever is displacing the substrate, along with whatever material is on the surface.

Thermal Equilibration- it's common that once a system is set up to let it equilibrate for 30 to 120 minutes (or more!). When in fluid, this process can take longer, but it's worth the wait for processing the data after the fact because there is less drift in the data. For example, the author used to set up his fluid expts up and let it equilibration for at least 2 hours before collecting force curves.

Work in Fluid- (if the sample allows – i.e., doesn't swell) Do this to avoid large adhesion (i.e., jump to contacts) which will make the point of contact determination even more subjective.

IF Working in fluid- when acquiring force curves in fluid (which is common) it's a good idea to let the system equilibrate for a couple hours. The most dramatic deflection drift will occur in the first 20 minutes, but drift will continue to occur as the thermal gradients approach equilibrium.

Choosing velocity *in fluid*- choosing too large a velocity when working in fluid can cause **hydrodynamic effects.** Figure 11.2.9.1 shows an example of this- as the velocity of the cantilever increases from 1 to 20 um/s, there appears to be some hysteresis between the approach and retract cycles- this is a hydrodynamic effect, and is something that should be avoided (in most appliations) due to error in hysteresis between approach and retract cyles.

NOTE: *Velocity is the calculated speed of the piezo based on its factory calibration.*

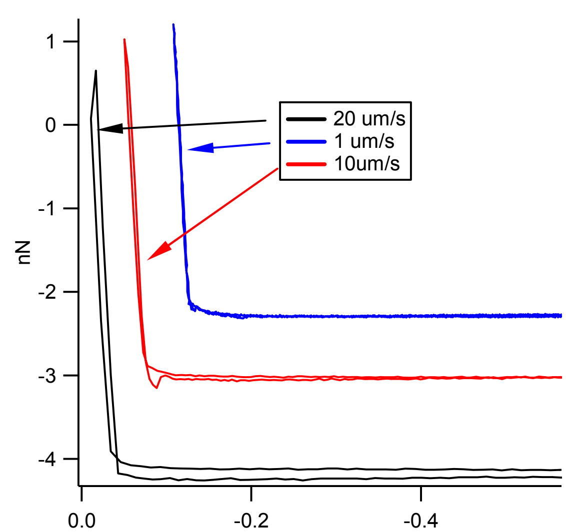


Figure 11.2.9.1: Hydrodynamic effects can occur in fluid with fast force curve acquisition cycles.

11.3: Force Mapping:

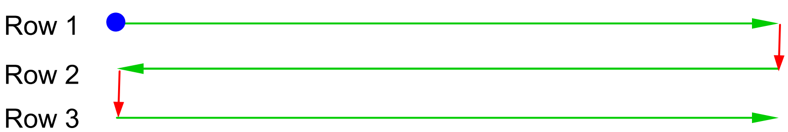
Force mapping is when force curves are acquired in an XY raster pattern to construct an image of adhesion, force, young's modulus, compliance, etc. In real time, only adhesion and height can be displayed in real time, but offline analysis can yield young's modulus or other fits, but are too prohibitively costly in terms of processing memory during the acquisition.

Early versions of the MFP3D software relied on some code that Chad Ray (Duke Chemistry alum) – He wrote a macro to acquire force distance curves in an array- but is no longer supported in Igor 6 or the newer installers- if interested, pls contact the author.

MFP-3D Force Mapping:

The fifth tab in the Master Panel called the *FMap* panel (Figure 11.3.1A) allows the acquisition and calculation of Force Maps acquired from the MFP-3D, and can do this in **REAL TIME**. This code can acquire up to 100,000 force map points, and even more if your have a hot rod computer.

The curves are collected in a 'Frame Down' raster fashion (starting point blue dot) in a 'across the fast scan, move to next slow scan line, across in fast scan' iteration:



The opposite is true is doing a 'Frame Up' Force map.

▪For the MatLab users out there, exporting is an important consideration for post processing due to the way the files are exported.

The FMap Panel has three subtabs that combine similar controls found for imaginig in the main tab, and the force parameters in the Force Tab. *The help menu defines these parameters pretty well.*

Scan: This subtab defines the XY scan area and operates similar to the *Main* tab (Figure 11.3.1A).
Force: This subtab defines the piezo velocity and trigger points (Figure 11.3.1B).
Calc: This subtab defines what channels are to be displayed (Figure 11.3.2).
Data column: this is for the X axis data to be displayed/ captured
Data B column: this is for the Y axis data to be displayed/ captured
Section column:- chose extension or retract portion of curve to be analyzed for the map.
Auto Name: author not sure what this does- aside from it's vaguely obvious description.

The lower portion of the *FMap* tab is for capturing data, similar to the Main tab.
▪The 'Delay Update' nor 'Slow Scan Disabled' features do not work yet. *Look for it to be added in later versions.*

▪Continuous Maps ☑ checkbox: this will collect force maps continuously in the same scan range region until told to stop

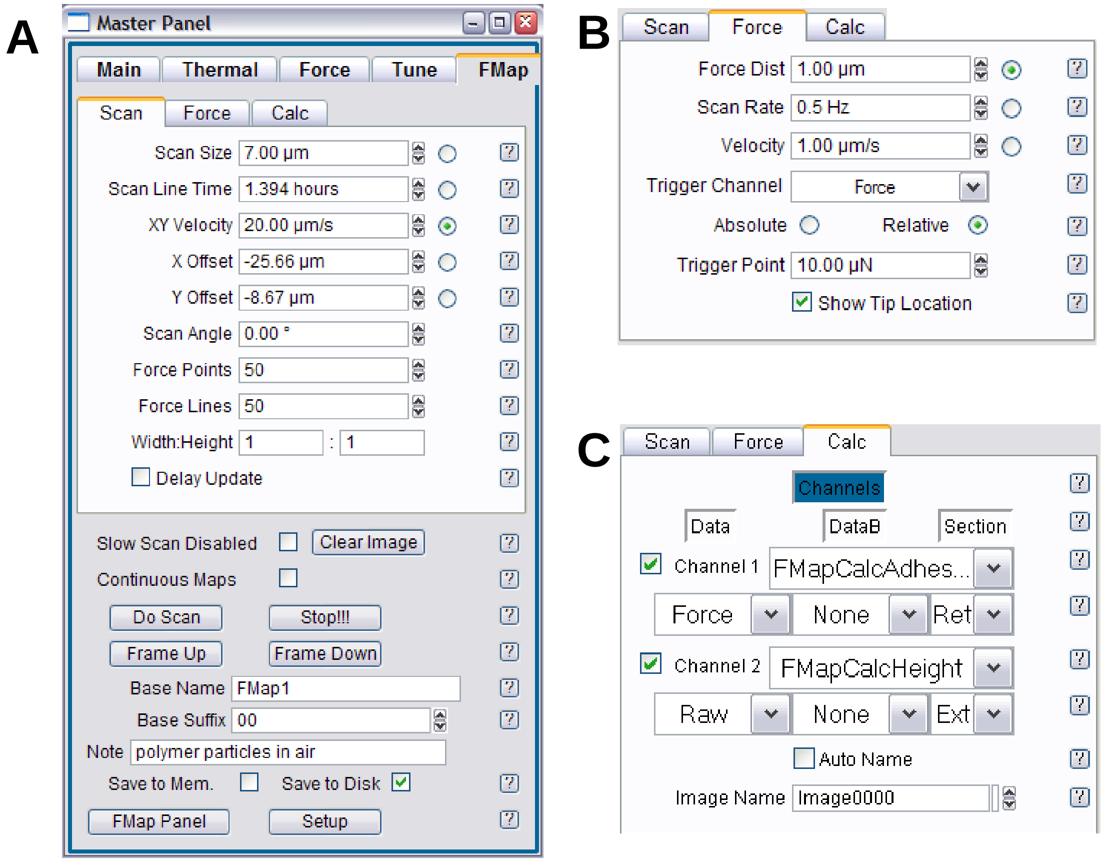


Figure 11.3.1: Force Map tab: A) showing map parameters; B) showing triggering parameters; C) Calc. sub tab defines data channels per respective axis.

Procedure for force map acquisition up-

✓ Define whether to perform Force distance acquisition in contact mode or AC mode (AC Force curves- see Section 11.4.7).

✓ Define the scan area for the Force Map; if acquiring a force map directly after imaging an area, the 'Scan Size' setvar will already have that area dialed in the 'Scan Size' setvar; the same occurs for the X&Y offsets.

✓ Define the velocity of the translation between points in XY- this is remarkably similar to scan rate, but a setvar with frequency units is not offered.

✓ Define a Scan Angle the tip will follow during the acquisition (i.e., if 0°, then the X axis will be parallel to the length of the cantilever; 90° will be normal to the length of the cantilever).

✓ Define the number of points and lines. This code is designed to handle 256 x 256, but the coder has had it beyond that- he suspects higher performance computer makes this occur with less hiccups.

▪ The 'Scan Line Time' setvar will tell how long it will take to acquire the Force Map based on the parameters defined (all the XY translations and time to acquire each force curve).

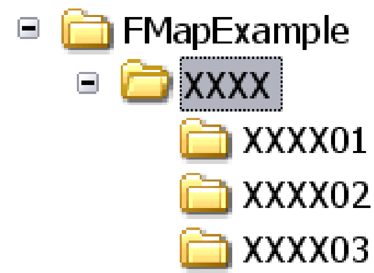
▪ The Width: Height is the aspect ratio of the scan area, just as in imaging.

✓ Define trigger points and tip velocity in the *Force* sub-tab (Figure 11.3.1B); they will be refreshed in the Force tab as well; and *vice versa*.

✓ Confirm the data is being saved some where; The author suggests creating a separate folder for this- the curves will then be saved in separate folders that contain all the points per each line (i.e., in the 50 x 50 example below, 50 different folders were created). This is no big deal, for the Calculation part, just load the folder created (*see Section 11.5.1*).

Without defining the path, the data will dump into the MFP3D data folder on My Documents (date specific).

▪ Every time a new force map is begun, a new folder will be added to the root folder. For example, if your root file name is XXXX, then the first time a force map is begun, a folder called XXXX01 will be created in the root; that folder contains folders containing the points from each line/row of the map. Beginning a new force map will create a new folder called XXXX02, or so on.



▪ The author has experienced some difficulty when calling the folder 'FMap'- probably because FMap is in the code (although this is just speculation because the author doesn't know how to code). *Name it something other than this- FMap1 works*.

✓ To acquire a Force Map in real time, the Calc subtab needs to have the proper data inputs defined to display them as the Force Map is being acquired. Something that looks like an MFP-3D 'Display Window' appears during acquisition, with tabs that correlate to what Channels that are selected in the *Calc.* subtab (see Figure 11.3.3).

✓ Click 'Do Scan', 'Frame Up' or 'Frame Down' to begin Force Map Acquisition.

✓ Go get a sandwich, or take a nap.

▪ As the Force Map is being acquired, the red dot that indicates tip position is displayed on the image channel window. Figure 11.3.3 shows a screen shot example of a Force Map being collected in real time. The red area at the top of the image is where data points hadn't been collected yet.

NOTE: if using a functionalized tip, or you don't plan on imaging before the Force Map acquisition, click on '**Do Scan**' in either the *Main* tab or the *FMap* tab to expand the image channel panel **before engaging**- the tip will begin scanning even though it isn't on the surface, and no damage will occur to the tip. This is only mentioned because it can help the user get a special feel for where the tip (in XY) is during the experiment.

▪ A real time screen shot of a Force map (Adhesion channel) of some polymeric particles that were involved in a seemingly failed attempt at some kind of micro molding using a Si tip is shown in Figure 11.3.3.

To calculate Young's modulus, or an Oliver Pharr type map, see Section 11.5.X for the full process.

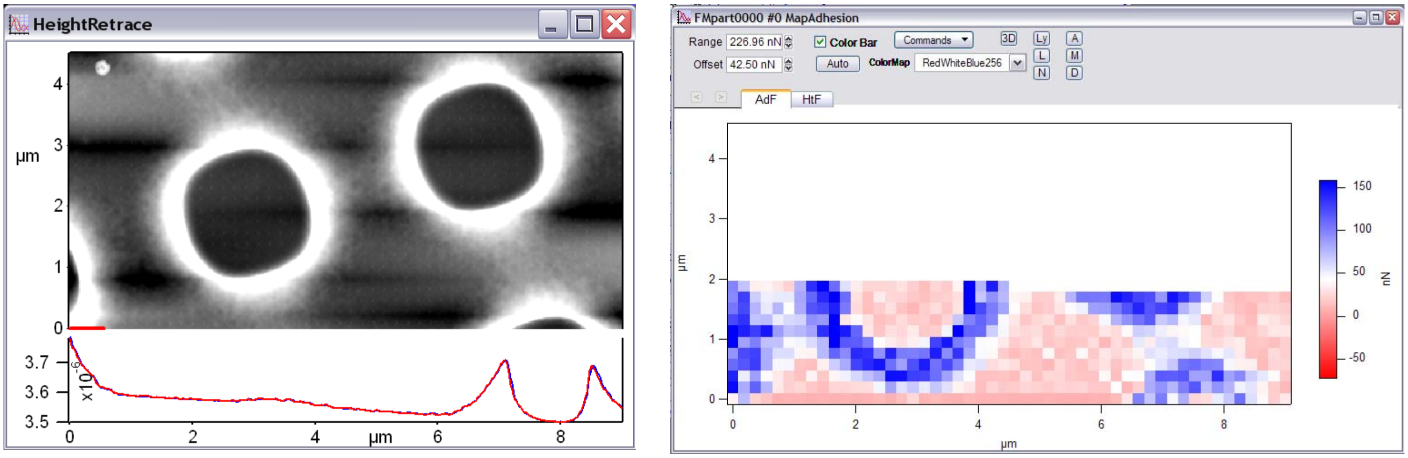
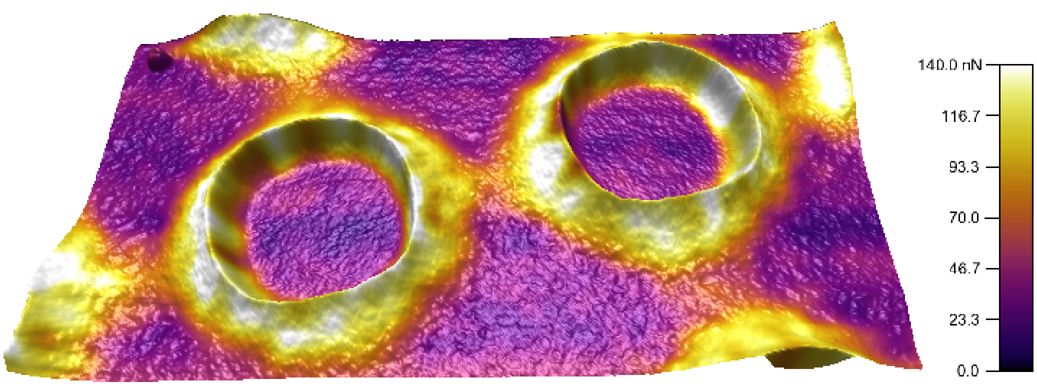


Figure 11.3.3: Screen shot of real time Force map acquisition: Pixels are updated after each subsequent acquisition. Below: ARgyle rendering of adhesion channel painted on Z sensor.



Force Curves can be monitored in real time by listening to the transducer (headphones). When the tip makes contact, the free air (non contact) static noise switches to a quiet/ dampened during contact tip sample contact, then back to the white noise static sound upon retraction.

11.4: Misc. Acquisition Operations:

Pulling based techniques-
11.4.1: 'Fishing':

'Fishing' involves trying to hook a molecule on a surface with the tip via some sort of adsorption, then pull on it to measure some kind of dissociation event, generally within that molecule. Examples are pulling the tertiary domains apart within a protein. Quite often the success rate of this is low, and takes many attempts, *hence the term 'fishing'.* Fishing on the 3D can be done using the hamster wheel to update the start distance in real time- further it can be done in the forward pull (red) mode, or start on the surface in the reverse pull (blue) mode., which allows some reaction time to occur as the tip sits at the surface

Procedure-

✓ Confirm that Forward pull (Red Mode) radio button is activated in the Force tab. (*see Section 11.2.1*).

✓ Use a small trigger point value (i.e., couple nm's worth of Deflection), especially if the tip is functionalized to avoid grinding the tip chemistry off when it's on the surface.

✓ Click the 'Single Force' button such that a force curve is performed to find the surface.

✓ Select 'None' from the Trigger point pull-down menu (i.e., turn off trigger point; Figure 11.2.7.1).

✓ Activate 'Start Distance' radio button.

✓ Use the Hamster wheel to finely move the Start Distance closer or further from the surface such that the tip makes contact with the surface right at the end of the Force Distance cycle. This can take some practice to get the hang of it.

✓Use the Function toggle switch to activate a single force pull by pushing the lower portion down (Figure 11.4.1.1). If there happens to be some Z drift, adjust the Hamster wheel in the appropriate direction. *NOTE: There is no toggle switch to do this on the remote hamster on the new ARC2 controllers (Figure 1.5) it'd be nice if there was.*

Figure 11.4.1.1: Downward Function toggle to execute force curve when working in Force tab.

✓ Repeat as necessary.

▪ Figure 11.4.1.2 shows some examples of fishing for a titin construct physisorbed to a gold Au substrate in fluid, using a gold Au coated tip. Panel A) shows a force curve that doesn't touch the surface; B) a slight increase in the start distance (80nm) allows the tip to just touch the surface; a further increase in the start distance (50nm) brings the tip in contact with the surface long enough to produce an event, although not very periodic; C) increasing the start distance another 10nm shows a decent titin pull (i.e., periodic sawtooth pattern).

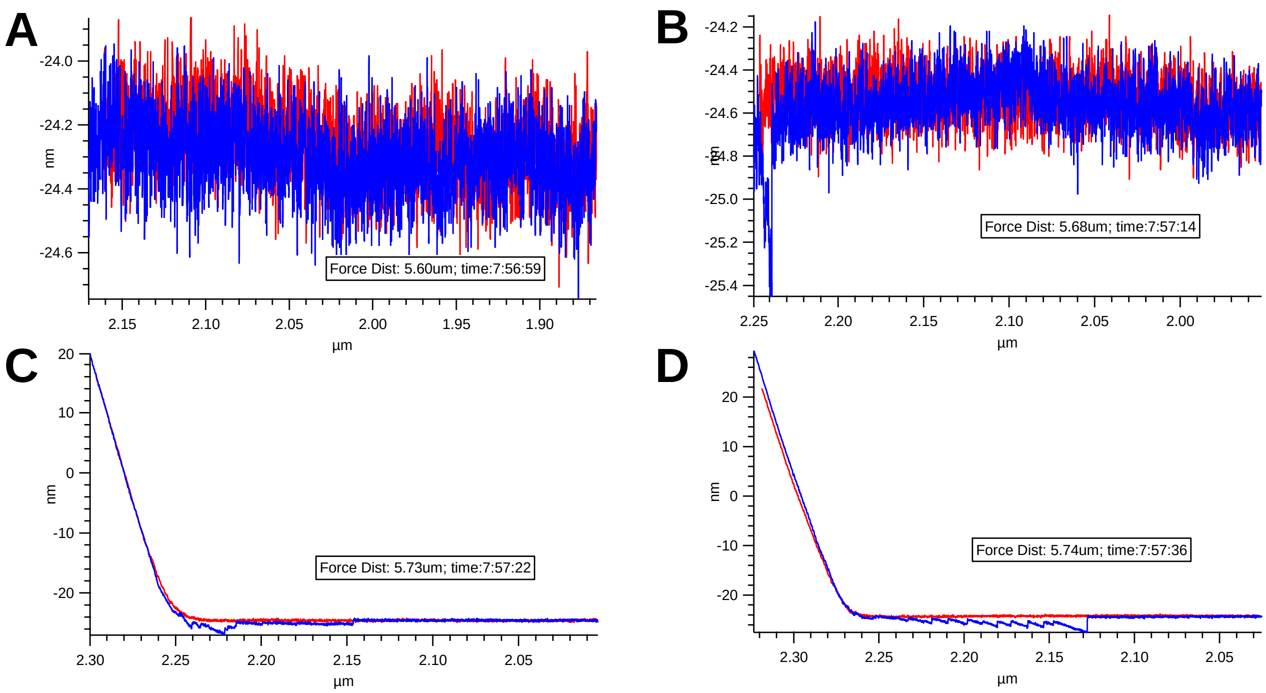
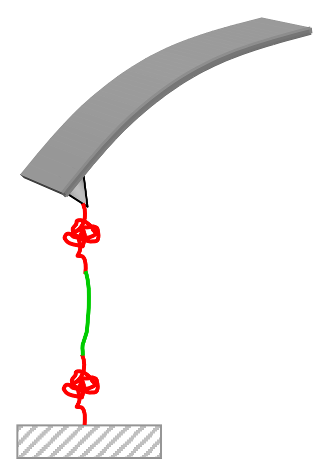


Figure 11.4.1.2: Examples of sequential force distance curves acquired via 'fishing' for a Titin Construct. All curves acquired with a force Distance of 297.83nm; and velocity of 297.83nm/s. Force distances and time indicated in each force curve panel; A) tip doesn't make contact with surface; B) increase force distance to barely touch surface; C) increase Force distance futher to show some events; D) increase Force Distance slightly more to get nice titin pull. *Data courtesy of Jason Cleveland (AR) and Jan Hoh (JHU).*

11.4.2: Force Clamping:



Force clamping is an advanced force curve acquisition technique that is designed for molecules tethered to the tip and surface. At some user defined negative cantilever deflection in the retract cycle of the force curve, the cantilever will maintain this negative deflection under feedback (i.e., cantilever end bowed towards surface). Depending on this negative deflection set point (pulling force), the molecule can dissociate somehow, or maintain that deflection. Quite often, force clamping is used to study the interactions between individual tertiary domains in a protein, or the force of a ligand-receptor interaction. A qualitative illustration is shown to the right, a three domain protein representation is shown, with the center domain unfolded to it's primary structure.

▪There are many parameters in the Force Clamping Panel that are arrived at by trial and error depending on the molecule or receptor - ligand combination. Without the proper molecule to tether to the tip and surface a, it has been difficult for the author to appropriately describe in this the beta version.

The intent to describe this section more accurately in the final version of this manualette, although coming up with appropriate samples is difficult.

✓ To open the Force Clamp Panel, Goto Programming → Load User Func.

▪The LoadUserFuncMacro dialogue appears-

✓ Select ForceClamp.ipf.

✓ Click the ‘Continue’ button.

▪ This will load the Force Clamp Panel (Figure 11.4.2.1).

▪ Typically, long before successful clamping commences, some standard force curves should be obtained to determine the types of parameters to be used (i.e., force distance, trigger points; sample rates, etc.). Sometimes a ‘fishing’ approach can more easily allow the arrival at these parameters.

✓ Perform some force curves to get an idea of appropriate trigger points, Dwell, velocity, and other parameters that are needed. The author’s experience is a Start distance (from the surface on the retract cycle) must be determined from acquiring regular force curves to assign the ‘Pull Off’ value in the Force Clamp Panel – this will be when the clamp feedback (i.e., negative deflection) kicks in.

Another advantage of performing regular force curves before activating clamped curves is to find an area on the surface that has a molecule to grab. Depending how ‘dilute’ the surface is, this could take a bit of time and patience. Try moving in XY if no hooks occur in the first several tries.

At this point, it is best to read the help menus for Force Clamping. A protocol will be developed for the official version 10 release of this operational manualette. Contact support@asylumresearch.com if you really need to use this in your research.

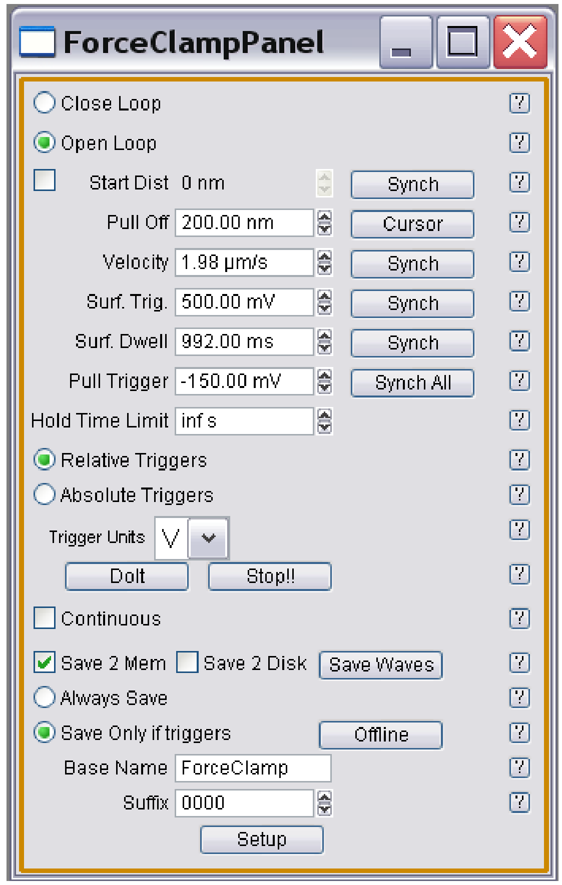


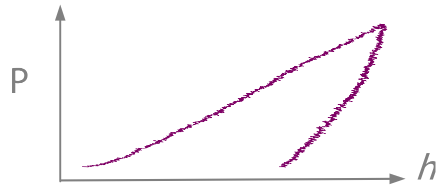
Figure 11.4.2.1: Force Clamp Panel.

11.4.3: Force Maverick-

The Force Maverick was an experiment of sorts, designed for seriously advanced users allowing custom programmed force curve acquisition acrobatics to be made- The author does not, nor many AR applications people, know how to use it, and probably won’t learn soon (due to lack of interesting materials to learn on), and the author of it wants to see it die in the code. The reason is hasn’t yet is because it hasn’t been broken in the code yet.

There is an extensive help section on how to use this, with numbered procedural directions for us. Contact support if you think the maverick is the way to perform your advanced force experiments- chances are most can be done with the existing force techniques described above.

11.4.4: Cantilever based indentation:



Cantilever based indentation is a decent (relative) way to look at compliance of materials, but it is subject to great error due to the concomitant longitudinal and lateral cantilever movements as the piezo pushes the cantilever / tip towards the surface- this additional movement cannot be easily characterized, ultimately compromising the quantitative modulus value determined in the analysis because of the unknown tip-sample contact area during the indent. All that being said, cantilever based indentation can be rather useful for making relative approximations of sample compliance, and modulus, etc., when approached with these sources of error in mind. *References that have been most helpful to the author's very weak understanding of this topic are listed at the end of this section*.

This section will introduce some of the most basic concepts of nanoindentation of materials, empirical considerations one needs to make regarding the cantilever/ tip selection, the loading parameters one will use on a material depending on what kind of recovery behavior it displays such that data can be collected with a proper fitting model to use in the determination of something (semi-)quantitative. It will also describe how to navigate the AR indenter panel software, and some examples of various materials from the AR user base that the author has interacted with. That being said, this description will not include all the scenarios one may encounter when attempting cantilever based indentation.

Depending on the material in question, some degree of elastic and / or plastic deformation in materials from the applied load will exist – Getting a sense of which (and how much) is important for arriving an indentation parameters for arriving at the most accurate quantitative value obtainable from cantilever based indentation.
Why? Mostly because the contact area approximation has great contribution to many of the fitting models. *(Other factors, such as total indentation depth can be easily backed out from the Z sensor data (if the indentation movements ware large enough (> ~80 nm).*

For example, a material that has some plastic deformation makes it possible to image the indent via AFM (using Z closed loop Z sensor), and hence back out a contact area that can be plugged into the analysis algorithm in the MFP3D software. That being said, applying too large a load wil cause the aforementioned (deleterious) convolving longitudinal and lateral tip 'plowing' while the tip loads the sample in Z- this contact area will be too large to use in the fitting algorithms. Conversely, a material that shows largely elastic deformation (& hence full recovery) will have no indent to image after the fact, forcing the analysis to rely on an approximation of the tip area relative to the total indentation depth. This can be difficult since some AFM tips can have asymmetric pyramid sides (due to compensation of 10° to 12° degree pitch.

Hence, one of the most important pre-experimental / acquisition considerations is the selection of the probe: This is because indentation is (at minimum) a two spring system, and the probe properties should be selected such that it is negligible enough for the eventual modulus approximation.
▪The tip material is important because it must be must have a much larger modulus than the material to be indented. Silicon tips can be used for many biological and polymeric materials, but may not be effective on harder materials. Diamond (solid or coated) and silicon nitride tips are widely available. Spheres or various modulus can be glued to levers for using Hertzian contact mechanics (also see Section 11.4.5).

▪ The cantilever spring constant is also very important- if a material is hard enough such that the lever deflects more than the material indents, that is a source of error at minimum, because it will have similar contact regime distance *vs.* deflection slopes similar to the calibration on an infinitely hard surface.

It is also important to mention the (sometimes) delicate interplay between elastic and plastic deformation in the material being indented (*elastic* will recover from the removed load, while *plastic* does not). The indenting probe pushes down into a material; reaches some trigger point/ defined load; may or may not have some dwell period to monitor various forms of creep; and finally unloads - remarkably similar to a standard AFM force curve (but under closed loop sensor control). What can occur during loading is the probe pushes to the total indent depth, but during unloading the material recovers (elastically) back to some degree (temporally based on the indent parameters), leaving a final indent depth (h_f) more shallow than the total indent depth (h). *This can be described as a simple power law relation-*

$$P=\alpha(h-h_f)^m$$

Where:

P is Load; typically in the force dimension (N)
α and m are empirically determined fitting parameters associated with indenter shape
h is the total indentation depth
h_f is the final displacement after complete **un**loading, and also determined by fitting.
There are various other h's depending on the fitting model being used.

Figure 11.4.4.1 gives a schematic view of the basics: A) shows the various indentation depths (h) in a material that deforms plastically; B) typical load/ unload curve for a material that has some plastic deformation.

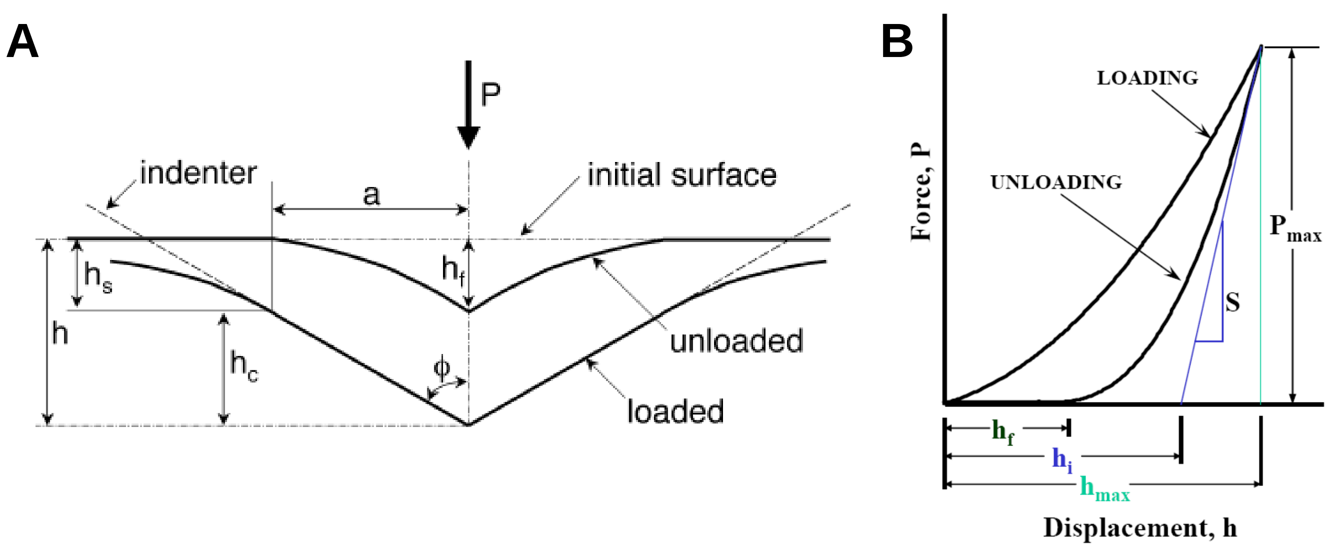


Figure 11.4.4.1: Qualitative schematic commonly used in nanoindentation to illustrate difference between maximum indentation (h) and indentation depth after load is removed (h_f); B) A common shape for load vs. displacement curve of a material that has plastically deformed. *Figure adapted from Oliver, W.C., Pharr, G.M., J. Mater. Res. 2004, 19 (1) p3-20.*

▪ General qualitative responses of various materials under applied load can be seen in Figure 11.4.4.2. Panel A shows a fully elastic system; there is no hysteresis between the load and unload components, indicating the material fully recovered while / as the load was removed. Panels B and C Show various amounts of plastic deformation (less and more, respectively), based on the degree of hysteresis between load and unload components. Panel D shows some material response/ rearrangement event occurred

during the *unload* cycle; Panel E shows some material response/ rearrangement event occurred during the *load* cycle. Panel F shows the that material is moving significantly at the initial unload, indicative by the negative slope of the unload component.
Note to obtain curves like this with the MFP3D, the indenter Panel must be used (see Section 11.4.4.3 below)

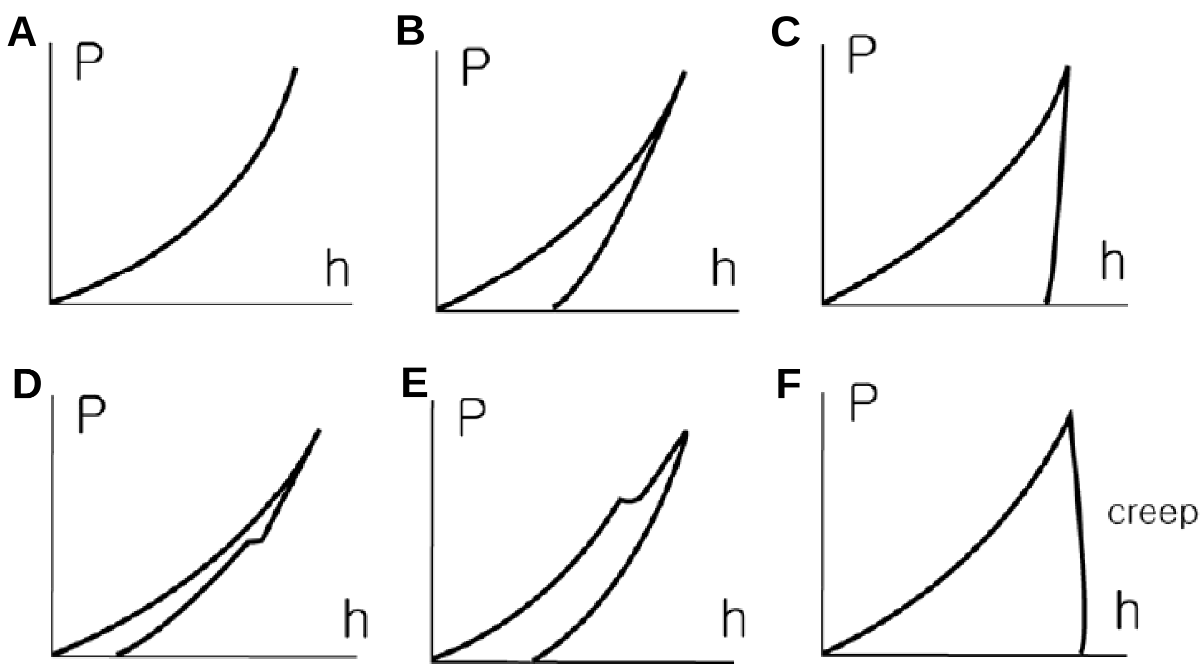


Figure 11.4.4.2: Various load/unload curves for materials of varying plastic deformation: A) fully elastic; B) some plastic deformation (fused silica); C) more plastic deformation (steel); D, E) some material rearrangement from applied load; F) Creep from polymer.
Adapted from web.kaist.ac.kr/~nano/NT512/Nano_indentation.ppt

Additionally, to add to occurrences during plastic deformation, some materials will deform in such away that material gets pushed upwards along the surface of the probe to accommodate the oppositely applied load (called pile up); *OR* may just sink in all around the indent region, leaving nonlinear indents on the final indent looking (called sink in). One could imagine this will convolute the contact area approximation, and why using a closed loop afm is the best way to back out a contact area. This is also why one always should perform every indent at a different area, unless there is complete confidence there is neglible plastic deformation.

Indenter Probe Geometries-
There are three common indenter geometries used for cantilever and vertical nanoindentation-

Cone:	m= 2
Sphere (parabolic);	m=3/2
Flat punch:	m=1

Values of m have been also determined experimentally, and errors in the theoretical models are discussed (Gong ref).

▪The MFP-3D software has two common fitting approaches: Hertzian (for analysis of loading) and Oliver-Pharr (O-P) which fits the initial unloading portion of the curve. Empirical acquisition considerations will be discussed below for both.

References:

Vertical indentation-

▪ Oliver, W.C., Pharr, G.M., "Measurement of hardness and elastic modulus by instrumented indentation: Advances in understanding and refinements to methodology", J. Mater. Res. 2004, 19 (1) p3-20. (REVIEW)
▪Gong, J., Miao, H., Peng, Z., "Analysis of the nanoindentation data measured with a Berkovich indenter for brittle materials: effect of the residual contact stress ",Acta Materialia 52 (2004) 785–793.
▪Mencik, J., "Determination of mechanical properties by instrumented indentation", Meccanica (2007) 42:19–29. (REVIEW)
▪ W.C. Oliver and G.M. Pharr, J. Mater. Res. **7**, 1564 (1992)
▪ VanLandingham, M.R., "Review of Instrumented Indentation", J. Res. Natl. Inst. Stand. Technol. (2003), 108, p249-265. (REVIEW)
▪ Schuh, C.A., "Nanoindentation Studies of Materials", Materials Today 2006 9 (5) p32-39.
▪VanLandingham, M.R., Villarrubia, J.S., Guthrie , W.F., Meyers, G.F., Nanoindentation of Polymers: An Overview", Macromol. Symp. 2001, 167, p15-43. (REVIEW)
▪Fischer-Cripps, A.C., "A review of analysis methods for sub-micron indentation testing", Vacuum 58 (2000) 569-585. (REVIEW)
▪ Fischer-Cripps, A.C., "Critical review of analysis and interpretation of nanoindentation test data", Surface & Coatings Technology 200 (2006) 4153 – 4165. (REVIEW)
▪ Mosesonm A.J., Basu, S., Barsoum, M.W., "Determination of the effective zero point of contact for spherical nanoindentation", J. Mater. Res. (2009) 23 (1) p204-209.

Cantilever based:

▪Lin, D.C., Dimitriadis, E.K., Horkay, F., J. Biomech. Eng. "Robust Strategies for Automated AFM Force Curve Analysis—I. Non-adhesive Indentation of Soft, Inhomogeneous Materials", (2007) 129, p430-440.
▪ Lin, D.C., Dimitriadis, E.K., Horkay, F., J. Biomech. Eng. " Robust Strategies for Automated AFM Force Curve Analysis—II: Adhesion-Influenced Indentation of Soft, Elastic Materials", (2007) 129, p904-912.
▪Lin, D.C., Horkay, F., "Nanomechanics of polymer gels and biological tissues: A critical review of analytical approaches in the Hertzian regime and beyond", Soft Matter 2008 4 p669-682. (REVIEW)
▪Masterson, V.M., Cao, X., "Evaluating particle hardness of pharmaceutical solids using AFM nanoindentation " International Journal of Pharmaceutics **2008** 362 p163–171.

Some useful web sites that provided some understanding:

http://www.its.caltech.edu/~ae244/Lecture10_110107.pdf
www.csm-instruments.com/en/webfm_send/42/1
http://www.microstartech.com/

the author recognizes there are more useful references

11.4.4.2: Empirical Considerations:

▪ ***Calibrate* spring constant, and InvOLS values**: Use an infinitely hard surface (cleaved mica; very clean glass) before indenting on sample; DO NOT touch LDX, LDY or PSD knobs on head while changing samples (keeps *InvOLS constant*). **Try to calibrate in air if possible**. Always calibrate InvOLS in the medium you are working in.

▪*Sample Prep:* DO NOT use two sided tape, or an adhesive that will not cure rigidly- an additional spring or source of XY creep does not need to be added into the convolved.

▪ *Tip material vs. sample material hardness:*- if the tip material is similar in hardness/ modulus to the sample, a two spring system is in play, further adding to the error- especially is soft. In indentation, the indenting tip should be much harder than the sample (Si has a Moh's hardness scale of ~2- 3).

▪*Cantilever selection*: pick a lever stiff enough such that it doesn't get pinned by the sample to cause stick/ slip events in the loading cycle (Figure 11.4.4.4A); nor have the sample deflect it, rather than be indented (Figure 11.4.4.3)

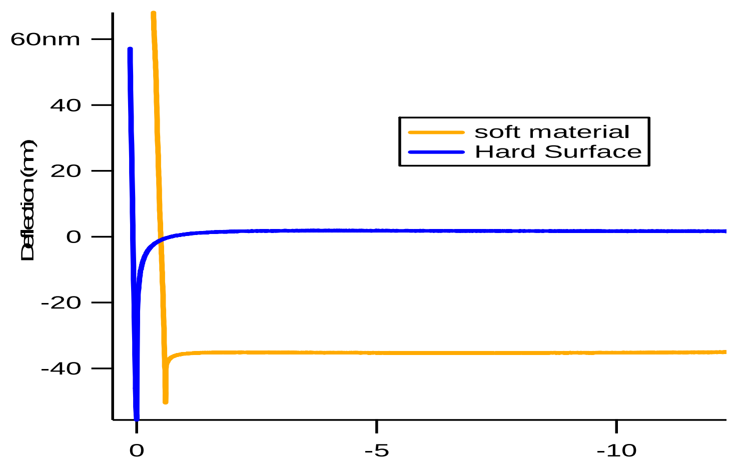


Figure 11.4.4.3: Example of too soft a lever being used- hard surface contact regime very similar to force on material- means cantilever cannot apply enough force to push into it.

▪System equilibration- if working in-fluid, let the system equilibrate for 20 to 30 minutes.

▪Collect data from the Z sensor channel (i.e., make sure LVDT is selected under the 'Save' column of the Master Force Panel).

▪**Surface roughness**: roughness adds to contact area that it difficult to define. If possible, it is always better to avoid very rough surface when indenting- this can complicate the contact area, but also may pin the tip as the load is applied, causing regions of negative deflection with applied load (See Figure 11.4.4.4).

▪A cantilever can get pinned (longitudinally) by a material that is plastically deforming, and will contaminate the analysis effort greatly. NOTE: this is different than the situation found in Figure 11.4.4.2E, where the material had a response to the load; although they may look similar. As a cantilever gets pinned by the material (Due to it's floppiness or surface roughness), the piezo will continue to push- the problem herein is the analysis algorthim requires the difference of the deflection signal and the Z sensor data as the X axis (indentation) vs. Force: Often this will yield negative slopes, and if fitting by O-P, the software doesn't know what to do with: it thinks the deflection is decreasing with increasing piezo extension (load). An example is seen in Figure 11.4.4.3A This is not always easy to spot, and why seeing how the data will be fit before collecting days worth of data is a good habit to acquire such that one can arrive at the best indentation parameters.

De-convolving this would be very challenging and laden with error. Additionally, during analysis, these areas in the approach curve where the cantilever gets pinned (arrows) as the Z piezo continues to push against the material often results in negative deflection (cantilever becomes bowed under tension), which give negative slope in the Force vs. Indentation plots, causing it to not be fit because negative slope tricks the software into thinking the cantilever is moving away from the surface (remember extending piezo).

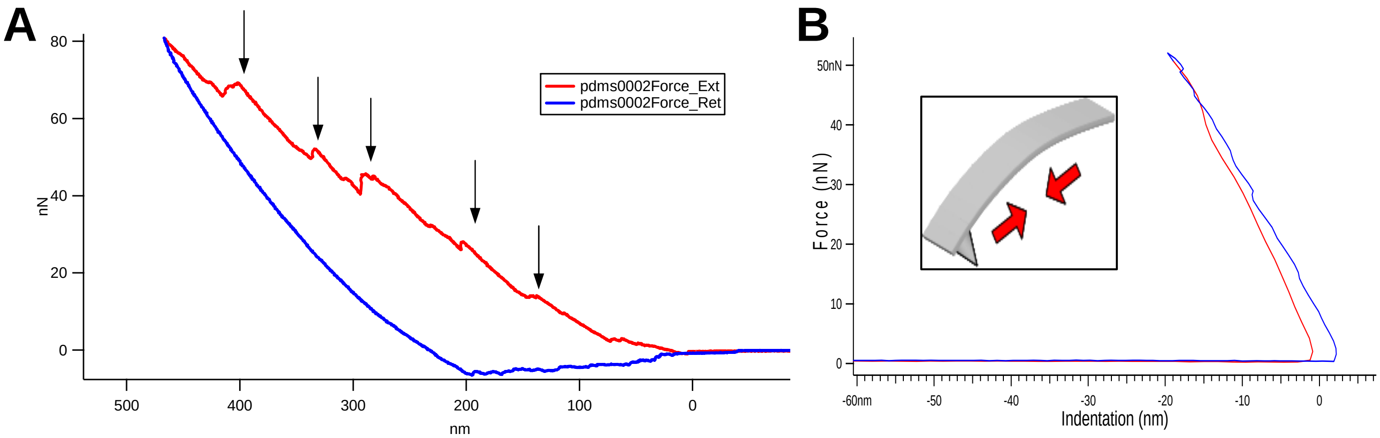


Figure 11.4.4.4: example of silicon cantilever based (k=1.45N/m) indent on PDMS where tip stick-slips in the approach (red), while retract looks like a smooth unload (blue). Force (Y) vs. distance (X); B) Effects of negative deflection that can occur when cantilever gets 'pinned' as piezo continues to push towards surface: Indentation vs. Force gives negative deflection- the controller thinks the cantilever is moving away as piezo extends (applies load), which confuses it.

▪ **Indentation depth**: shallow indent depth (10's on nm's maximum) is that spherical fitting parameters can be employed during the analysis.

Shallow indents show less tip 'plowing' than large indent depths- Figure 11.4.4.4 shows how a Silicon tip indenting into some PDMS- which gave a negative slope in the unloading curve because tip became pinned as piezo continues to drive it into surface (Force vs. indentation). This negative deflection doesn't make sense to the computer during analysis (it thinks the tip is moving away from the surface with increasing piezo extension which clearly shouldn't occur). This is likely due to a combination on mostly plastic deformation and tip plowing into the surface.

▪**Number of data points collected**: (generally) the more points the better: increase from default 2kHz to a minimum of 4kHz data points, but 10 kHz is not be out of the question for the more serious fitting protocols- As you change the number of sampling points, *make sure the low pass filter setting is sufficient for the planned fitting and acceptable S/N ratio. Collect curves at different LPF values to get a sense if it will work for you fitting. Too low a LPF will give data that might adversely affect data fitting after the fact. Refer to Figure 11. 2.2.3 and Figure 11. 2.2.4 (under empircial considerations) for examples of varying sampling rate and/or LPF bandwidth settings.*

11.4.4.3: Procedure:

Before Indenting, it is assumed that the following has occurred: (refer to chapters in the procedural manualette v10):
✓ Load tip (Ch3), align SLD on cantilever/ zero PSD (Ch4)
✓ Decide on type of cantilever spring constant and tip shape you plan to do your expt with- this is important.
✓ Perform a 'Gentle engage' to preserve tip apex. This can be done in AC or Contact mode- much more difficult to do with stiffer levers.
✓ Confirm the imaging mode is set to 'Contact'.
✓ Find InvOLS and cantilever spring constant on infinitely hard surface – so you know roughly what kind of loads to apply, and or deflection voltage. (ch9). Floppy levers can give you some challenging surface charge issues for k determination.

✓ Set up the experiment. Does an AC mode image need to be acquired first for point and click force curves?

Software Operation:

There are two ways to approach cantilever based indentation in the MFP3D software:
❶ use the *Force* tab of the Master Panel;
❷ use an indenter panel; better suited for harder materials.

1) Using the force tab exclusively:
This approach is remarkably similar to a regular force curve. Trigger points can be used as loads; Dwells can be used as holds or creep studies. The authors issue with it is sometimes the indent (X) vs Force (Y) plots sometimes don't look the way one might do to fit an indent curve.

2) Use the *Force* tab in conjunction with the Indenter Panel:
With this approach the force tab can be used to apply a very small trigger point (i.e., 1 nm deflection trigger), then the cycle defined in the activated indenter panel applies the load until it reaches its end and completes the Retract curve.

▪ Keep in mind there are two ways to do the indentation controls: 1) use the AR Indenter Panel; 2) use Indentation Master Panel accessible through the Mode Master menu dialogue (see Section 2. fX).

The AR Indenter Panel:

① When the indenter check box ☑ is activated, this panel uses Closed loop Z sensor to apply the load (after the initial force trigger used to find the surface)
So here's how this normally works: you 'find' the surface with a very gentle trigger force/ point to engage the tip, but hopefully not indent at all; then the desired indent

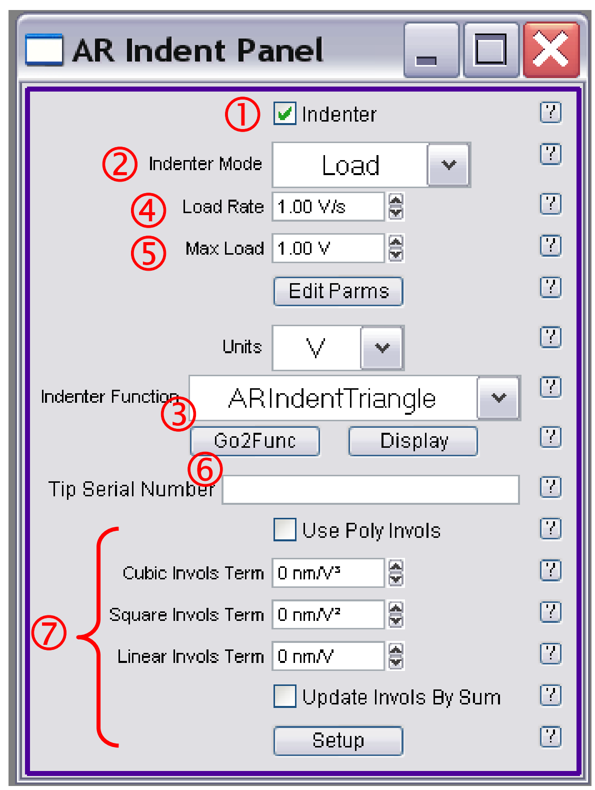


Figure 11.4.4.5: AR Indent Panel

parameters from the AR Indenter panel kick in to do the real loading.

② The indenter mode pull-down menu gives important options regarding the feed back-
▪**Load:** Feedback of the Deflection voltage (aka constant stress rate in the vertical indenter community)
▪**Displacement:** Feedback of LVDT sensor voltage
▪**Indentation:** here the LVDT signal from Deflection- gives the indentation depth (best for those types of expts); However, these types of expts require more attention during acquisition.

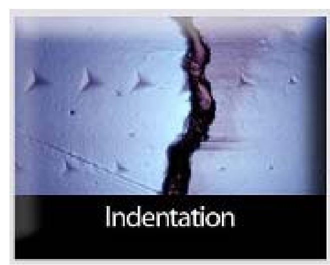
③The **Units** pull down let's the user choose between Volts, distance (m) or Force (N)
This lets you choose your loading rate ④ and maximum load ⑤ in a dimension you feel comfortable with.

⑥Indenter function pull down- this let's you choose between a triangle (voltage/ loading) waves, add a dwell (for creep) or use the AR Function editor for custom routines- very flexible- AC and DC modes. The pull-down menu options are:
ARINdentTriangle: linear loading and unloading at the same rates.
ARINdentTriangle2: linear loading and unloading at different rates.
ARINdentTriangleDwell: linear loading and unloading at the same rates, with a hold (dwell) in between
Function Editor: Allows customer waves to be built, including static and dynamic load and hold applications; see Section 11.4.6 for more about the function editor.

⑦ Disregard these- *The tip serial number and poly InvOLS is for the vertical nanoindenter flexure module accessory option.*

2) the Master Indentation Panel: this panel more or less combines 3 or 4 different panels used in the description. It can be convenient…

✓ Click on the mode master indentation button upon software startup, it opens up this large panel. Keep in mind that it also sets parameters used for our vertical indenter- a much different spring constant and resonant frequencies than cantilever based expts. Change parameter setvars based on the cantilever stiffness in expt; also, this is the only way to get to this panel; using the smaller indentation panel is a much scaled down version.



The **Indentation Master Panel** (Figure 11.4.4.5) is an example of making an AR User Panel (See Section 14. 14), and saving it so it can be a custom Mode Master Panel. It's construction involved copy/paste actions form various other panels.

The Master Indentation Panel real estate is laid out as follows:
Navigator- Imaging: imaging parameters are entered here
Approaching Surface: these are the parameters for regular force curves- same controls as the Force tab in the Master Panel
Data Acquisition: this is like every Save tab in the software.
Indentation Positioning: this is for point and click indents; similar to the 'Go There' substab from the Force panel.
Calibration: the two important calibration parameters are are displayed here; For AFM based indentation, disregard the 'Use Poly InvOLS' (i.e., lower half of sub-panel). It's for the vertical indenter accessory.
Force Map: this is for making modulus maps- similar to the FMap tab in the Master Panel.
Indentation Method: this is were the loading parameters are entered; the same things one finds in the Indenter Panel described above. The Indenter checkbox ☑ must be activated to apply these parameters (typically under closed Z loop control).

Analysis: this shows the Reduced Modulus series- The 'Review' and 'Make Elastic Tab' buttons bring up the respective panels to analyze the data to some common fitting algorithms.
Status Meter: this is the S&D meter updated in real time. This panel is so big, it can take up a full flap panel monitor- but it's nice to monitor deflection and the Z voltage with out having to constantly bring forward the S&D meter after adjusting something in this gigantic panel.

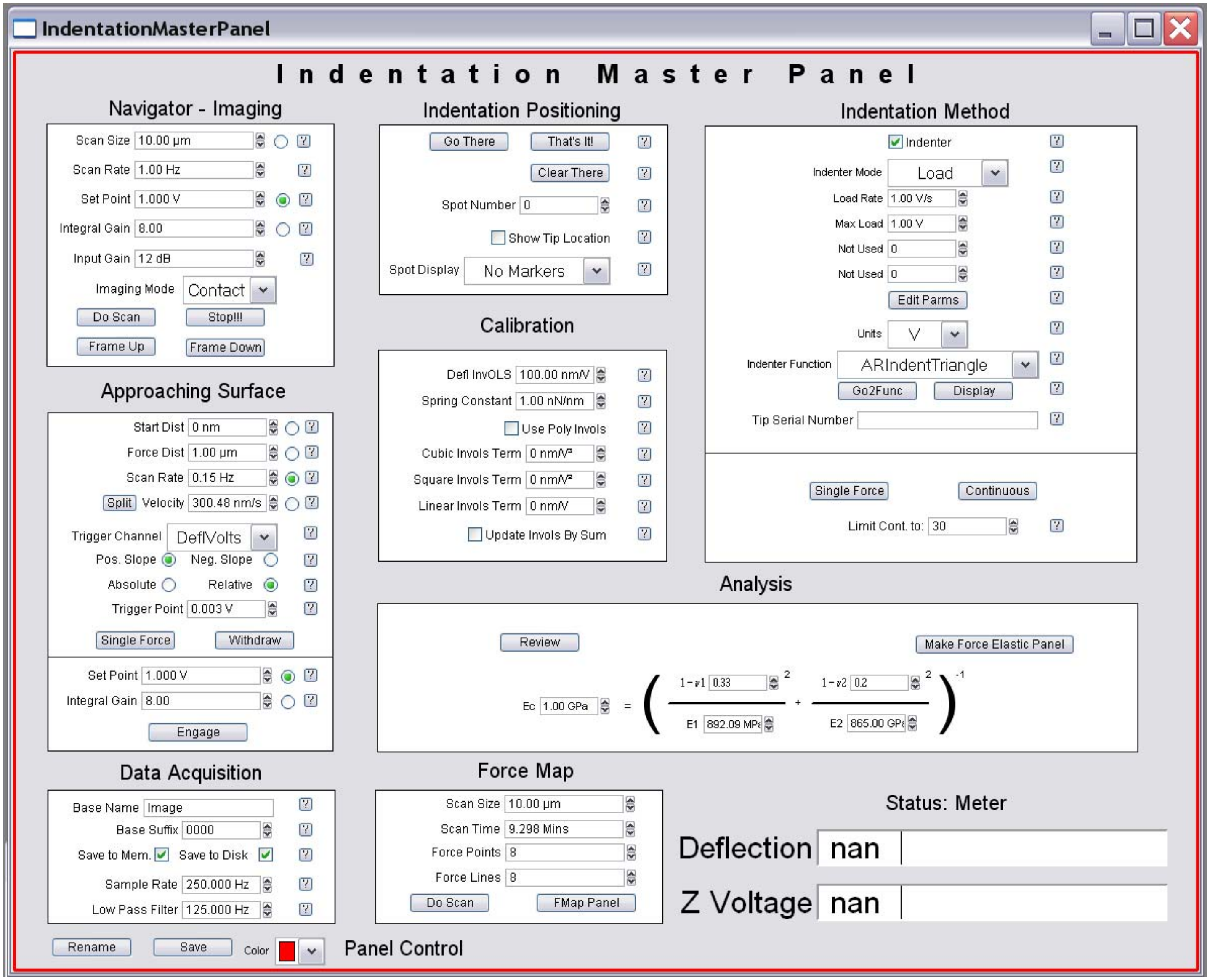


Figure 11.4.4.6: The Master Indentation Panel has most pertinent experimental parameters in one convenient place.

▪An example of a cantilever based indent using the AR indent Panel is seen in Figure 11.4.4.7. It was acquired from a 'point and click' user defined force curve on a polymeric particle, using a 40.07 N/m Si cantilever in air (InvOLS 60.60 nm/V). The intent was to be able to compare the approximations of fitting the load data with Hertizian mechanics, and the unloading data with the Oliver Pharr algorithm (See Section 11.5.X- Elastic tab). Therefore the approach was to apply about a ~5nm deflection trigger with the force tab (this would allow hertizan fitting), then kick in the closed loop indenter to apply ~1.2 µN load/ unload cycle, before the retract cycle kicked back in.

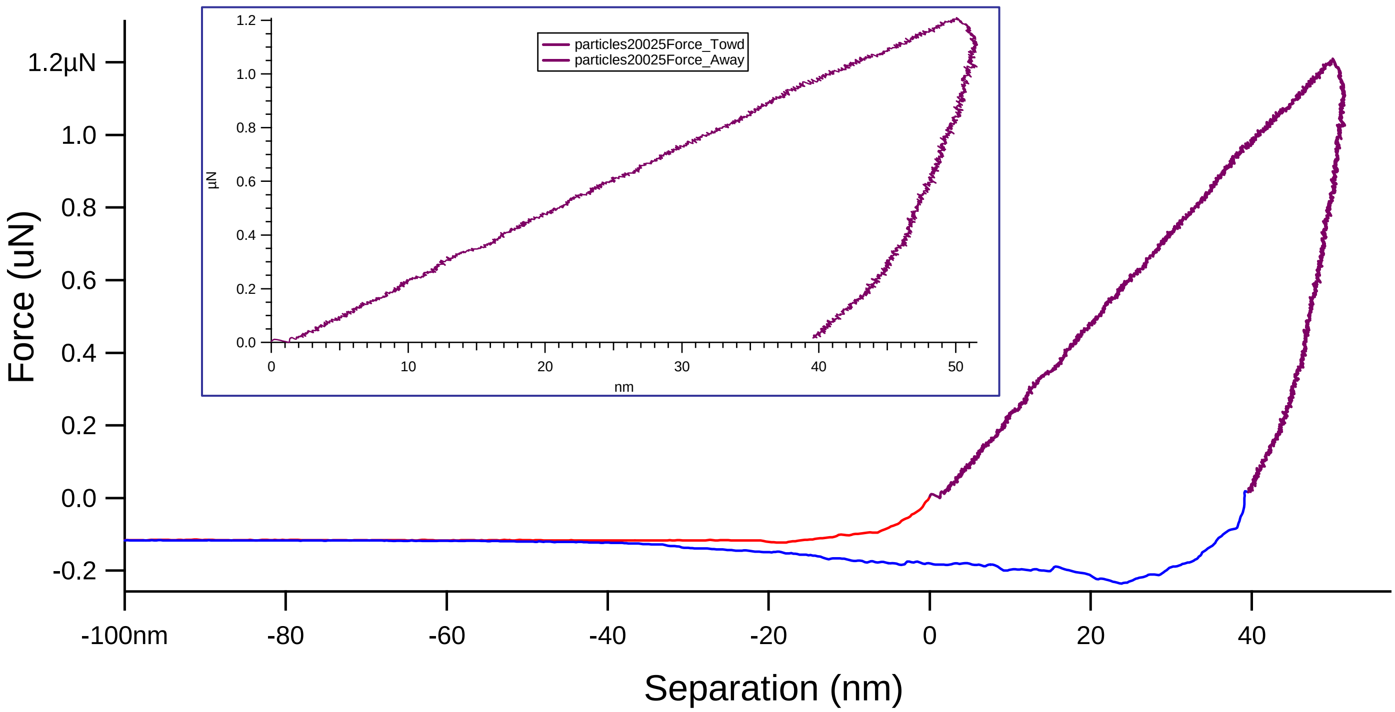
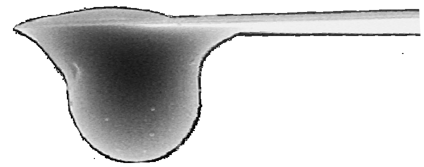


Figure 11.4.4.7: Cantilever based indent using AR indent panel on a polymeric particle (Si; k = 40.40 N/m). *Sample courtesy of Tim Merkel (Desimone lab; UNC-CH Chemistry).*

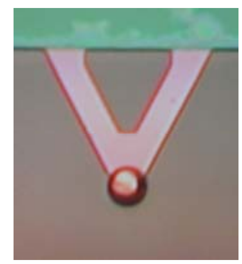
11.4.5: Colloidal Probe Microscopy:



Colloidal probe microscopy is a force spectroscopy technique in which a spherical probe is mounted onto a cantilever, and used (in most cases) to push on a soft material. The sphere allows Hertzian contact mechanics to be applied to the analysis.

Some reviews and useful papers regarding colloidal probe microscopy:

▪Bonaccurso, E., Kappl, M., Butt, H.-J., Current Opinion in Colloidal & Interface Science, **2008** *13* 107-119.
▪Vezenov, D., Noy, A., Ashby, P., J. Adhesion Sci. Technology **2005** *19* (3-5) p313 – 364.
▪Leite, F.L., Hermann, P.S.P., J. Adhesion Sci. Technology **2005** *19* (3-5) p365 – 405.
▪Christendat, D., Abraham, T., Xu, Z., Masliyah, J., J. Adhesion Sci Technology **2005** *19* (3-5) p149-163.
▪Tormoen, G.W., Drelich, J., J. Adhesion Sci Technology **2005** *19* (3-5) p181 – 198.
▪Kappl, M., Butt, H.-J., Particle & Particle Systems Characterization **2002** *19* (3) p 129-143.
▪Lin, D.C., Horkay, F., "Nanomechanics of polymer gels and biological tissues: A critical review of analytical approaches in the Hertzian regime and beyond", Soft Matter 2008 4 p669-682. (REVIEW)



Some assumptions for acquisition and Hertzian analysis include:

▪Load $\propto \alpha(\text{depth})^m$, where is a function of the geometry & the elastic properties (Young's modulus and passion ratio); and *m* represents the shape of the pressure distribution.
▪ is fully elastic (this doesn't always occur empirically, or at least on the time scale of the load/ unload cycle)
▪Pressure distribution same a spherical indenter shape
▪Radius of spherical indent < radius of sphere
▪Frictionless
▪Bodies large compared to volume under contact
▪Isotropic
▪Spherical indenter contacting flat surface
▪Cantilever spring constant Is NOT similar to material being pushed- *want cantilever k much larger.*
▪Elastic indenter much stiffer than surface

*The **analysis** of compliance curves is described in **Section 11.5.2I** under the Elastic subtab description of fitting the data.*

A Note on Cantilever Calibration with Spheres: it is usually more challenging to calibrate the cantilever spring constant (via thermal method) with an affixed sphere due to the increased Surface Area (*especially in air*), and the materials spheres are typically made from. An example of *large* surface forces acting on a sphere can be seen in Figure 11.4.5.1- *notice large jump to contact and ridiculous adhesion to surface before snapping back to free air.*

There are two ways to avoid large attractive forces that will affect the virtual deflection calibration: ❶ If working in air, calibrate virtual deflection and the thermal tune far off the surface (several hundred microns) to avoid long range charges between the sphere and surface that premature the deflection cantilever; ❷ Work in fluid, although the much lower Q of cantilever can make fitting the curve from thermal method more challenging.

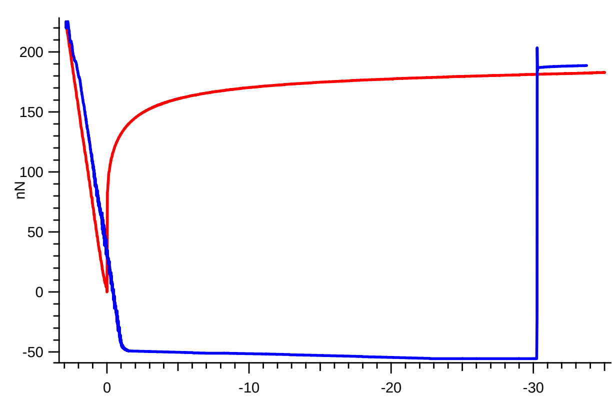


Figure 11.4.5.1: Large surface forces can act on a sphere affixed to cantilever (in air; *k ?*)

For acquisition, some considerations:

▪ Not to indent more than a quarter of the diameter of the sphere affixed to the cantilever- this is for the Herztian mechanics based analysis.

▪ Choose the cantilever spring constant so that it can apply a load to the sample, rather than (fully) deflect with the load- This can be determined from the Force vs. Indentation plot : If the contact regime has a slop that goes to infinity, then the lever is likely fully deflecting under the load, rather than deforming the sample Figure 11.4.5.2 shows an example of this- in Panel A), it isn't really clear that the lever is fully deflecting, however, when force is plotted against indentation (or separation), the slope looks like a right angle to the free air (non contact) portion of thhe curve.

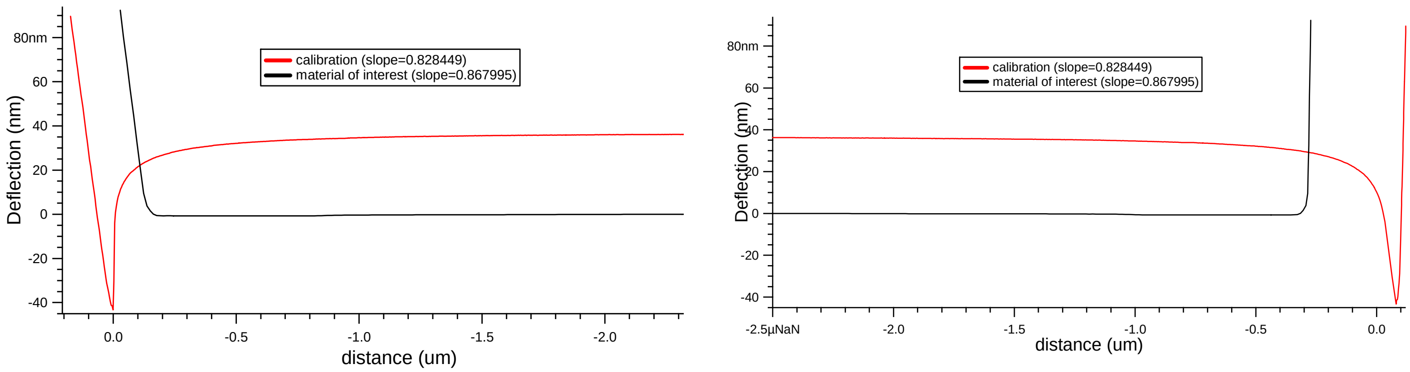


Figure 11.4.5.2: Force vs. distance; B) Force vs. Indentation (deflection (PSD signal) is subtracted from the LVDT signal.

▪ If viscoelasticity is a consideration, perhaps a dwell period at some constant load with an oscillating (dynamic) waveform is in order (see Section 11.4.7 to make custom waveforms in the AR Function Editor)

▪ Confirm that the piezo velocity isn't too fast to cause any hydrodynamic effects (see Section 11.2.9)

▪ To acquire force curves, similar advice would be given that is presented in the previous section- cantilever based indentation (11.4.4). Typically, this are fluid based experiments because the surface area and hence charge of the sphere cause huge tip sample attractive forces.

Colloidal probes are available two ways:

❶Commercially available from NovaScan (Iowa), sQube (Germany) and probably some other vendors;

❷Can be (home) made in the lab, especially if your MFP-3D has some sort of bottom view optics. The author believes the necessity of learning how to do this should be relative to how many are needed for the research. With enough practice, it can be done efficiently without lacking quality, but the first few take awhile and seem to have a larger margin of error in the assembly and aesthetics.
NOTE: Tipless levers are also available from most vendors

Regarding sphere materials, things to consider are:
❶ Material's thermal expansion coefficient is an important consideration (Borosilicate has a favorable one);
❷ Material's modulus (don't want it too soft to act as another spring in the system);
❸ Mono/ poly dispersity (i.e., as close to sphere shape as possible);
❹ Material charge- most polymers are charged, and glass products- can contribute to attractive/ repulsive forces.

Preparing Colloidal Probe tips:

There are different ways to prepare them, however, the author will describe how they are made in Stefan Zauscher's lab (Duke MechE; thanks to Jeffery Coles and Eric Darling for showing me how to make them). It takes some practice to achieve coordination.

The set up is shown schematically in Figure 11.4.5.3A- a pulled capillary (mounted in an XYZ micropositioner) is oriented such that some portion of the end of it can be focused upon with the objective of a standard optical microscope. A small amount of glue is placed on a glass slide and set onto the microscope stage (UV curable Norland optical adhesive #81- *UV cures in about 15 min.*, but is workable for some time).

✓Using the X,Y & Z translators on the microscope, the stage is moved up to the pipette to get a small amount of glue- what normally occurred is several small droplets formed along the pulled pipette, which is fine because the can all be used.

✓ Next, the slide with glue is moved aside, and another standard microscope slide is set on the stage with the probe(s) laying tip side up. The stage is translated such that the cantilever (to have sphere mounted) is brought up to a glue droplet- you'll know when the lever of interest gets close because it will come into focus with the droplet on the pipette (same focal plane); it's OK if the cantilever deflects slightly because the tip shape doesn't have to be preserved. Only a small amount of glue is needed- See Figure 11.4.5.3C.

✓Next, mount the uncured glued cantilevers into the cantilever holder of the MFP-3D; with a sprinkling of the spheres to be glued to the cantilevers on a clean glass slide over the scanner plate in the MFP3D. NOTE: Glass slide should be very clean (Piranha; rinsing with copious amounts of DI water; N2 dry; oven dry).

▪Spheres are made from all kinds of materials- in the author's tutorial, the Zauscher lab used 10 um borosilcate spheres (Duke Scientific). Unless tip-less cantilevers are being used, confirm that the pyramid height isn't taller than the sphere to be glued, or there will be some difficulty picking it up behind (or in front) of the tip.

✓ Using the top & bottom view optics, the probe can be translated over to a sphere on the substrate and the head lowered to make contact. This is remarkably similar to the Cleveland added mass method of spring constant calibration. It's best to have the tip engaged (in contact mode) so that when the head is lowered to make contact, there is some forgiveness from the feedback loop, AND the deflection can be monitored in the S&D meter.

✓ During this pick up stage, monitor the Deflection voltage in the S&D meter- it will deflect to a positive voltage when the tip is lowered onto a sphere. Switching between top and bottom view optics can make this registration process a little easier.

▪ Sphere placement can be in front of or behind the tip. The placement of the sphere can affect the analysis assumptions- be sure to have a plan for analysis before preparing these probes.

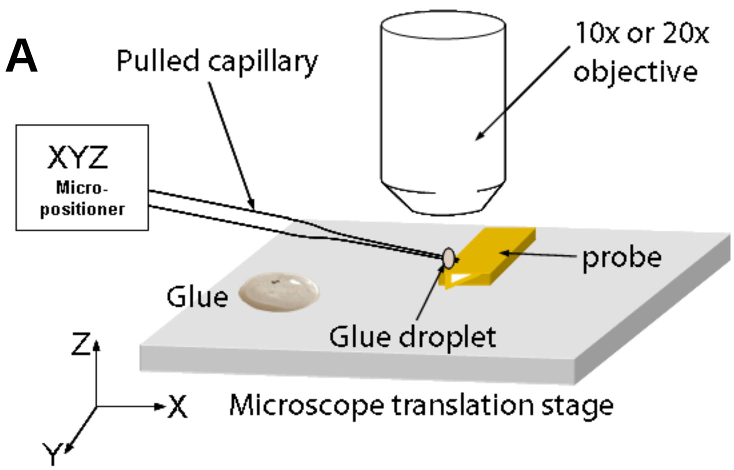
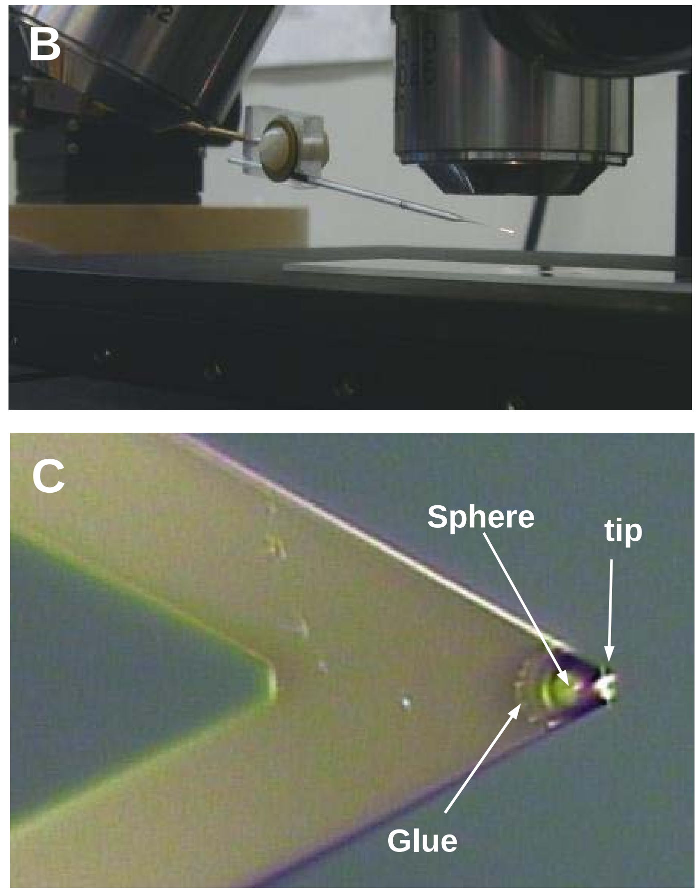


Figure 11.4.5.3: placing glue on cantilevers using optical microscope stage: Focus on pulled capillary containing glue droplets- use microscope stage to bring up to static glue drop until slight deflection is seen in cantilever; B) image of capillary under objective: Example of amount of glue need at end of cantilever (Veeco NP SiNx).



▪ Figure 11.4.5.4 shows an example of a Force vs. Separation curve acquired with a 5µm sphere on a NovaScan SiNx cantilever (k= 0.32 N/m).

▪ Fitting compliance curves with basic Herztian mechanics found in the MFP-3D software is described in Section 11.5.XX– the author is aware that analysis can get pretty involved using more complex mathematical models, but this is a good place to start for those in the crowd that want to dabble with this to make a relative measurement.

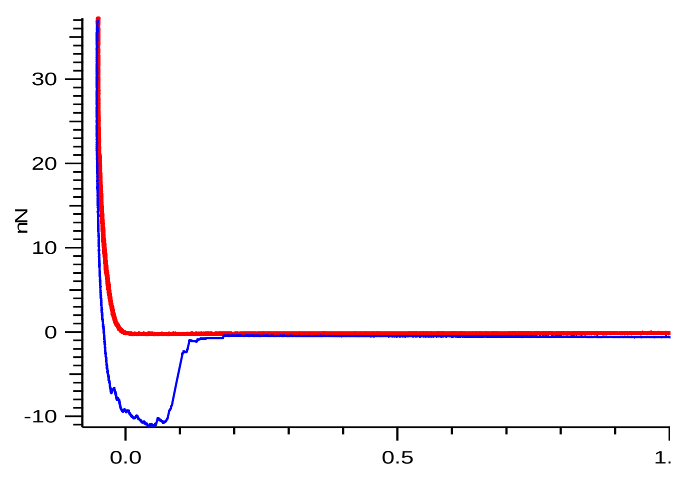


Figure 11.4.5.4: Force vs. Separation plot of a colloidal probe on some soft material.

11.4.6: Dynamic (AC) Force Spectroscopy-

Dynamic force spectroscopy acquires force curves while in AC mode. The Z piezo still performs all the same things as it does in contact mode force spectroscopy (pretty much everything described until this point), but the shake piezo is also being driven such that the cantilever is oscillating, as in AC mode. The interest in Dynamic force spectroscopy is that net attractive and net repulsive forces can act on the tip, just as it does in AC mode imaging (see Section 6.1.4).

Another advantage of an AC force curve is that a specific cantilever oscillation (peak to peak) can be dialed in to 'find' the surface; then perhaps followed by some other routine.

NOTE: Some define 'Dynamic' Force spectroscopy also as doing many contact mode force curves at different velocities to back out loading rates- which may also be an important consideration to be made for a series of force experiments.

As the drive amplitude is varied, the degree of net attractive and net repulsive interaction the tip has with the surface can change. Figure 11.4.6.1 shows various AC force curves acquired on mica at varying drive amplitudes (Figure 11.4.6.1A). Notice the amount of net repulsive and net attraction the tip has with the surfaces varies with distance from the surface.

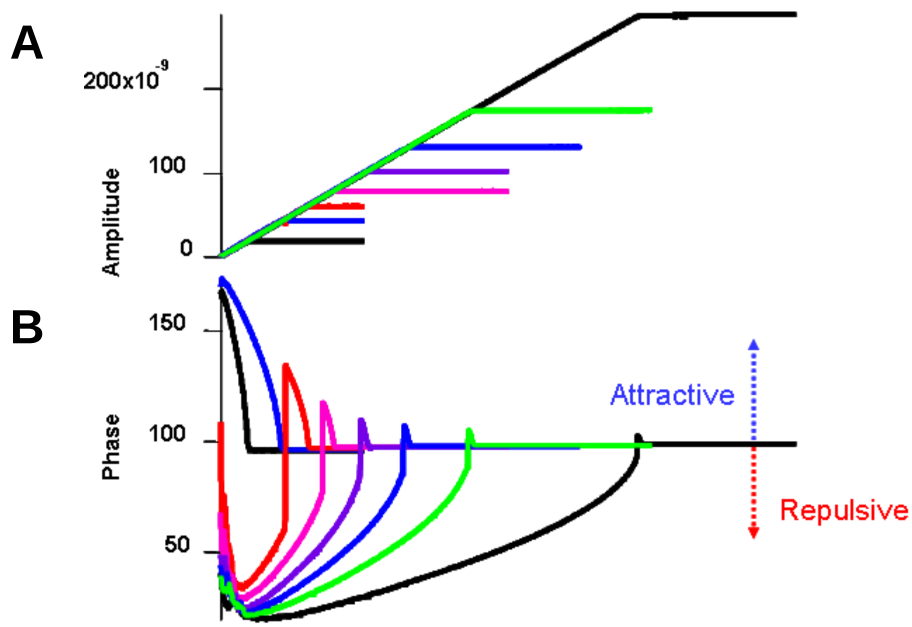


Figure 14.6.2: Scan range box in AR Video Panel: A) setting tip location; B),C), D)- different scan areas and XY offsets (indicated below CCD image. *Figure courtesy of Roger Proksch, Asylum Research.*

The data in Figure 11.4.6.1 was obtained by Roger Proksch, one of our most active researchers at AR regarding AC force curves, and DualAC techniques – so of course this is a very clean looking data set. Figure 11.4.6.2 shows the author's attempt at a similar data set. Using an Olympus AC240 Si lever and a clean glass slide as a substrate, varying the drive amplitude gives the responses seen. Here , it is important to point out that the force distance definition is important- notice the 'Phase freakout' close to the surface on approaches.

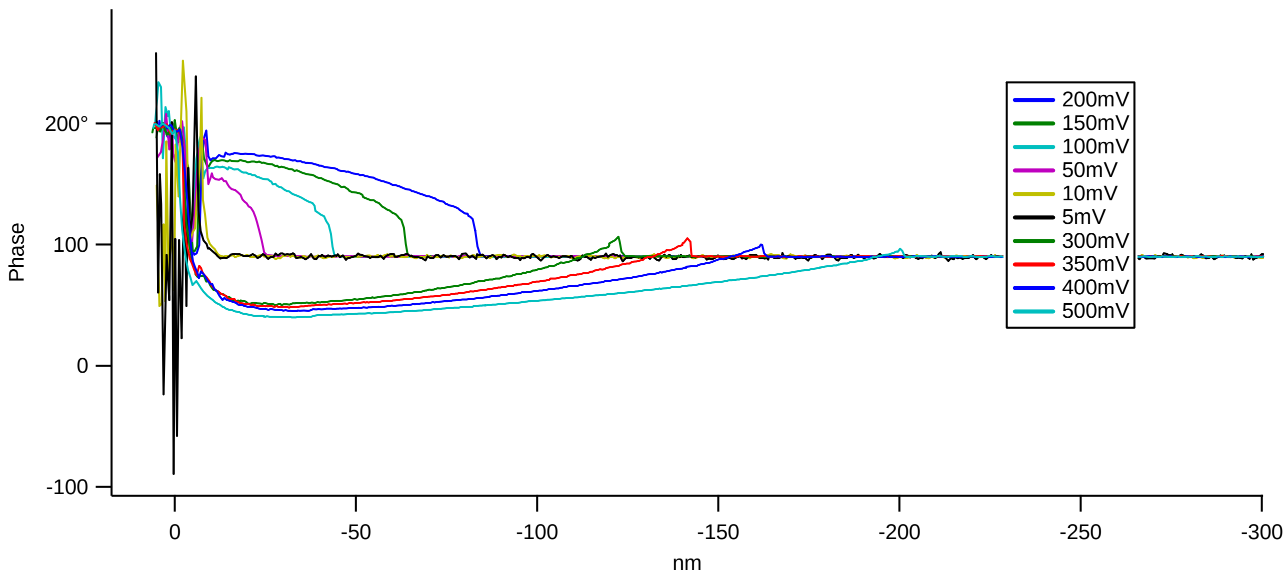


Figure 11.4.6.2: AC force curves on glass as a function of Drive Amplitude

The author's next step was to do some 'point and click' AC force curves at constant drive amplitude on a hetergenous surface known to have different net repulsive /attractive properties. Figure 11.4.6.2 shows some of these 'point and click' AC force curves defined from a phase image of water based latex paint (Sherwin Williams). The sample was imaged with an Olympus AC 240 (k~1.6N/m; ~70kHz) in air in repulsive mode; the free air phase was ~64 ° (depicted by orange dashed line). The force curves were acquired at constant (drive) amplitude (same as the image) and the trigger point was 5 nm to reduce the amount of Phase bi-stability (mode hopping) under applied load. *There are clear differences on the surface*- notice that points 2 & 4 (black and blue, respectively) are similar during the approach (extension) curves, there is an initial attraction to the surface, then repulsion; at some point, the tip becomes closer to the surface, it flipped back to attractive mode. Point 3 (red) never goes into the repulsive regime. *Aside from these observations, the author will not comment further on interpretation.*

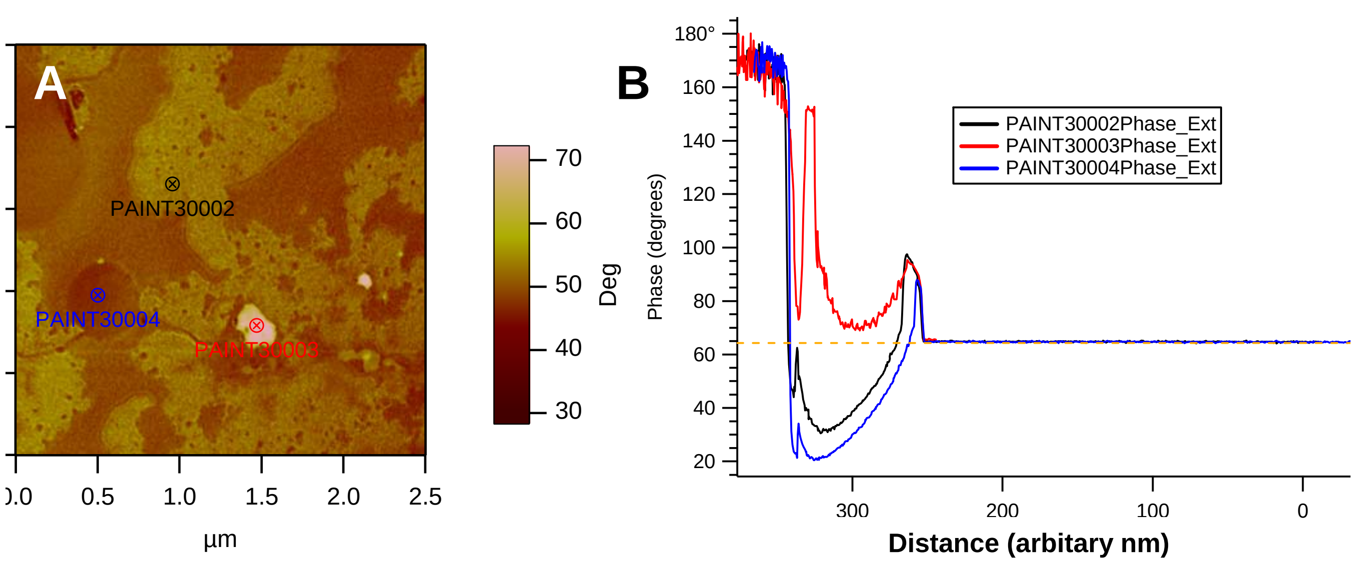


Figure 11.4.6.3: AC point and click Force curves on heterogeneous sample: A) Phase image of paint sample with location of user defined force curves; B) individual AC force curve Phase vs. distance (Z)- orange dash line indicates Free Air Phase values of ~64 degrees. (yes, that is the di color table on the 3D)

11.4.7: The MFP-3D Function Editor

The MFP-3D software has something known as the 'Function Editor', which is very useful for applying custom made waveforms for force spectroscopy, indentation (and various electrical) techniques.

To open, go to Programming → Function Editor. A panel like the one shown in Figure 11.4.7.1 will appear.

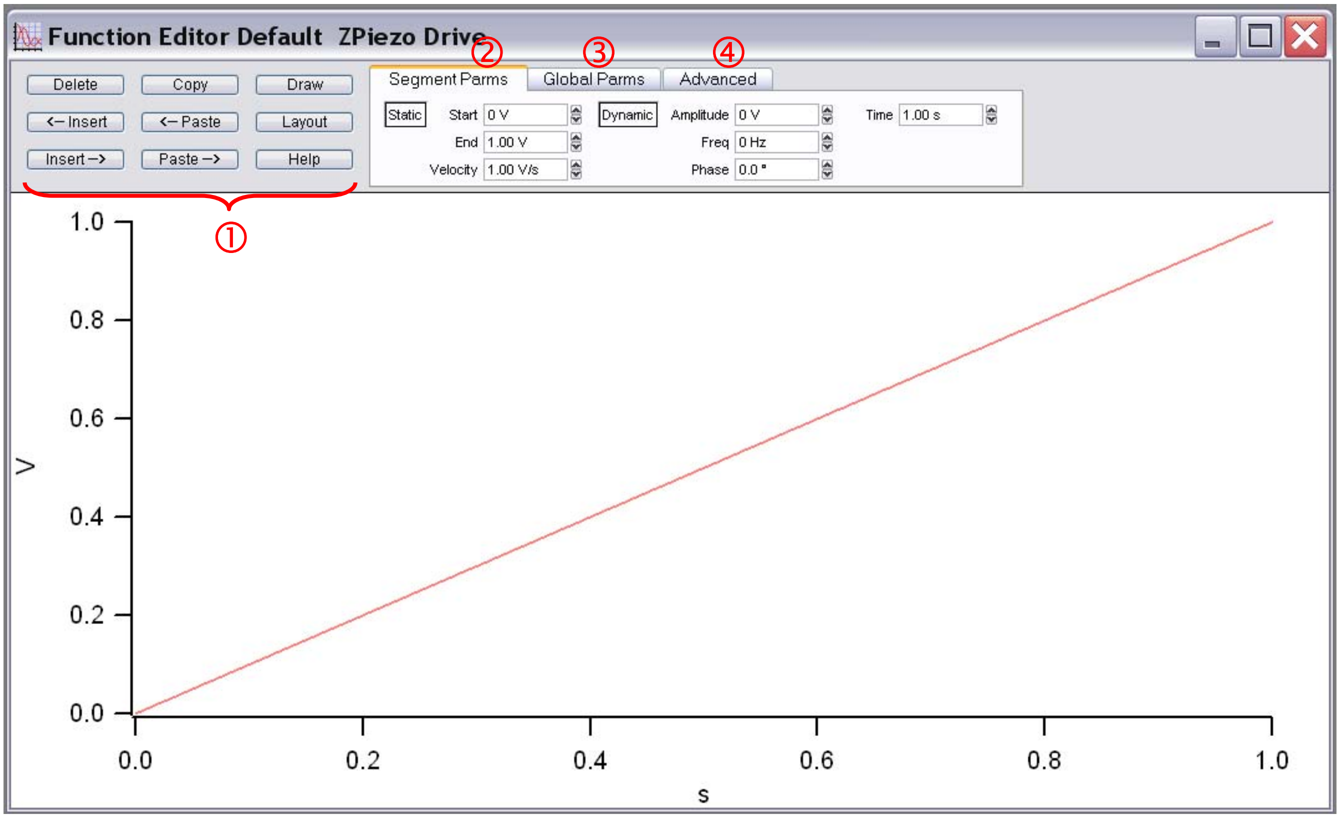


Figure 11.4.7.1: The AR function editor allows custom wave forms to be applied to the Z piezos, or tip sample biases, for advanced force spectroscopy expts.

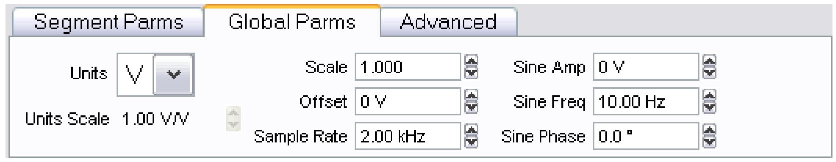
①The buttons to the right of the panel allow the segments to be copied then pasted to the left or right of the copied segment.
Insert- will insert a segment to the left or right of the forward-most (red) segment, having the same constant values as the red segment its attached to.
Paste- paste a copied segment; will have the same parameters as copied segment.
Copy- copy a segment. Segments must in activated (red) with mouse click to copy them
Draw- allows 'free hand' line segments that are strung together.
Layout- this dumps image of function to an Igor Layout (*which are awesome!*).

Whichever segment is displayed as red is the forward most segment to be manipulated via the buttons or setvars.

② *Segment Parms subtab-*
Static: this is for DC voltages applied to the piezo (stack); the 'Start' and 'End' Piezo voltages, and velocity can be user defined.
Dynamic: this is for AC voltages applied to the piezo (shake); the amplitude, Frequency and Phase setvars can be adjusted accordingly. This is useful for oscillating the tip for viscoelastic materials.

③ *Global Parms subtab-*

This scales and offsets the signals in the function generator



'**Units**' pull-down allows choice between Volts (applied to piezo) or meters (accurate with calibrated InvOLS). This can also be useful if using the AR Function Editor for applying electrical biases to tip or sample if doing an electrical technique (*not described here*).
'**Units Scale**' will give the sensitivity (InvOLS) displayed if meters selected for Units.
Scale- Allows the generated function to be scale be some setvar factor; axes will rescale.
Offset- Will offset the signal on the Y axis.
Sample Rate- what frequency (# of pts/sec) the data is collected at.
Sin Amp- Allows the sinusoidal amplitude to be scaled.
Sine Freq- Allows the frequency of the Sine signal to be scaled.
Sine Phase - offsets the Phase from 0 to some other user defined value.

④ *The Advanced subtab-*

The author is not advanced enough to describe this competently.

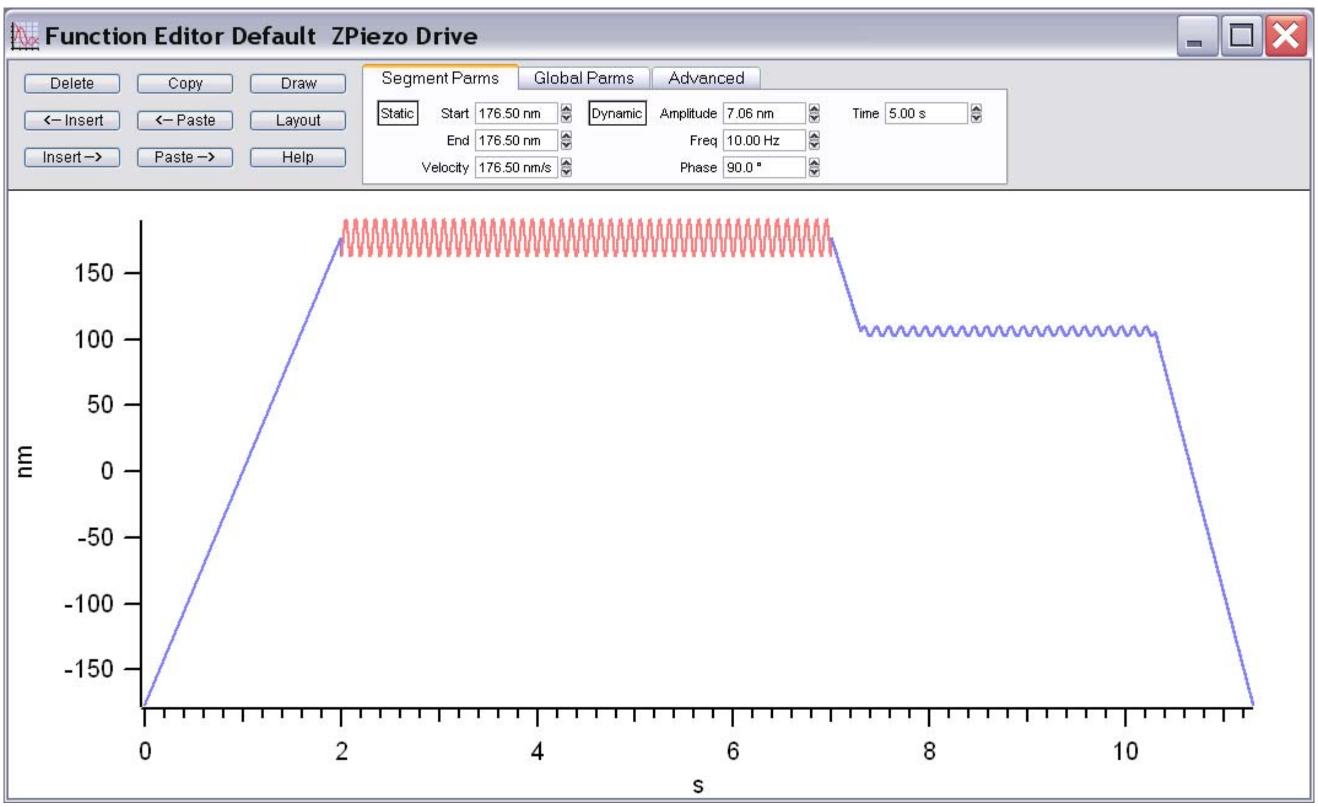
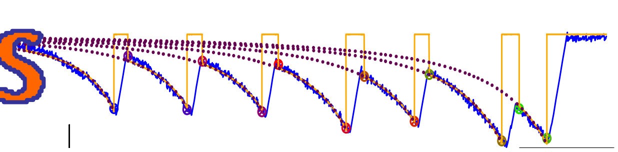


Figure 11.4.7.2: a custom wavefom created by the author for an example.

▪ Figure 11.4.6.2 shows a simple function that was generated in the Editor- many segments were added and set to tell the cantilever to deflect to 176 nm, hold for 5 seconds with an oscillation of 10 Hz with 7.06nm peak to peak amplitude at 90° phase, pull back a bit and hold again for 3 s at 8 Hz with 31.52 nm peak to peak amplitude 0°, then return to a negative deflection. Note that this was just made up and unlikely to work in deflection unless some major adhesion force could grab the tip for a 150nm deflection in a real expt.

11.5: Analysis



The MFP-3D software has some force curve analysis built into it. The force display panel has a lot of features, so the author attempts to tell what he knows about these features.

11.5.1: Loading force data:

✓ To load curves, click on the 'Review' button in the Force tab of the Master Panel; this will bring up the Master Force Panel (Figure 11.5.2A.1) and the The 'ARPathDialogue' will appear asking where to load the curves from (Figure 11.5.1.1) .

✓ If the displayed path is pointing towards the desired files, click 'That's It' button.

If The AR path dialogue doesn't have the desired path in it- do one of three things-
❶✓ Use the 'Select Path' pull down menu- there are ~20 previously used paths in a chronologically opened list- *perhaps yours is in there?*
❷✓ Use the 'Default Paths' pull-down menu- this will give a chronological list of dates the instrument was used (i.e. the data file in 'My Documents' that gets created automatically).

❸✓ Click the 'Browse' button which will bring up the 'Path to Load Data' dialogue.

✓ Click the 'Browse' button which will bring up a 'Browse for Folder' dialogue.

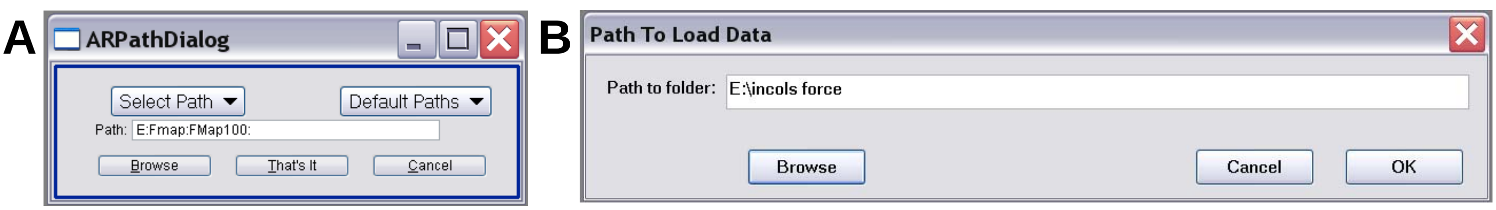
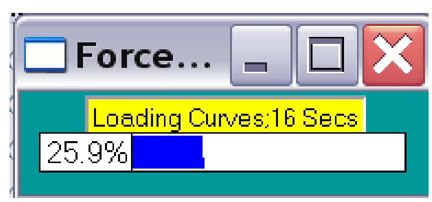


Figure 11.5.1.1: Loading force curve files in the MFP-3D software: A) ARPath dialogue inquires location stored data location; B) Clicking Browse offers a Path to Load Data dialogue points to stored data if different than ARPath.

✓ Select the desired folded and click the 'OK' button; this will refesh the Path in the 'Path to Load Data' dialogue.

✓ Click the 'OK' button; a small loading meter will pop up while Igor is loading the curves indicating progress and remaining time.



▪The last ARPath Dialogue location will be stored until next time a file load request is made.

11.5.2: The Master Force Panel: The Subtabs

This panel has many options built-in for force curve analysis in the MFP-3D software. It consists of nine tabs, each will be described below. As always, the help menus are pretty good at describing what the functions do; yet sometimes are not all that procedurally descriptive.

11.5.2A: The *Display* subTab:

The Display tab is where curves are loaded, axes chosen and curves (either individual or multiple) are listed to be displayed.

①**Load Curves**- Described in Section 11.5.1.
② **Multiple Axis** selection- from this pull down, multiple Y axes can be displayed: up to five Y axes can be displayed, just as with the Force Channel Panel.
③ **Y axis data to be displayed**- data that is listed in black is saved data from the initial experiment- the Force Channel Panel’s left most Save Column is where this comes from (i.e., green dots in Figure 11.2.6.1).

▪ Values that are gray (i.e., cannot be selected) are values that were not activated in the Force Channel Panel, and hence not saved data.

▪ Clicking the ‘Select 0’ button below this unselects any previous Y axis selection(s) when one’s mind has changed- makes it easier than scrolling through the menu to see what was selected for Y axis display.

④**X axis to be displayed**- this pull down allows the user to choose what X axis data will be displayed (i.e., raw, LVDT, indentation, separation, Force, Deflection Volts, raw volts, Drive, Drive voltage, time). Each has their own special place in data representation.

▪ Indent and Separation X axis plots the Y axis as LVDT- Deflection; the difference between the two is that indent plots the event on the right, while separation plots the event on the left.

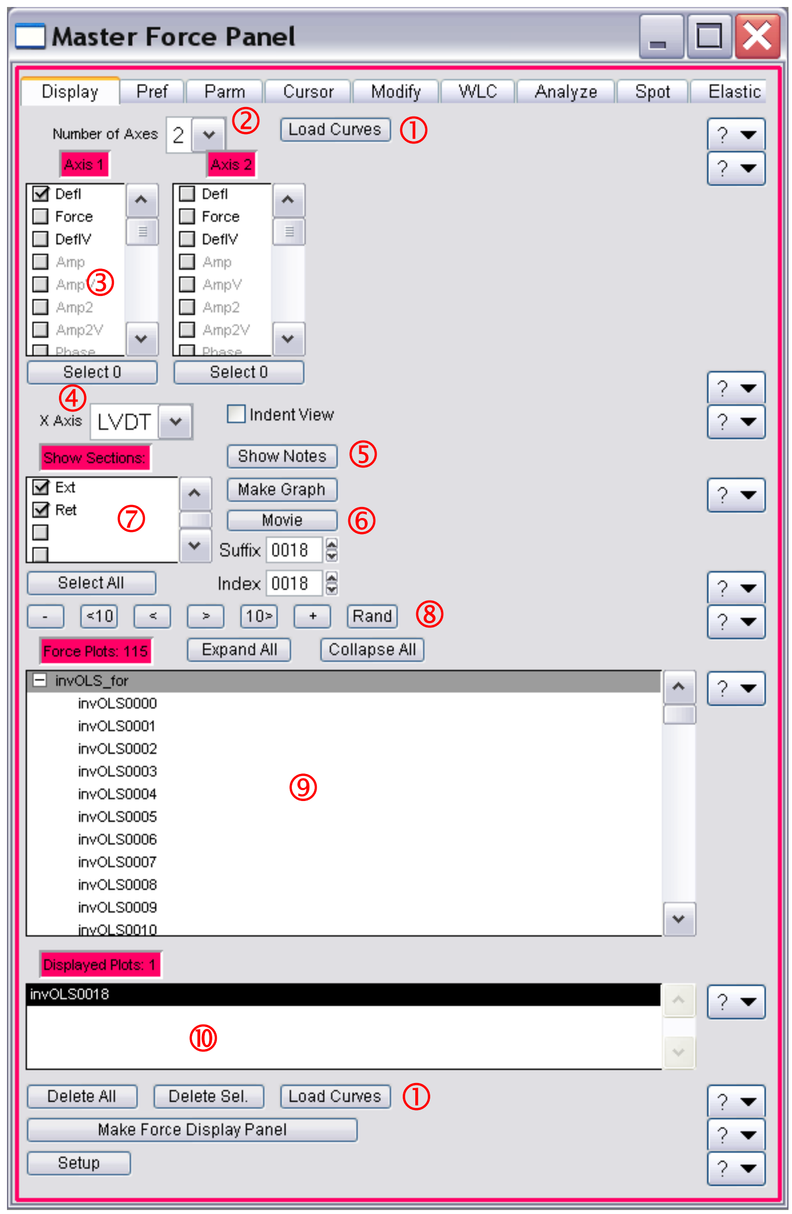


Figure 11.5.2A.1: The Display tab of the Master Force Panel. Curves are loaded, plot axes are chosen and curves displayed in this tab.

▪ By clicking the 'Indent View' ☑ checkbox, the X axis is switched to 'Ind', and automatically puts the surface on the right side of the plot, as is done with indenter data.

⑤**Show Notes**- this brings up an Igor spreadsheet of the global variables that has every saved imaginable saved parameter in it for your perusal pleasure.

⑥**Movie**- this allows the user to sit back and watch Igor scroll through the force curves (at a user defined rate) so you can concentrate on looking for interesting curves events, without incessantly pushing the advance button. *See the end of Section 11.5.2A for more on the 'Movie' button.*

⑦**Show Sections**- this allows the user to display extension, retraction, Dwell, and clamp (depending on the expt.); For example, if you are only interested in the extension part of the force curve, just have 'Ext' selected.

⑧**Scrolling buttons**- these buttons allow the user to scroll forward or backward through the saved force curves; either individually or in leaps of 10; or randomly by clicking the 'Rand' button- this is nice for statistical purposes.
The '+' button highlights additional / sequential multiple curves that are displayed in the Force Review Graph. The '–' button removes the last curve selected in a multiple non-sequential curve display.

⑨**Force Plots**- this area is where all the loaded force curves are;
✓Click on a filename to load the curve into the Force Review Plot; Clicking Shift or Ctrl allows multiple curves to be selected and displayed.

✓ To **select multiple sequential curves**,
❶ Highlight the first curve while holding the shift key down (Figure 11.5.2A.2A).
❷ Go to the last desired curve (which will highlight that individual; Figure 11.5.2A.2B).
❸ Go back to the first one (while holding down the shift key) and click on that one again- all the curves between them will be highlighted and displayed (Figure 11.5.2A.2C). If many curves are being highlight (i.e. loaded into memory), it may take a moment to display them; notice the Igor processing icon at the lower left of the software tray.

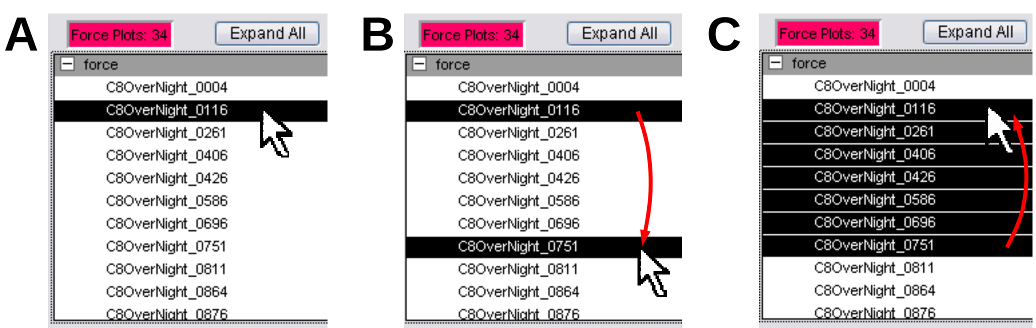


Figure 11.5.2A.2: Highlighting multiple, consecutive force cures: A) select the first; B) shift + mouse click on the second; C) shift + re-click on first highlights all between.

⑩**Displayed Plots**- This shows what force curves are displayed in the 'Force Review Graph (also highlighted in ⑨ Figure 11.5.2A.1).

▪ Once a curve is selected from the Force Plots section, the curve will be loaded into the Force Review Graph.

Figure 11.5.2A.3 is an example of a force curve on a hard surface with some relative trigger. Notice the buttons along the top of the Panel- these allow the typical export and save features that many of the data plots in the MFP-3D software offer.

✓A zero or contact point can be manually defined by placing the cursor at a point on the force curve and right mouse clicking- choose one of the offsets and it will adjust it to zero on the X, Y or both axes. In Figure 11.5.2A.3, the cursor was placed on the extension part of the curve, and offset in X&Y; the result is that point is zeroed (see inset).

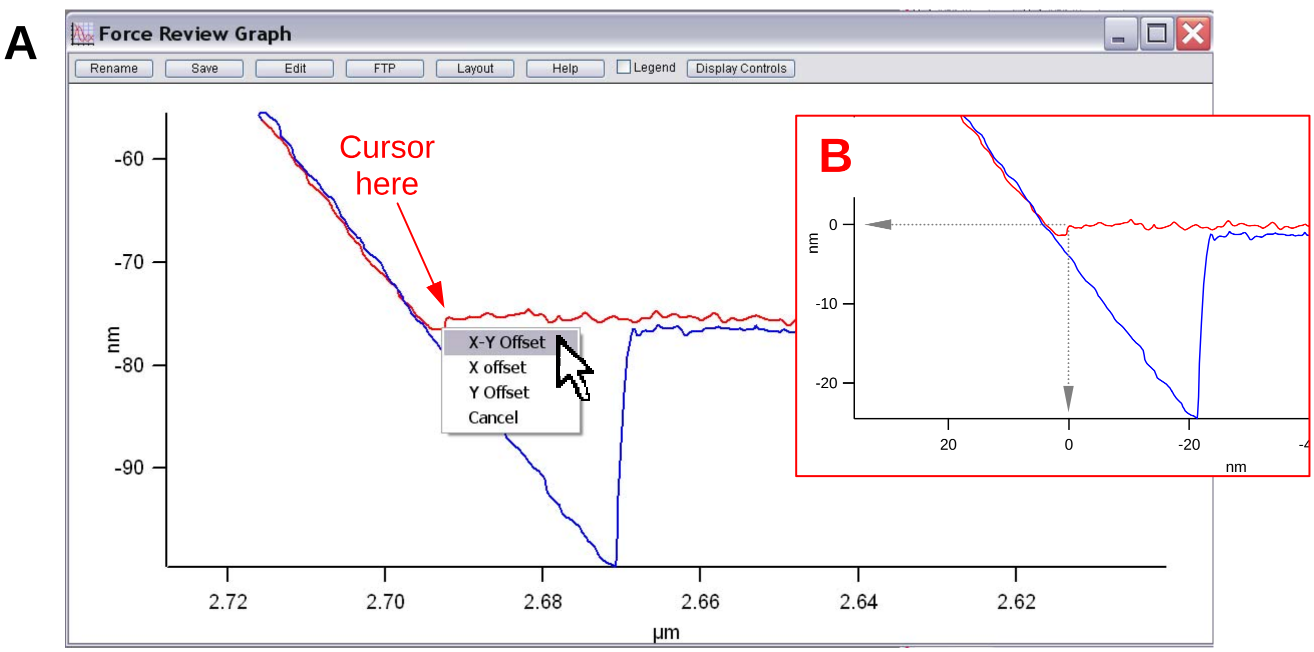


Figure 11.5.2A.3: Manually offsetting curves in the Force review graph: A) Place mouse cursor at curve location to offset to zero; right mouse click and select with axis to offset; B) result of X-Y offset on A).

More about the Movie Player-

⑥Clicking the 'Movie' Button brings up a small panel (Figure 11.5.2A.4).

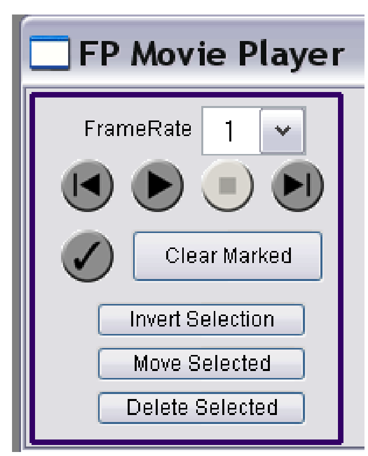
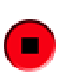


Figure 11.5.2A.4: The force curve review Movie Player Panel.

▪ **Frame Rate** pull down: 0.7 to 2 in 0.1 Hz increments; The author has experienced frame rates larger than 1.1Hz makes it difficult to stop the force curve you want to view at because it's tough to Interrupt Igor between frames to actually press the Stop button.

 To Begin, press the Play button; this will begin the process of shuttling through the loaded force curves.

 To stop, press the stop button.

 To mark force curves of interest, press the checkmark button; this stores the selected force curves in memory- accessible via the Data Browser.

▪ the 'Clear Marked' button- removes from data browser (?)

▪ the 'Invert Selection' button- This just swaps which files have been selected or remain unselected. If you want to select most of the files, it may be easier to just select the few you don't want, and then invert the selection.

▪ the 'Move Selection' button- this brings up a 'Question…' dialogue asking for a folder name to be created that will dump selected curves into this New folder. This folder is located in the subfolders string (see Figure 11.5.2A.5). Note that the extension, retraction and LVDT data will be displayed.

▪ the 'Delete Selection' button- this should delete a force curve from the memory holding the selected curves.

NOTE: clicking the help button in the Data Browser brings up a detailed Igor help .ihf file describing the data browser. *The author views the DB as a vast Igor underworld.*

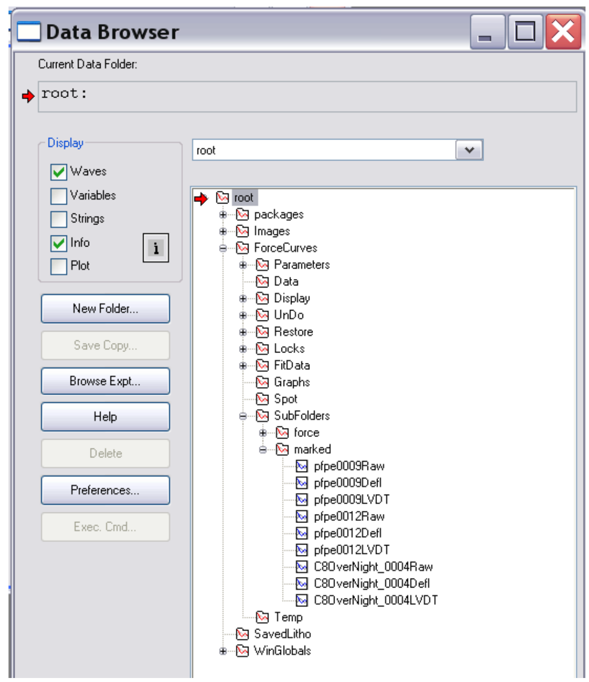


Figure 11.5.2A.5: Igor Data Browser selected force curves from Movie panel in the marked folder in the SubFolders .

11.5.2B: The *Pref* subTab:

The author had no experience using this tab because of code writing ineptitude. Let's move on…

11.5.2C: The *Parm* subTab-

This tab is useful for looking at force curve file parameters-

▪ Under each Title box are pull-down menus that can display various parameters. Next to each respective title box, a setvar allows a number of parameters to be shown. The author suggests exploring what each pull down offers in regards to stored parameters for the displayed waves (force curves), largely because it's not worth listed them here.

Figure 11.5.2C.1shows a Parm Panel the author set up to display desired parameters. Notice in XPT & Thermal, the setvar is set to zero because none of those parameters are of importance to the author in this example. Also notice that are three force curves' acquisition parameters displayed in the columns on the right.

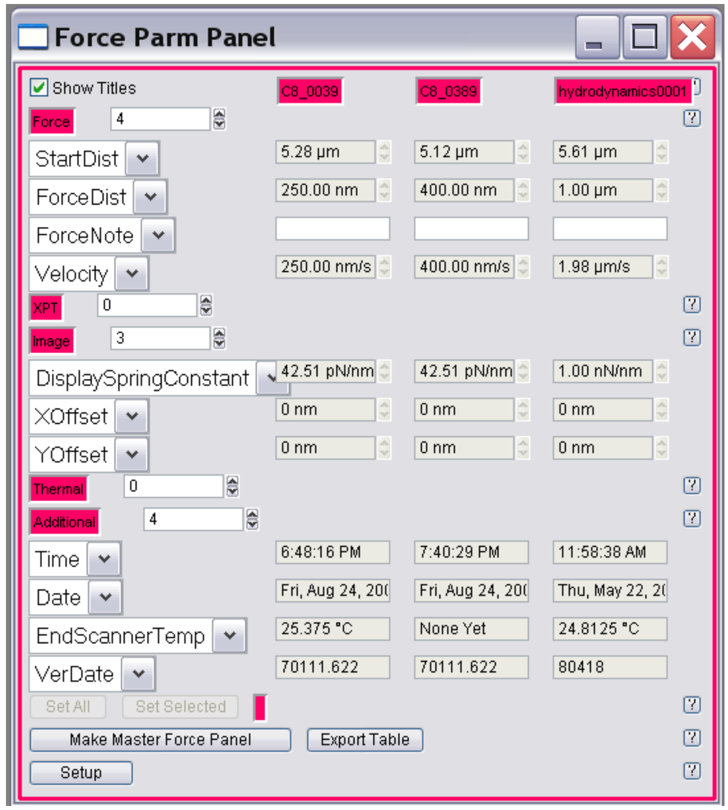


Figure 11.5.2C.1: The Parm tab shows stored parameters values for each open curve.

▪ The author will often make an additional Parm Panel, as in Figure 11.5.2C.1 *(notice no upper tabs!)*, so that curves can be selected in the *Display* tab without having to shuttle between Parm and *Display* subtabs to look at the parameters. This feature is great for working offline on the curves you are trying to remember what occurred during the experiment.

Changing Force curve parameters-

The *Parm* subTab also allows parameter changes to be made to the curves offline- say you didn't know the spring constant of the cantilever until the end of the experiment, but the saved data has the default value of 1N/m in it.

Procedure-

✓ With the proper parameter open in the title box, change the setvar value to the proper value- this will update the information immediately.

✓ To permanently change the parameter, click the 'Set Selected' button towards the bottom of the panel.

✓ To change *all* the parameters in the set, click the '**Set All**' button towards the bottom of the panel.

✓ Clicking the '**Export Table**' button at the bottom of the panel will create a small spreadsheet with all the parameters set up by the user

▪ Figure 11.5.2C.2 shows an example of two consecutively acquired force curves with the same parameters. Before changing the InvOLS value on the black curve, the slopes are the same (Figure

11.5.2C.2B inset); After changing the InvOLS to half its initial value, the slope of the contact regime is updated (Figure 11.5.2C.2B).

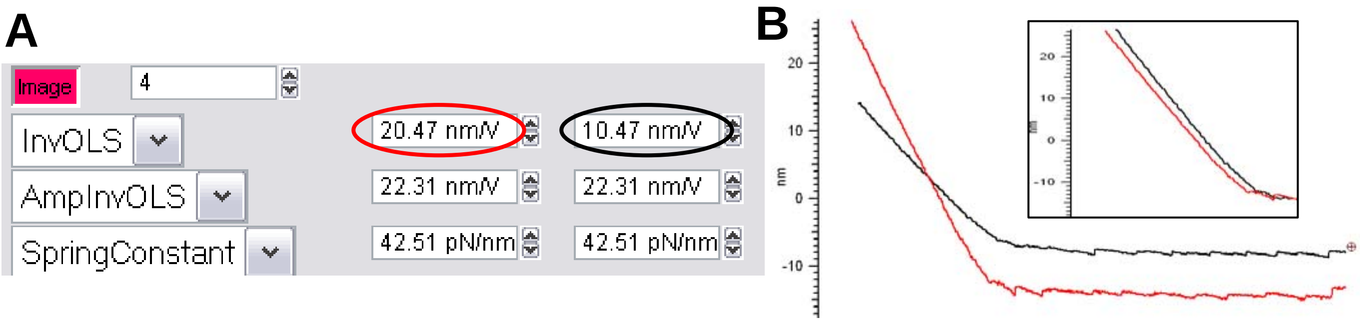


Figure 11.5.2C.2: Changing setvar values in the *Parm* subTab: A) two consecutive curves with same parameters, but changing black curve to roughly half the InvOLS value; B) result in slope of contract regime (Inset: same InvOLS values)

11.5.2D: The *Cursor* subTab-

This tab allows the user to install cursor 'locks' on the curve- these are typically used in conjunction with the WLC and Elastic subtabs, described / reiterated in Section11.5.2F & 11.5.2H, respectively.

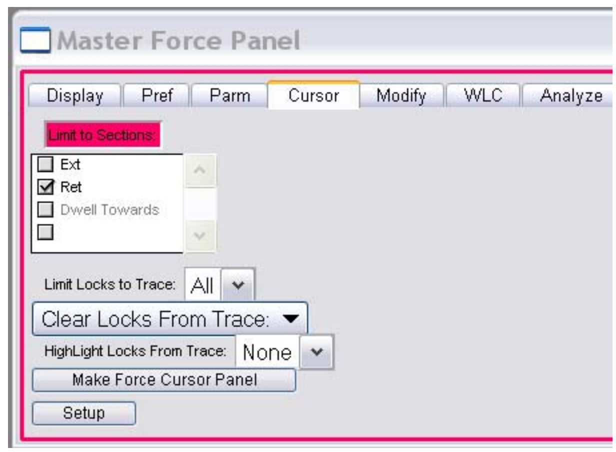


Figure 11.5.2D.1: The Cursor subtab – useful for installing cursor locks on force curves for function fitting.

✓ To place a 'lock' on a curve, place the mouse cursor in the desired location and click the left mouse button while holding the shift key down (Figure 11.5.2D.2A). Multiple locks can be installed per curve (see WLC, section 11.5.2F).

✓ To *remove* an individual lock, place the cursor on the lock and left mouse button while holding the Ctrl key.

✓ To remove *all* locks from a curve or multiple curves, choose the specific curve (or all curves) from the 'Clear Locks From Trace' pull-down menu; Click 'Clear Locks From Trace' button to activate this process (Figure 11.5.2D.1).

▪ The 'Highlight Locks From Trace' pull-down allows the 'locked' cursors to appear much larger- this is good for all those old folks out there that have trouble seeing smaller things - *like the author does* (Figure 11.5.2D.2B).

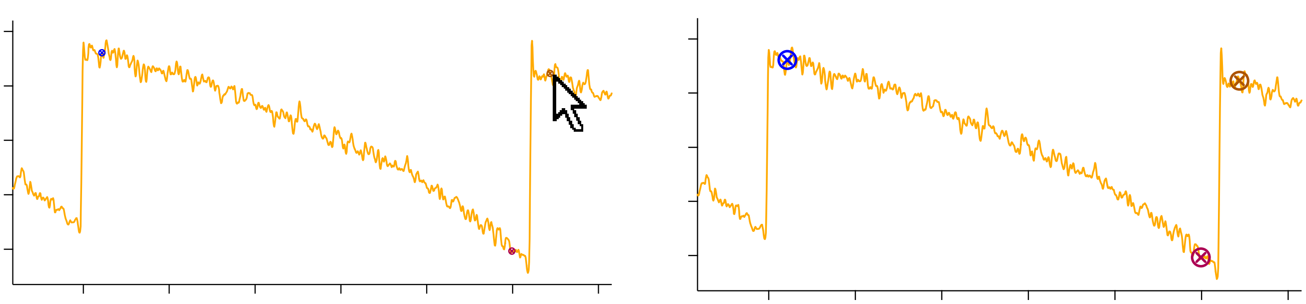


Figure 11.5.2D.2: Installing locks on a curve using the Cursor subtab: A) Shift + left mouse click at desired location installs cursor locks; B) Locks highlighted (increases lock size for better viewing).

▪ When multiple force curves are in memory, and have cursor locks installed, The 'Limit Locks to Trace' pull-down menu allows all the curves selected (if more than one), or selected to be chosen. For example- In Figure 11.5.2D.3, only the locks in curve 0004 (red) can be manipulated, while the ones in 0261 (blue) cannot.

11.5.2E: The *Modify* subTab-

This is a very useful subtab, and there is a lot going on here- points in curves can be zeroed in X,Y or both; virtual deflection (or other portion of curve) can be subtracted out; force curves saved to memory in an experiment can be saved to .ibws for each curve (if that wasn't done initially).

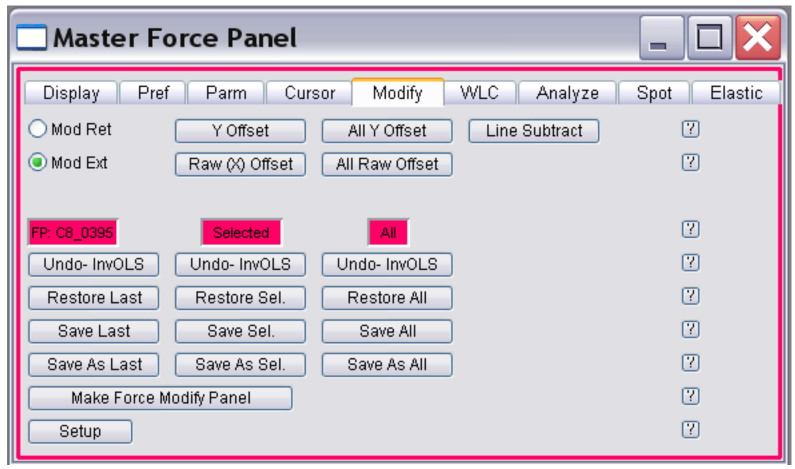


Figure 11.5.2E.1: Modify subtab.

the subtab is broken down into three general columns: under the left title box is the 'last' curve selected; the middle 'Selected' curve is or are the curve(s) selected- operations will be performed on multiple curves selected; the last (right) column will perform operations on all the curves in memory- this is nice for batch stuff.

Y & X (Raw) offsets:

There are non manual ways to offset the force curves, opposed to those described in Figure 11.5.2A.3 above:

Y offset: this operation averages the last 10 points of the free air, and makes that zero. Typically, if there is a small attractive 'jump to contact' in the extension cycle of the curve, the zero point will be just to the right of it.

▪ Figure 11.5.2.E.2 shows a before and after (inset) of Y offset of simple force curve.

✓Clicking the 'Offset all Y' button will offset all the curves that are loaded in the memory.

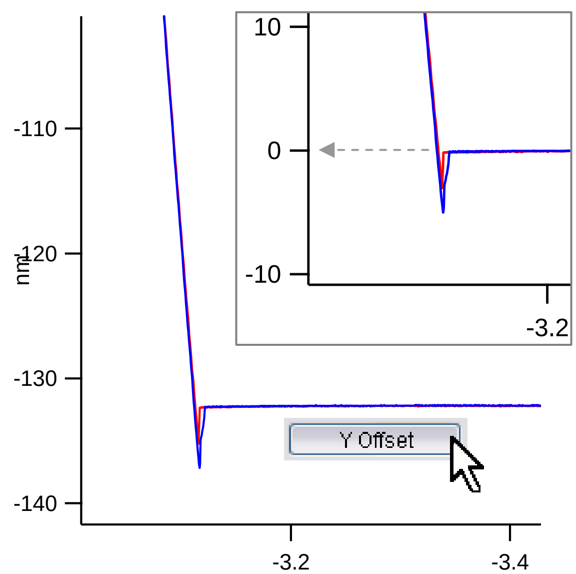


Figure 11.5.2E.2: Offsetting the Y axis in force curves using Modify tab: Click the 'Y Offset' button, which will offset the curve to zero on the Y axis (inset).

Raw (X) Offset: this operation defines the surface as zero distance, and defines the surface as when the deflection of the lever is the same as the free air.

✓Click the 'Raw (X) Offset' button.

▪ Figure 11.5.2E.3 depicts this with some author drawn lines to guide the eye. At the point of intersection, the deflection would equal the deflection of the free air (i.e., no positive or negative deflection in the lever).

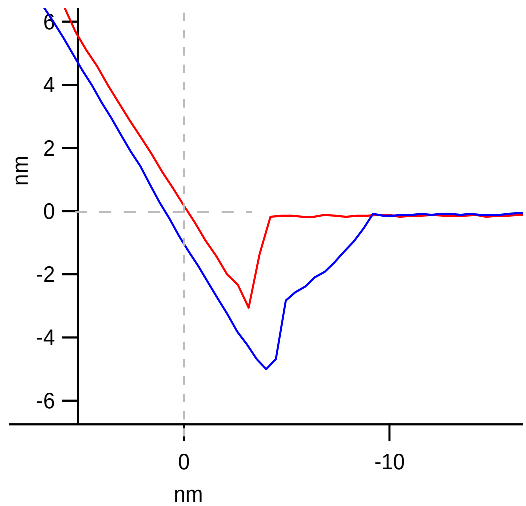


Figure 11.5.2E.3: Offsetting the X axis in force curves using Modify tab: Click the 'Raw Offset' button, which will offset the curve to zero on the X axis.

Line subtraction-

For occurrences in which the virtual deflection *(see Section 9.1)* was not calibrated and all the curves in a data set have a little bit of slope in the free air portion (Figure 11.5.2E.4A)- this can affect the accuracy of where the zero point of the curve is, and ultimately the magnitude of the force/ deflection/ etc. value calculated. Well this can be corrected in the Modify tab:

Procedure-

✓ Place the Igor cursors on the extreme ends of the linear region, preferably the extension part of the curve.

✓ Confirm that the Modify Ext. radio button is activated (Figure 11.5.2E.1).

✓ Click the 'Line Subtraction' button in the *Modify* subTab. The curve will become parallel to the X axis (Figure 11.5.2E.4).

▪ These modifications can be saved individually (Save Sel), or in bulk (Save All), depending on what operation was executed.

✓ Click the 'Save Sel.' button to save this change to the individual selected force curve. It will overwrite the raw data.

▪ Figure 11.5.2E.4 shows an example of a force curve collected on a MFP plus in the old Igor 5 software (i.e., before the virtual deflection calibration was featured in the software). Here, just the Extension part of the curve is shown.

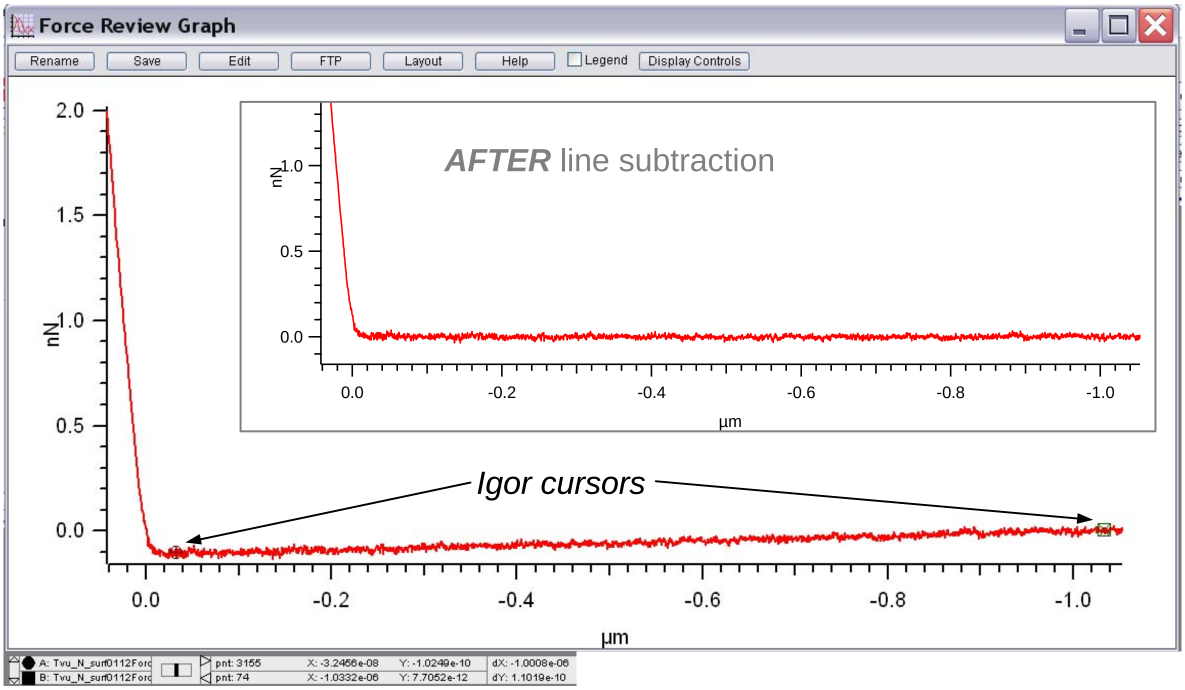
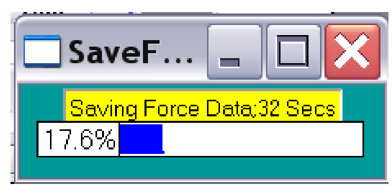


Figure 11.5.2E.4: Example of using line subtracting to remove slight virtual deflection from a force curve (acquired on older MFP-3D/ Plus software).

NOTE: Line subtraction doesn't work if Y axis is on Force- must be on deflection.

Saving curves from memory to ibw's: Sometimes an Igor experiment is saved, but the force curves were not saved to disk, just to memory. When opening up the experiment, the force curves should be loaded in memory, as seen in the lower box in the *Display* subTab.



✓ To Save the curves to individual .ibw's, click the 'Save All' button, which will convert all the curves saved in memory to individual .ibw's (igor binary waves), dumping them into what ever folder the initial experiment was saved in.

✓ To change folder directories, click the 'Save As All' button, which will bring up the AR Save Path dialogue awaiting the user to indicate a path to the desired folder.

If the curves weren't saved to memory in the experiment, there is little chance of recovery, seemingly.

11.5.2F: The *WLC* subtab-

This tab is where Worm Like Chain fitting occurs, in conjunction with locks placed on the curves described in the Cursor subtab (section 11.5.2D). This is generally used on force curves that show 'saw tooth' patterns- typically proteins whose domains are being pulled apart. Each one of these saw tooth dissociation events are commonly termed 'hitches'. There are two models (i.e., single or multi chain models) that can be fit, depending on if the sequential hitches are influenced by the preceding hitch, or not.

▪ Another description of this resides on the FAQ portion of the AR website: http://www.asylumresearch.com/Support/FAQ3D.shtml

Locks need to be installed on the hitches of the Force vs. Sep plot….

✓Load the force plot(s) to be fit. Accurate InvOLS and *k* are important, or the Force *vs.* Sep plot (see next step) will be compromised.

▪The demonstrative example in Figure 11.3.2F1 shows a titin construct (data *courtesy Jason Cleveland (AR) and Jan Hoh (JHU)*) with some sawtooth dissociation action (k=42.51 pN/nm; InvOLS=20.47nm/V). NOTE: only the interesting part of the retract curve of the deflection vs. distance plot for fitting the WLC is shown (Figure 11.5.2F2).

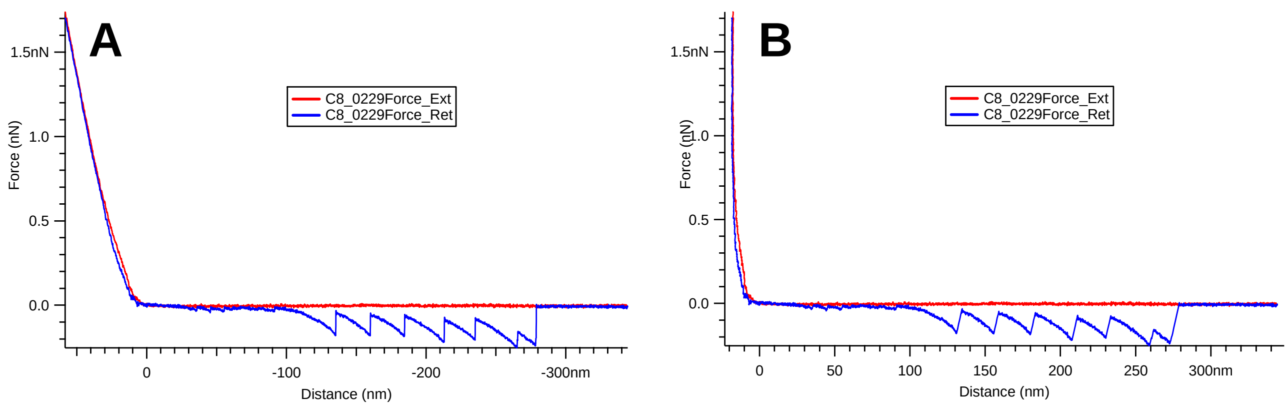
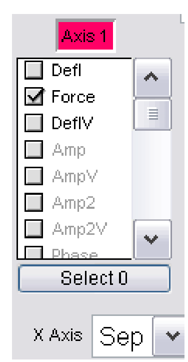


Figure 11.5.2F1: A) Force vs. distance; B) plotted as Force vs. Separation- contact regime is equal to unity.

✓In the *Display* subtab, plot as Force *vs.* Sep (Sep is short for tip - sample separation). This should make the contact regime of the curve have a slope of infinity (if on an infinitely hard surface).

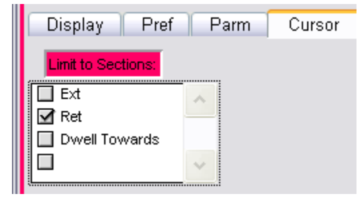


Separation is the LVDT sensor signal – Deflection signal

✓Offset X&Y to zero, either manually (via mouse cursor on curve, see Section 11.5.2A), or use the 'Offset Y' and 'Offset Raw' buttons (Section 11.3.2E) in the *Modify* subTab. This is an important step.

✓In the *Cursor* subTab, select the ‘Ret.’ (retract) checkbox.

✓ Expand on the hitch region because this is where most the dissociation events occur (Figure 11.5.2F2).



✓On the force plot, place the locks on the graph to define the stretching regions (Shift + left mouse click, Section 11.5.2D) - these are the lock points described in Section 11.3.2D: the error region of the fitting function must be defined, so pick one point where the polymer ruptures its attachment to the tip, and pick the other point at the lowest extension as the data looks like it will be fit. As the locks defining the hitches are installed, a fitting curve will appear (Figure 11.5.2F.2A), although not yet correctly fit until the ‘Fit’ button is executed.

▪ Multiple stretching events can be fit in a single force plot: pick two ‘locks’ (cursors) to define each stretching event (the software calls polymer stretching events hitches).

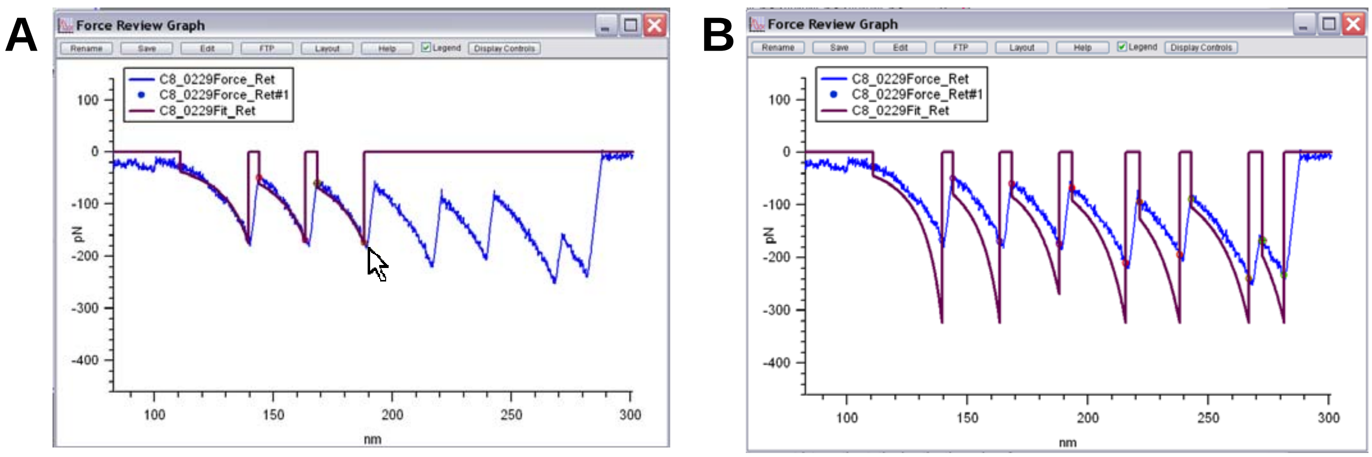


Figure 11.5.2F2: A) Installing locks on sawtooth pattern for WLC fit: as locks are defined, fit line appears and is updated; B) all locks installed, but WLC not fit.

✓Go to the *WLC* subtab. There is a lot going on here- the author will try to explain- the software help menus are also very useful here:

① Multi Chain model/ Single Chain WLC models:
To determine whether the multiple hitches in the force plot to be fit via either single chain or multi chain, consider the following: *Is the observed response due to one chain with domains or loops (Single Chain); or is each hitch the result of stretching a separate chain (Multi Chain)?*
That is-
▪**Single Chain**: You are stretching only 1 chain and each observed elastic response is the result of that single polymer chain unfolding

▪**Multi Chain**: Each observed elastic response is the result of an independent polymer chain that is not interacting with the other polymer chains that are being stretched in parallel.

⇨If you think you have a single chain, then you want to unselect the Multi Chain ☑check box (upper left corner, Figure 11.5.2F.2). Single Chain is when the latter hitches are influenced on the preceding hitches. In this example, Single chain is being used.

⇨If you think that each hitch is from a separate chain, then select Multi Chain☑ checkbox. Multi Chain is when each hitch is not dependent on the preceding hitch.

②**Single Persistence length** ☑ checkbox- this should be checked if (you think) the domains of the same size are being pulled apart. Uncheck it if they are not.

③ **Temperature**- can influence the calculation: this information can be obtained at various places- the *Parm* subTab panel; or if using a heater accessory from the environmental controller (heater) panel.

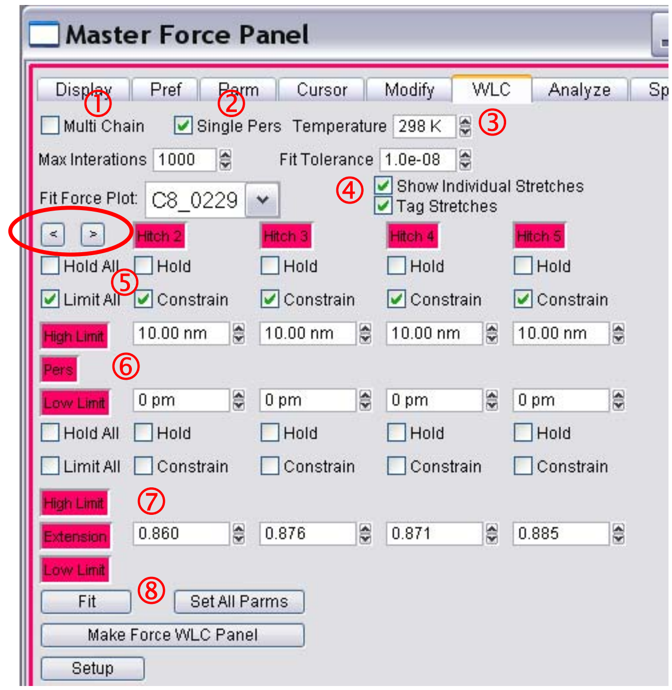


Figure 11.5.2F3: WLC tab.

④ **Show Individual Stretches** ☑ checkbox- this will put a dotted elastic response fit line on hitch (*see Figure 11.5.2F4*).
Tag Stretches ☑ checkbox: this will label the contour length (L_C) on each fit (*see Figure 11.5.2F4*).

⑤ Hold and Constrain fit parameters checkboxes:
▪ **Hold** means that the fitting function will hold that parameter constant and not fit it.
▪ **Constrain** means that it will keep the fit parameter between the upper and lower limits (the limit controls show up after you constrain one of the parameters.

⑥ The **Pers** is the 'Persistence Length'; it is basically a measure of the polymer's stiffness. Lower persistence lengths have much more non-linear responses.

⑦The **Extension ratio** is the fraction of the contour length that the polymer chain is extended.

The **Fit parameter** is the extension ratio where the polymer chain ruptures its attachment to the tip (max extension ratio). So you can get the contour length of the chain from the rupture length divided by the max extension ratio.

⑧ 'Fit' button- this executes the fit calculation command once the fit parameters have been defined.

✓ You can also tweak the fit with the Max Iterations and Fit Tolerance setvar controls. The author doesn't have enough experience fitting WLC to offer additional insight into these fitting features.

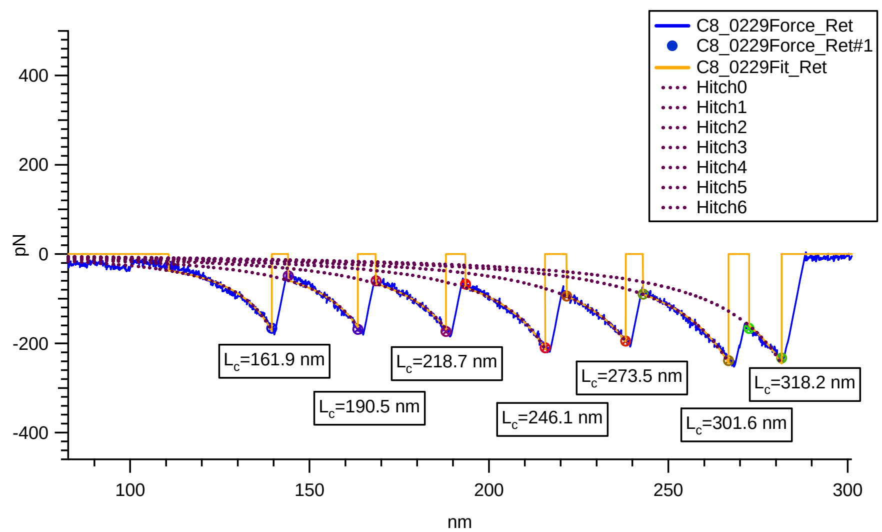


Figure 11.5.2F4: Fit WLC model on Titin construct sawtooth dissociation force curve. Single Pers was used.

▪Once the fit is complete, it will have labels and fit functions similar to Figure 11.5.2F4. The values of the hitch columns can be shuttled using the arrow buttons in the WLC tab (red circle Figure 11.5.2F3).

11.5.2G: The *Analyze* subtab-

This panel can do many curve analyzing functions-this is where histograms tabulating some property of series of force curves and Force maps are generated.

▪The upper portion of the *Analyze* subtab is for performing basic statistical processing on force curves (mostly histograms), which can then be fit with Guassian, Lorentzian or other functions.

① **Calculate** Pull-down: select what Y dimension for the batch analysis calculation

②**X Parm** pull-down: this defines what the X Axis will be

③ **Use All FPs** ☑ checkbox : uses all force curves loaded in memory for the calculation

④ **Select FPs** button- this brings up a alternate panel to select specific curves (see Figure 11.5.2G.2.

⑤**Export Display to Scatter** pull-down: Options are 'Append' or 'Overwrite'; this will make a scatter plot of all the force curves in the data set. From what the author can tell, a scatter of each point per given Y is shown across all the force curves is given, and also a standard deviation (or some kind of error bars) is also presented. Not having a lot of experience with this function, it's best to experiment with it yourself.

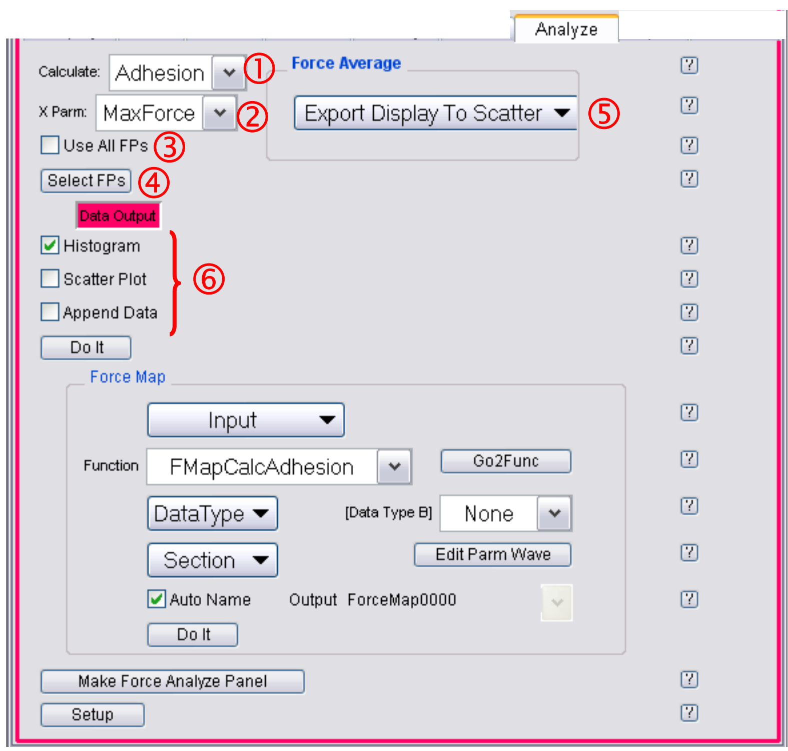


Figure 11.5.2G.1: Analyze subtab is where histograms and FMaps can be batch processed.

⑥**Data Output:** select Histogram/ Scatter ☑check boxes depending on how to present data. Use Append data if recalculating. *Multiple checkboxes can be activated at one time.*

The lower portion of the *Analyze* subtab is for compiling Force Map style data images (described below).

Procedure-
✓Load the force curves from the *Display* subtab. Use the technique shown in Figure 11.5.2A2 to select continuous/ sequential curves.

✓Offset X&Y in the Modify sub Tab (see Section 11.5.2E.1). To do this, click the '**Offset Y**' then '**Raw (X) Offset**' buttons in the *Modify* subtab- this will offset only the curves that are selected in the *Display* subtab.

EXAMPLES: Adhesion (MaxForce) batch process, and averaging InvOLS:
The author has acquired a data set of ~100 force curves on a hard surface taken in air (Figure 11.5.2G.3A) – such that the next two examples can be shown on the same data set: Adhesion (MaxForce) and average InvOLS.

ADHESION:
The MFP-3D software can do simple batch force curve analysis processing for measuring the dissociation event in a series of curves, however it measures the largest minima- maxima pair/distance per curve. The maxima is an average of the last 5 or 20 points in the 'free air' (non contact) portion of the curve, and the minima is the largest event in the retract portion of the curve. Keep this in mind if using this application for multiple dissociation events it may not be all that useful.

Procedure-

✓ In the *Analyze* subtab, select 'Adhesion' from the 'Calculate' pull-down menu.

✓ Select MaxForce from the 'X Parm' pull-down menu.

▪ If *ALL* the loaded curves in memory are what need to be analyzed, activate the 'Use all FPs' checkbox;

▪ If only select curves from the curves in memory are to be analyzed, they can be selected manually from the Display subtab (See figure 11.5.2A2); *OR* click the '**Select FPs**' button, which will bring up the 'select FP by folder panel' (Figure 11.5.2G.2).

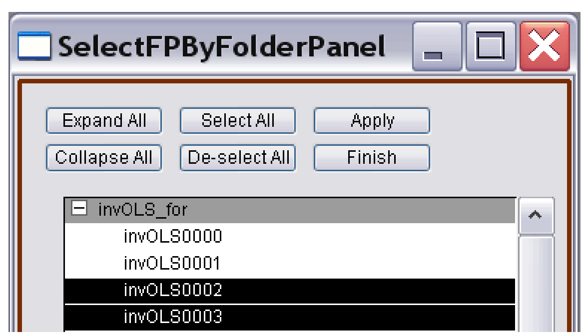


Figure 11.5.2G.2: Selecting Force Curves by folder panel.

✓Click the Histogram ☑ check box.

✓Click the '**Do It**' button; Igor will process, and produce a histogram (Figure 11.5.2G.3B).

▪ Within the Histogram window, a variety of information can be acquired or exported. Fits can also be acquired from the 'Fit Type' pull down (Figure 11.5.2G.3B).

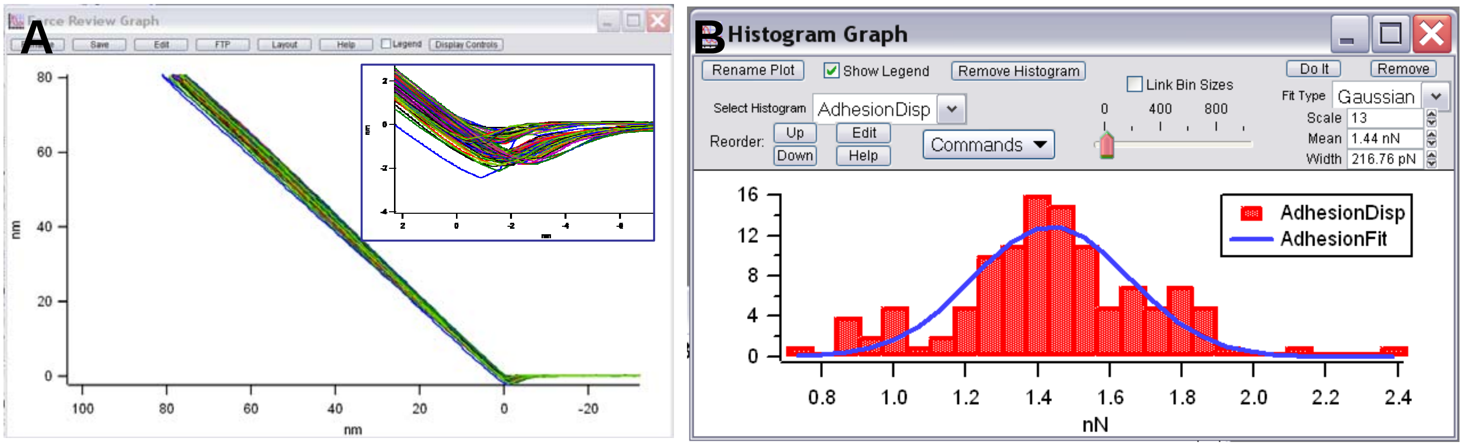


Figure 11.5.2G.3: Adhesion (MaxForce) batch processing: A) ~100 curves collected with continuous curves (Inset: shows region of interest for MaxForce calculation; B) resulting histrogram of Max pull off Force, with Guassian fit (blue).

InvOLS averaging:

▪Since many of force experiments depend on statistical occurrences, a statistical average or some other value for the InvOLS may sometimes be necessary. Often there can be a slight variance in the InvOLS value across a series of curves, and for various reasons depending on the system (equlibration time, surface asperities, SLD health, etc.). In Figure 11.5.2G.3A, the variance in the slope of the contact regime (which is InvOLS) is clearly seen. In this example, the author suspects the system was far from thermal equilibrium because data was merely collected as an example for this document.

Procedure-

✓ In the Master Force Panel, load the curves that you want to average the DC InvOLS on.

✓ Confirm that only the 'Ext' ☑ checkbox is activated, indicating that you only want to look at the extension part of the force curve considered in the calculation (Figure 11.5.2G.1B).

✓ Plot the Force curves as LVDT (X) versus Deflection Volts- typically this is done to calibrate the spring constant since the force constant of the cantilever isn't known.

✓ In the *Modify* subtab, click the 'Y offset' and 'Raw (X) Offset' buttons to zero the selected curves (see Section 11.5.2E.1). This maay take some processing time depending on number of curves.

✓ Install the Igor Crtl + I cursors on the contact regime part of the Extension curve. Take note of the Y axis values- these will also be used in the Analysis tab to designate the 'Deflection Range' during the averaging (Figure 11.5.2G.4A).

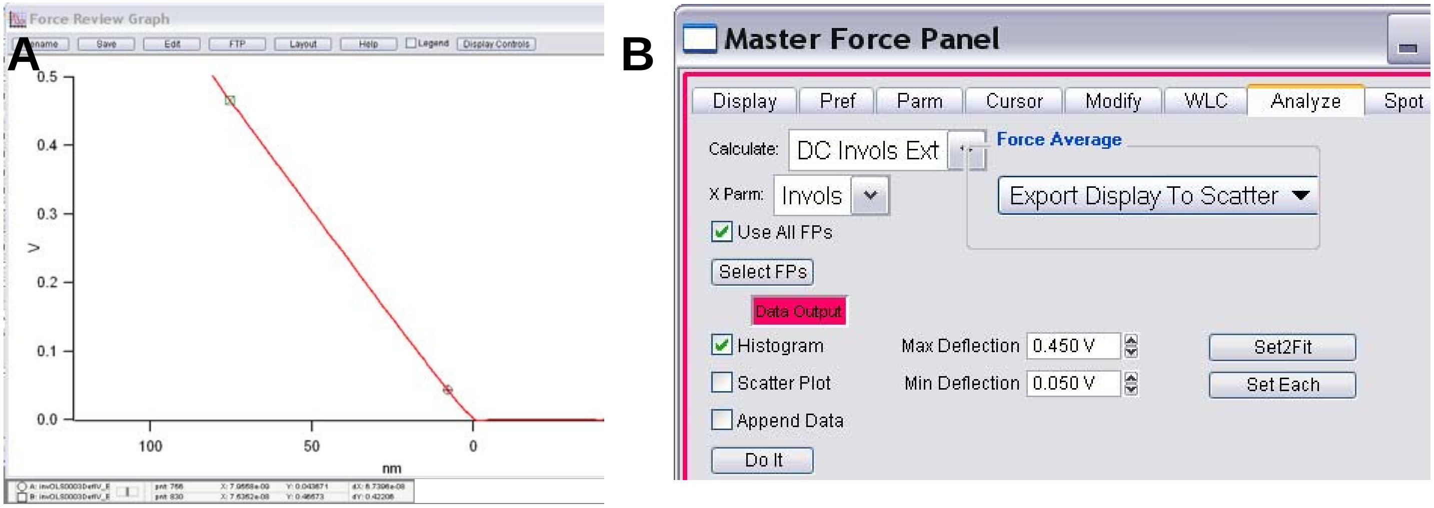


Figure 11.5.2G.4: Averaging InvOLS to display as histogram distribution: A) determine Deflection range on extension part of one of the curves to be averaged; B) enter these range values into the deflection range and Offset setvars in the Analysis tab of the Master Force Panel. Set up Analysis tab similar to above for averaging InvOLS.

✓ Go to the *Analysis* subtab of the Master Force Channel.

✓ Under the 'Calculate' pull-down menu, select DC InvOLS (i.e., InvOLS in contact mode, select AC InvOLS if you are doing some Dynamic (AC) Force Spectroscopy).

✓ Under the 'X Parm' pull-down, select 'InvOLS' (InvOLS is what the Histogram must have on its X axis).

✓ (in older software) Enter the appropriate suffix range in the 'Start' and 'Stop' Indices.

✓ Under the 'Max Deflection' setvar, enter the value of the Igor cursor closer to the trigger point (Figure 11.5.2G.4A).

✓ Under the 'Min Deflection' setvar, enter the value of the other Igor cursor (Figure 11.5.2G.4A).

✓ Check the 'Histogram' ☑ Check box- this will calculate the histogram.

✓ Click the 'Do It' button- a histogram should be generated that will show a distribution of InvOLS values (Figure 11.5.2G.5).

▪ In Figure 11.5.2G5, a Gaussian fit was applied, although other fit types are available in the 'Fit Type' pull-down. Notice the Mean and Width of the histogram fit is displayed, and that the Bin size can be changed using the slider bar.

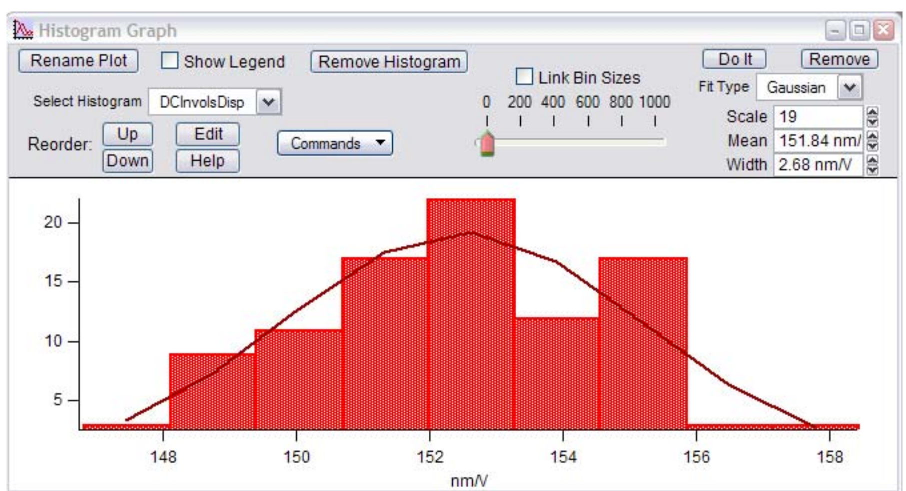


Figure 11.5.2G.5: Histogram of InvOLS values. The fit type pull down allows different fitting functions to be applied- for an example, a Gaussian fit was applied (*not that the histogram looks Gaussian*).

Force Map Analysis:

Follow this protocol to create force maps for collected/ stored data-

✓ Click the 'Review' button in the Force tab of the Master Panel.

✓ A dialogue will appear asking where to retrieve the data from-

▪ After a moment, the curves will load, and each individual file will be visible in the Force Plot List of the *Display* tab in the Master Force Panel.

✓Go to the *Modify* subtab, click the 'Offset all Y' and 'Offset all Raw' buttons- to 'zero' the curves about zero.

✓ To produce a Force Map, go to the *Analyze* subtab. Notice at the bottom of the panel, an area called 'Force Map' (Figure 11.5.2G.6B).

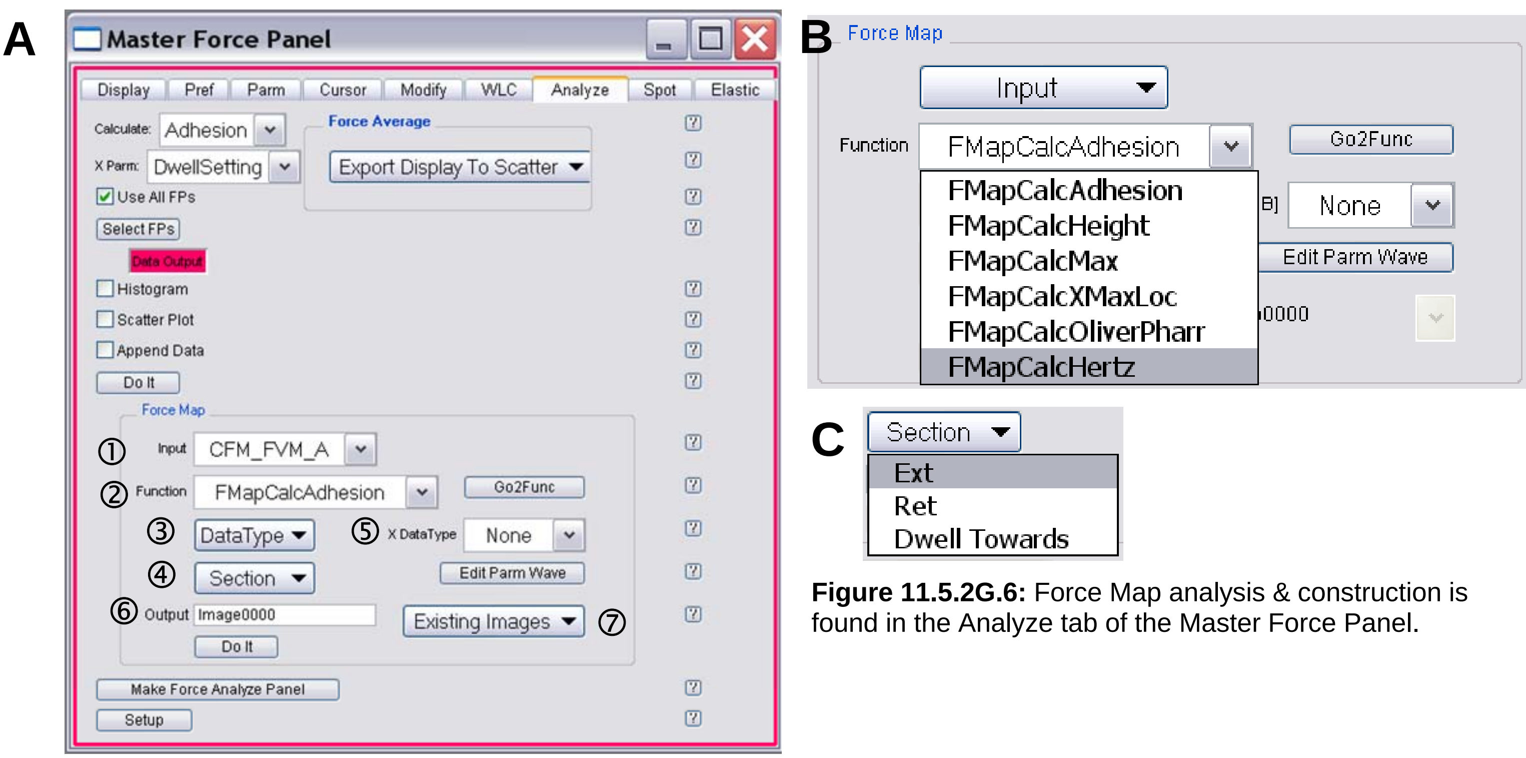


Figure 11.5.2G.6: Force Map analysis & construction is found in the Analyze tab of the Master Force Panel.

① **Input** pull down: select name of folder where the FMap of interest is stored.
② **Function** pull down: select Y data type (adhesion, height, max, O-P, Hertz).
③ **Data Type:** this is Y data.
④ **Section:** Extension (Ext) or Retraction (Ret).
⑤ (X) **Data Type B:** this is another Y data channel.
⑥ **Output:** filename can be changed here.
⑦ **Existing Images** pull-down: this is removed in later versions of 080501 MFP-3D software.

Procedure-

✓ In the Input pull – down menu, confirm the data is from the folder just loaded containing the force curves to be analyzed for the Force Map (Figure 11.5.2G.6B).

✓ In the function pull-down, choose what kind of data to be analyzed (*see* 11.5.2G.6B).

✓ Choose what portion of the force curve to be analyzed form the section pull-down menu (i.e., for FMapCalcIndent, extension should be selected; if looking at FMapAdhesion, Retract should be chosen (11.5.2G.6C)).

✓ Choose what dimension to display the X & Y data in from the X pull-down menus.

▪ The author finds it easier to leave the output image name as what the software calls it. You can easily change this name in the List panel (see Section 12.1.2) after the Force Map has been created.

▪ Figure 11.5.2G.7 shows an example of a Force Map using chemical force microscopy over a µicroContact printed pattern of a carboxylic acid terminated thiolate and methyl terminated thiolate, with the carboxylic acid terminated thiolate on a Au coated tip in pH 4.0 buffer (standard pH meter buffer, with out indicator color). The Force Map calculated with the MFP-3D analysis Panel is seen in Figure 11.5.2G.7A; the LFM image of the same area immediately after the force Map was acquired can be seen in Figure 11.5.2G.7B, and clearly correlates the areas of acid hydrophilic (higher force pull off forces in FMap and dark in LFM image) and the hydrophobic areas (lower forces in FMap and brighter colors in LFM image).

▪ *See Section 13.4.2 to insert the Force Map layer into an image channel display.*

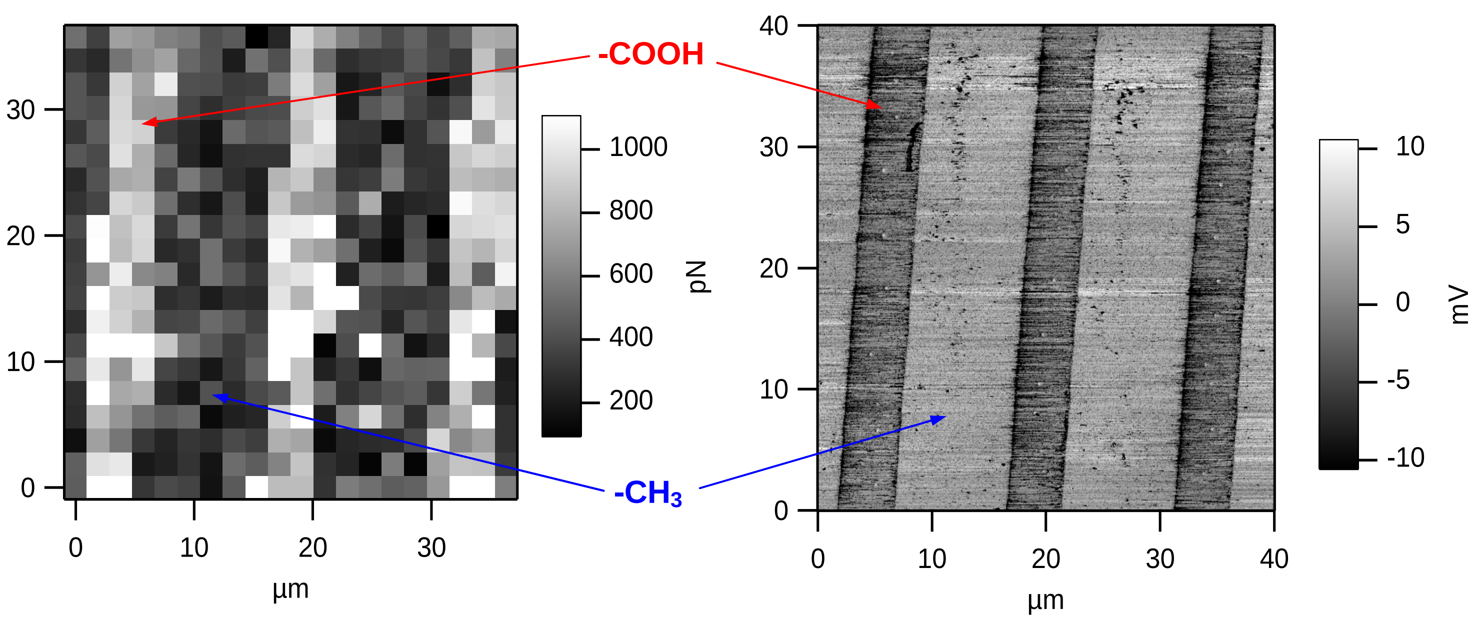
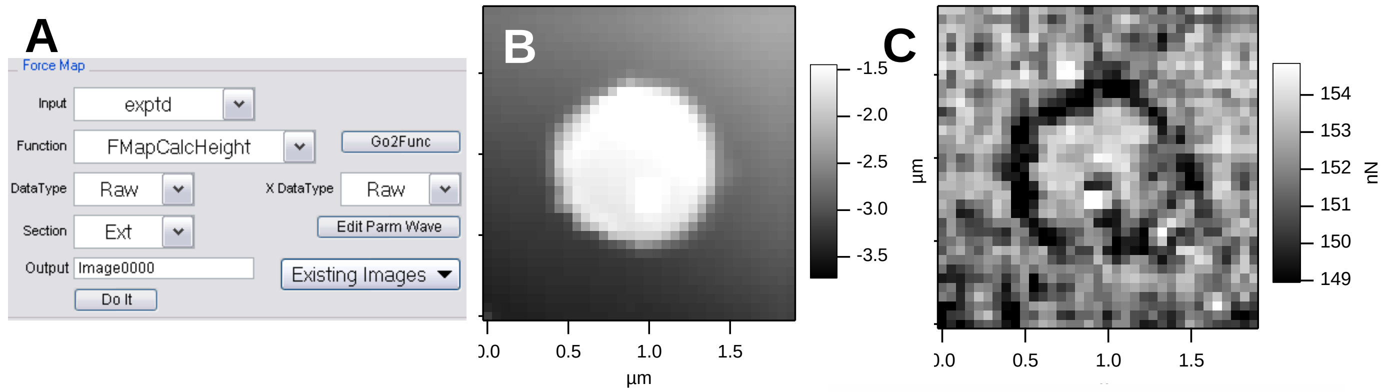


Figure 11.5.2G.7: A) Force Map from a chemical force microscope experiment in which carboxylic acid thiolates (brighter pixels) and methyl terminated thiolates were pattern (via µCP) with a carboxylic acid thiolate functionalized Au coated SiNx tip; B) subsequent LFM image of pattern utilizing same scan area. See Figure 13.13 for an example of the Force Map painted onto the LFM image using ARgyle.

▪ Figure 11.5.2G.8 is an example of a Force Map on a polymeric particle. Here, two simultaneous Force maps were calculated using height, and adhesion. In C), since the tip wasn't functionalized with anything, only edge effects are seen in the adhesion map (dark perimeter ring). The particle did not deform under the load of the ~2N/m cantilever with the trigger values used for the acquisition, the calculated indent force map didn't show very exciting data.



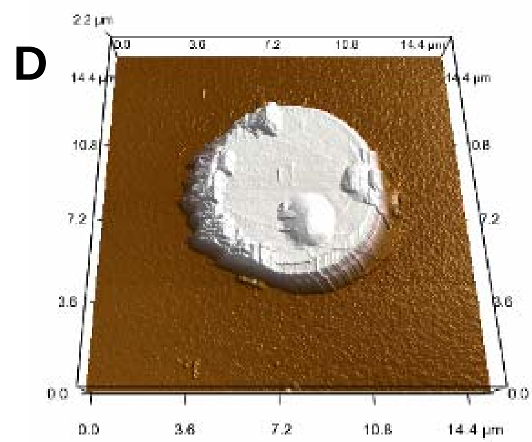


Figure 11.5.2G.8: Force map of a molded polymeric particle: A) force map parameter settings for B); B) force map calculated Height using extension portion of the curves; C) Force Map calculated Adhesion using retract portion of the curves; D) Z sensors height image rendered in 3D (ARgyle; Chapter 13).

Coming in late October- calculating modulus map from FMap data set

11.5.2H: The *Spot* subtab-

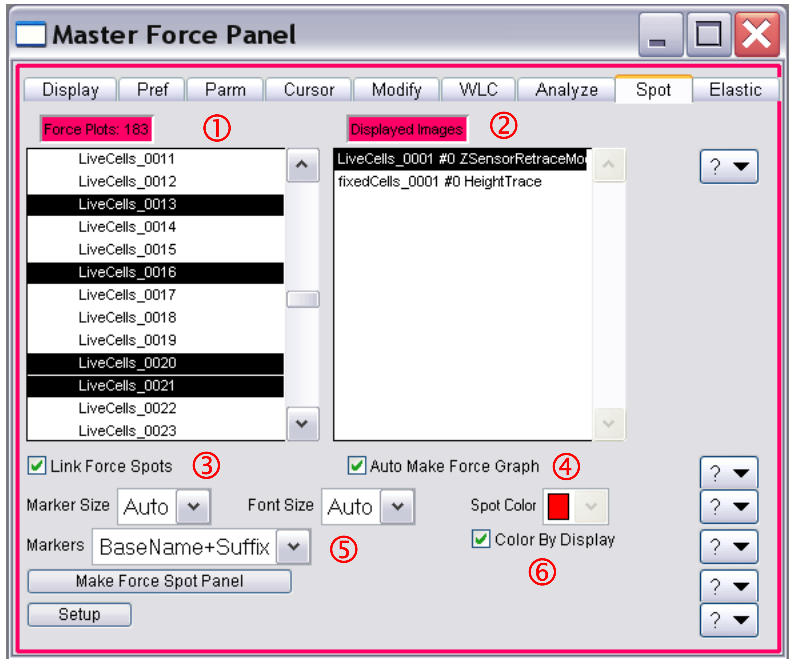


Figure 11.5.2H1: Spot subTab links point and click SPM spectroscopy curves to the image.

The *Spot* subtab links force curves (or other SPM spectroscopy curves) acquired at user defined locations on a previously acquired image via the 'Point and Click' feature from the 'Go There' subtab (see Section 11.2.4).

Features of the subtab-

① Force Plots: number of curves loaded into memory. Select force curves similar to Display tab.

② Displayed images loaded into memory.Select image for cooresponding Force curve linking.

③ Link Force Spots ☑ checkbox: check to have locations of force curves to be displayed on image.

④Auto Make Force Graph ☑ checkbox: generates a Force Review Plot.

⑤Markers pull down menu: lets the user label full filename, or just curve suffices under location markers. The Marker and Font size can be adjusted via the above pull-down menus.

⑥ 'Color by Display' ☑ checkbox: this correlates the color of the curve in the Force Review graph the same on the markers on the image. If not checked, it just is the color designated in the 'Spot Color' pull down.

The author's example is with some data courtesy of Dr. Schlenoff (FSU Chemistry) collected by Keith Jones. It's a cardiac cell collected with AC mode in fluid, then subsequent force curves were collected to look at the compliance of the different areas. The files and parameters in Figure 11.5.2H1 at different user defined locations were used for Figure 11.5.2H2.

✓ Load the images (see Section 12.1).

✓ Load the 'Point and Click' Force curves that correlate to the image(s).

✓ Highlight the image taken just before the force curves were acquired.

✓ Check the 'Link Spots' ☑ checkbox.

✓ Check the 'Auto Make Force Plot' ☑checkbox.

✓ Select the force curves desired to be linked on image. Zero them in X&Y if needed (Figure 11.5.2H2B). this will put the spots at the XY locations on the images.

▪ Clicking the 'Color by display' checkbox will make the markers on the images the same color as the force curves in the Force Review Graph. Not essential, but nice for presentation.

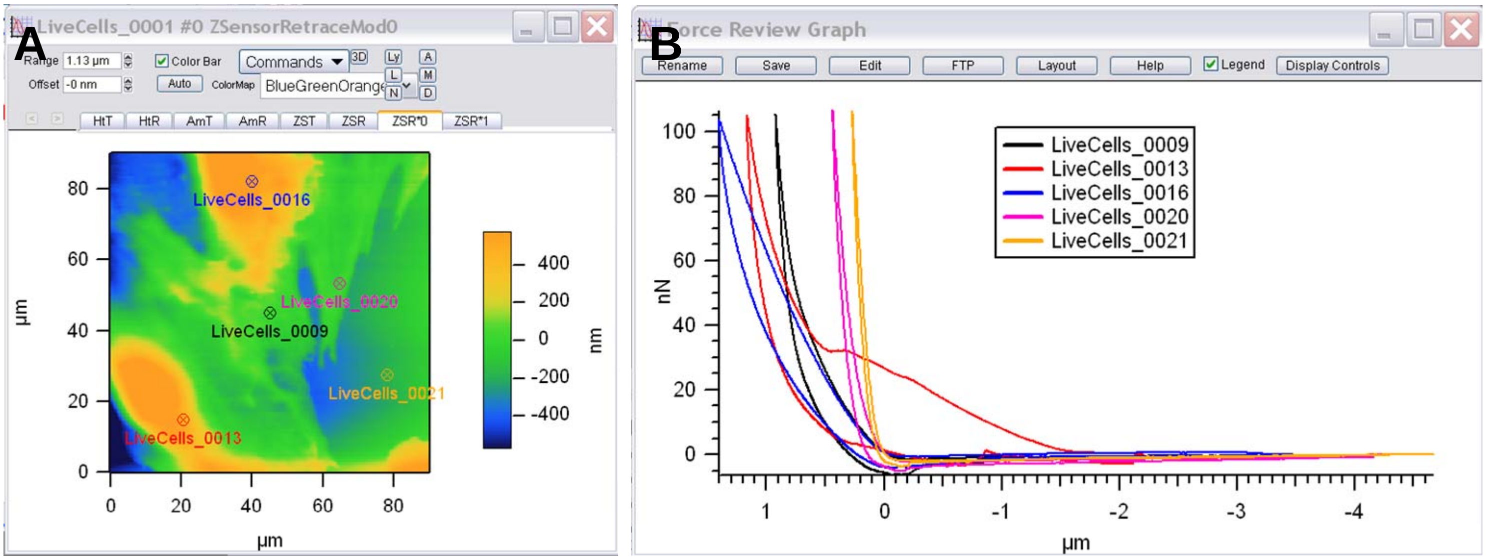


Figure 11.5.2H2: Plotting the locations 'point and click' force curves obtained at user defined locations in offline analysis: A) the stored AC mode image with force curve location labels; B) the respective force curves.

The Parm sub tab allows the spot number to be shown under the Force Parms pull down menu.

What if you can't remember which image belongs to the force curve(s)??? *that happens*.
If the image is the same XY area and same offsets, it will put the markers up without a problem. If the image is the same size, but different offsets, OR if the images are different sizes, then it's pretty easy to spot that it's the incorrect image: Often the image will be rescale (unfavorably) and something like Figure 11.5.2H3 will occur. This rescaling issue will also occur if the image is a 'Partial Save'.

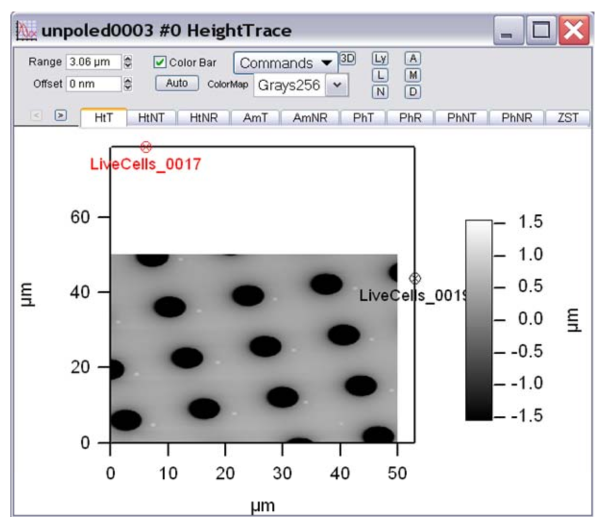


Figure 11.5.2H3: offscale images occur when attempting to correlate point and click force curves with images that they don't belong to.

11.5.2I: The Elastic subtab-

This section is currently under construction - due to the complexity of fitting various types of cantilever indentation data.

The elastic tab is where indent data can be fit for either Hertzian mechanics on the loading data; *OR* using an Oliver-Pharr fitting approach on the unloading data.

Let's start with the Hertzian mechanics sub tab of the Elastic tab- this analysis is applied to the loading wave. Keep in mind that this tab was initially designed for AR's vertical indenter, but can be used for cantilever based indentation as well.

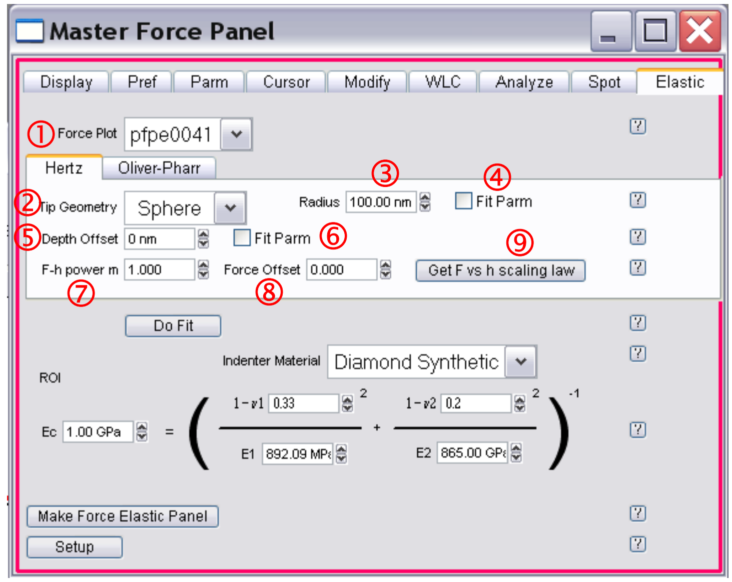


Figure 11.5.2I.1: Elastic tab with Hertzian mechanics subtab displayed.

① **Force Plot** pull-down menu- this allows the user to pick a specific force plot if multiple curves have been selected in the Display Tab (Section 11.5.2I.2)

②**Tip Geometry** pull-down: choose type of tip shape used- this is reflected in the exponent
Punch: cylinder; if the slope of the fit is < 1.25, choose this.
Sphere: use radius as diameter of tip apex.
Cone: often used for tips- For the cone model, it is the 1/2 cone angle notice the side angle setvar appears in place of 'radius'; the slope is > 1.75, choose this.

③**Radius setvar**: this is an approximation of the tip or colloidal probe sphere on the cantilever; this could also be determined from a line section of an imaged indent (if applicable); or from electron microscopy or tip de-convolution standard.

When 'Cone' is selected from the Tip geometry pull down, this setvar is labeled '½ Angle'. The default 36° is (presumably) for a four side SiNx pyramid, that have nominal 1/2 angles of 36°. Check tip specs to see what the tip pyramid angles are.

④**Fit Parm checkbox ☑:**

⑤**Depth Offset setvar**: this defines the contact point- it's rather subjective

⑥**Fit Parm checkbox☑:**

⑦**F-h Power m** (exponent) setvar: F is force; h is depth- in indentation lingo. The Ff-h *m* is the slope of the log (force) vs. Log (indentation). It should be between 1 and 2, and describes the geometry of the tip (if the hertz model applies). If it is out of range of 1 to 2, or is telling you a geometry that is clearly not true, you need to ask if your data can be described by the hertz model.

⑧ Force Offset:

⑨'**Get F vs. h Scaling Law**' button: This sets the tip geometry parameter and the F-h power *m*.

Indenter Material- this is important because it incorporates the modulus of the indenter material (for tips, usually silicon, Silicon nitride or some fancy diamond).

▪ To arrive at the 'Reduced Modulus' (Ec), the formula below incorporates the compliance of the tip and sample in a two spring series, with the E_1 and υ_1 are the Young's modulus and Poisson's ratio (respectively) of the sample material, and the E_2 and υ_2 are theYoung's modulus and Poisson's ratio of the indenter tip material. This yields the compliance (aka 'Reduced') modulus, Ec.

$$E_c = (1-\upsilon_s^2/E_s) + (1-\upsilon_i^2/ E_i)$$

The equation for the reduced modulus Ec is displayed. Ec is a weighted harmonic mean of the moduli of the sample, E *1,* and the indenter, E*2*. The weighing factor is 1 – $\upsilon i2$ where ν*i* is the Poisson ratio for sample (*i* = 1) and indenter (*i* = 2.)

'**Do Fit**' button: This button will fit the region between the 2 cursors you have placed on the force vs. indentation plot (which must be the first axis). It will fit the log of the force vs. log of the indentation to a straight line.

Procedure for fitting Hertz model-

✓Load the force curve(s) to be fit.

✓Click on indent View ☑ checkbox on the *Display* SubTab of the force review panel. This is mostly going through all the force plots in memory and offset them in X and Y so that the 0,0 (hopefully) means something.

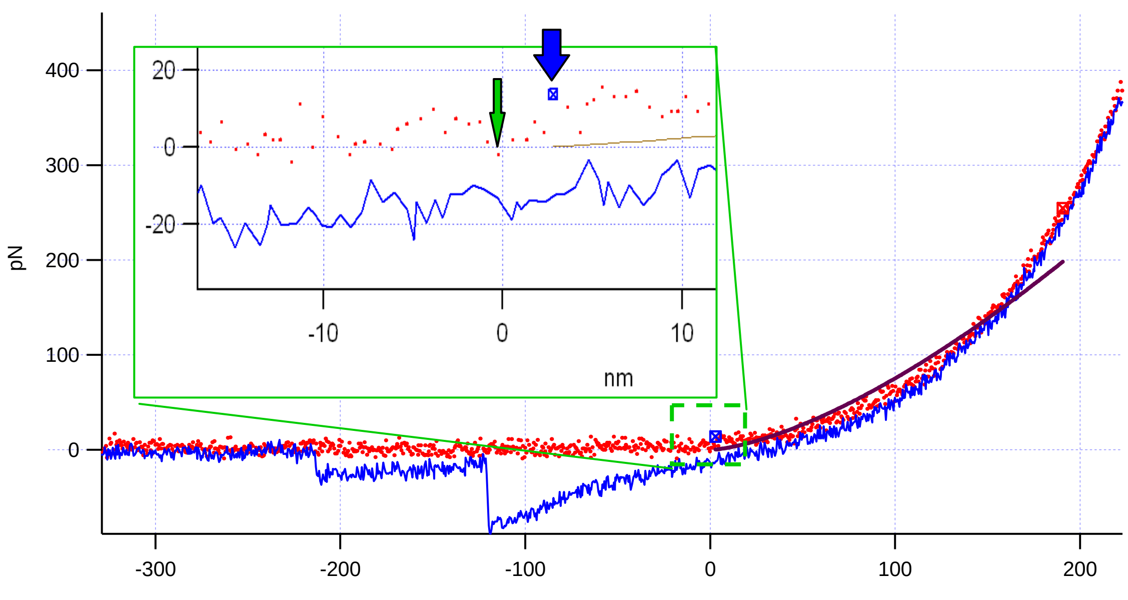


Figure 11.5.2I.2: Defining the X-Y zero point on fitting Hertz mechanics from a cantilever based indent on a bio polymer.*Inset*- zoom of zero and first cursor lock. Indent in water; k=0.815 N/m. *Sample courtesy DeSimone lab, UNC-CH chemistry.*

✓Confirm the zero point is properly defined (See Figure 11.5.2A.3). It is important that the zero point have a lower Y axis value than the first cursor lock (Figure 11.5.2I.2), or when the scaling law button is clicked, Igor will return a NaN to you (not a real number in Igor terms). *This is one reason why it’s good to increase the sampling rate during acquisition, just to provide more points when fitting.*

✓Select the force curve to be fit from the force plot pull-down- *IF* multiple curves are selected from *Display* subtab. If only one curve is loaded, then this will be the only choice under the pull-down.

✓Install the cursor ‘locks’: Hold down shift and left click at the beginning and end of the region you want to fit.

✓Go to the *Elastic* tab (the author normally makes an additional *Elastic* tab panel from the button in the lower left corner, to avoid constant switching between other force panel tabs).

✓Go to the Hertz sub tab.

✓Click on ‘**Get F vs. H scaling law**’ button. This sets the tip geometry parameter and the F-h power *m*. The F-h power *m* is the slope of the log (force) vs. Log(indentation). It should be between 1 and 2, and describes the geometry of the tip (if the Hertz model applies). If it is out of range of 1 to 2, or is telling you a geometry that is clearly not true, you need to ask if your data can be described by the Hertz model.

✓Make sure the tip parameter (1/2 angle for cones, radius for sphere), is correct.

NOTE: For cones, a significant indentation depth must have been used to have this fit be employed correctly- otherwise, the sphere is a better approximation for shallow indents. Check manufacturer packaging for nominal ½ angles. SiNx pyramids typically have 36°; Si pyramids from Olympus have 17°.

✓Make sure the fit describes the data.

✓Set the indenter material. If your indenter material is not listed, then enter the Young’s modulus and Poisson ratio of the indenter material in υ2 and E2. Notice many common materials are available under this pull-down and will update the υ2 and E2.

✓ Enter the sample's estimated Poisson ratio for υ1. If unknown, enter 0.33. The author will use Wikipedia or the literature to make approximations. The author doesn’t notice large changes in Ec values by changing this by 0.1- 0.15 (the average difference between many V’s found on tables).

✓ Click the ‘**Do It**’ button. The plot will be fit, and the reduced modulus and Sample modulus will be updated.

▪ E1 is the samples Young's modulus.

▪Below (Figure 11.5.2I.3) is an example of fitting some colloidal probe data taken by Alejandro Bonilla- AR’s indentation applications person. A 5 µm glass bead was glued to a cantilever with k= 0.32 N/m. A contact point was defined manually, and the cursors were fit to 10 nm indent depth. Panel A shows the Ind vs. Force data; Panel B shows just the Ext data, zoomed in on the fitting region of interest, with cursors installed, and result of fit: Panel C shows the parameters used in the Elastic tab for this fit, and the Ec value of 41.78 kPa (which seemed reasonable to the author since this was a cell of some sort).

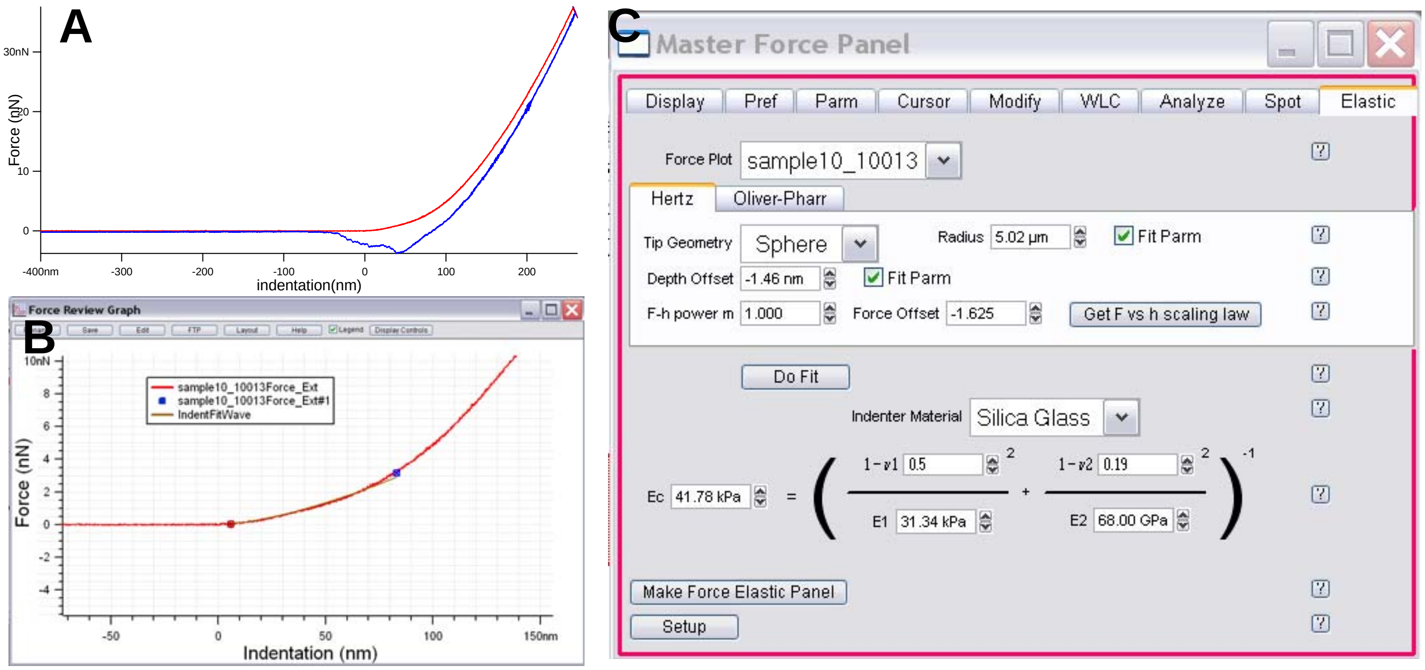


Figure 11.5.2I.3: B) Colloidal probe microscopy on some kind of cell with 5µm sphere affixed to cantilever: A) Force vs. indentation; B) XY offset to define contact point; install cursors (Blue at 10nm deflection: shown with fit from parameters in C) Hertz tab.; k= 0.32 N/m.

▪An example of cantilever based indent fit with Hertzian mechanics can be seen in Figure 11.5.2I.4 in which a bio polymeric particle (~3µm dia. x 1µm) was indented in water with a 0.815 N/m SiNx lever. The fit 100pN of load was fit with the cursor locks and the tip was approximated as a 30 nm sphere based on nominal tip manufacturer sharpness. Panel A shows the corresponding fit, while Panel B shows the Hertz subtab parameters used, and resulting sample modulus of ~11kPa.

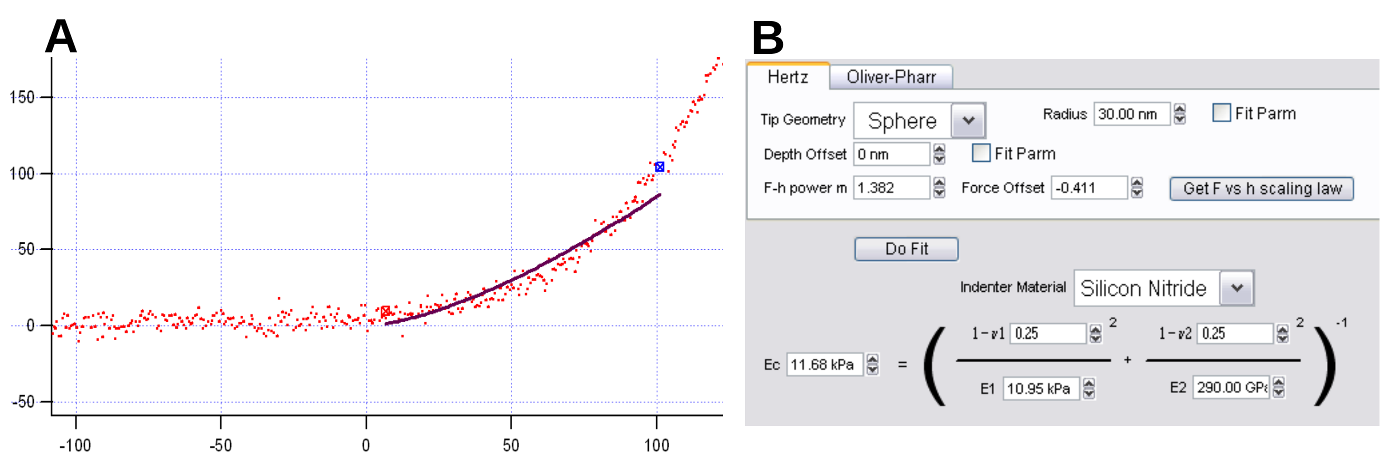


Figure 11.5.2I.4: Cantilever based indent on biopolymer particle Force (Y) vs Indentation (X): A) cursor locks at ~ zero point and 100pN, and corresponding fit based on parameters in B). SiNx lever in fluid with k=0.815N/m; InvOLS = 26.45 nm/V. Sample courtesy of DeSimone lab (UNC-CH Chemsitry)

To fit via Oliver-Pharr:

To be completed in late October 2009

ROI (i.e., 'Region of Interest')- this is essentially where the cursor locks are on the unloading part of the curve (works only when indenter panel is used- (i.e., doesn't work on retract part of curve controlled by the Force tab.)

Beta- 1.05- use

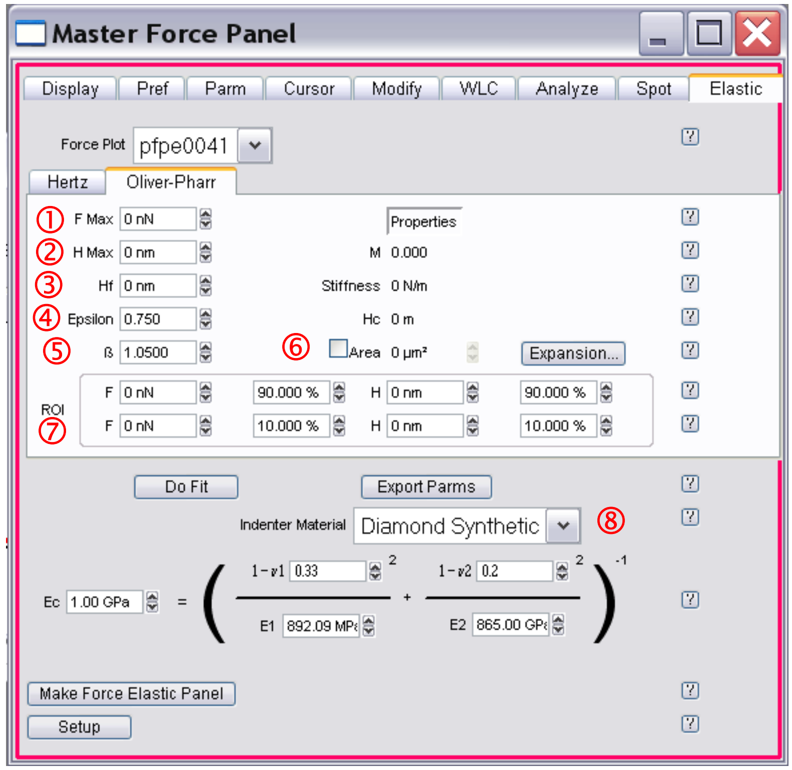


Figure 11.5.2I.X: Elastic sub tab with Oliver-Pharr fitting:

The O-P method is really just another curve fitting exercise to get a better estimate of the contact stiffness (initial slope of the unloading curve), from which you can determine E if you know the tip geometry. As long as you can get the O-P power law function to fit your unloading curve

11.6: Miscellaneous items:

11.6.1: Crosslinking/ tip functionalization/ sample prep:

this is incomplete at this but will be complete at the final version; thhe author includes it for novices.

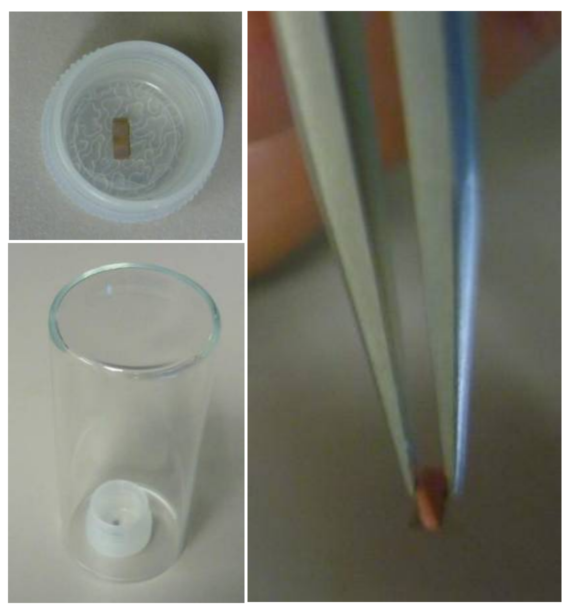
Many force adhesion or receptor-ligand experiments call for some functionalization of the tip. There is a great deal of chemistry that can be done at the end of a tip, although careful characterization should be performed on bulk surfaces before making any claims in publications.

That being said, there have been numerous reports of tip functionalization- many of which come from the AFM literature, based largely in part from the self-assembly and ELISA chemical linking literature. There are many companies that provide molecules, proteins and antibodies of interest, websites listed below. Typically molecular concentrations are in the millimolar (mM) range, with incubation times ranging from 10 mins to hours.

Some nice tutorials on sample prep and tip cleaning prior to functionalization:
http://www.jpk.com/tutorial/afm_sample_preparation1.htm
http://www.cma.fcen.uba.ar/files/prepmuestras.pdf
http://www.chem.duke.edu/~boris/research/force_spectroscopy/sample_preparation.htm

Vezenov, et al has a nice review about Chemical Force Spectroscopy (CFM), including a useful table describing tip functionalization – see Table 1 (p 319): Vezenov, D., Noy, A., Ashby, P., J. Adhesion Sci Technology 2005 19 (3-5) p313 – 364.

Functionalizing tips-
Tips generally have to be cleaned out of the box- many do this by gentle rinsing with a variety of solvents ranging in polarity, or with a UVO or Ozone cleaner, or both. Strong acid solutions like Piranha (be careful- the literature is not kidding with the obligatory statement describing violent reaction with organic material- the author has experienced this first hand, Read up on the handling and use of piranha solutions before ever using it). Anyway , piranha can make cantilevers brittle, and cause the reflective backside coating to flake off.



The author has placed the tips to be functionalized either in a small vial cap when using organic solvents (put probe in vial cap already filled with functionalization solution, and then place a larger vessel over it to prevent immediate evaporation- this works well for attaching a thiol to a gold coated probe; typically in 100% EtOH, commonly incubated overnight. The probes are held with a firm pair of tweezers (not fine pointed tweezers because they often scissor, launching the probe), and run a gentle stream of the solvent down the tweezers- avoid direct solvent stream on probe.

If using aqueous solvents, the author has found that putting the probe onto the underside of a small weigh boat with a thin layer of PDMS works great (this is a similar material that is used in a tip box- it's compliant and the probe substrate sticks to it very well). Drops of aqueous solvent (containing the molecular linker concentration of interest) create large contact angles, and generally stay put on the cantilever area of the probe. After incubation is complete, pick up entire weigh boat, and gently rinse- have solvent stream hit somewhere on the weigh boat so the probe doesn't take a direct hit.

To dry, the author generally wicks the bulk of the fluid away from the probe with the corner of a ChemWipe, then gently dries in a weak stream of N2.

Functionalizing substrates-
The chemistry can be similar here, but the author urges careful consideration on how to attach substrate into the AFM (glass slide, fluid cell, etc.). considerations involve when to secure the substrate- epoxies and superglues (cyanoacrylates) have VOCs in them, and can contaminate the surface during curing. Also, if the substrate has to stay wet after the chemistry is complete, this will also be an issue.

Surprisingly, dow corning vacuum grease works very well, and doesn't contaminate that many aqueous systems (see Figure 6.2.9).

Some places to get chemical linkers, and some useful tutorials as well:

▪Pierce biotechnology:
http://www.piercenet.com/Objects/View.cfm?Type=Page&ID=FE7F690D-58AE-4342-AE85-BA94DCA642F8

▪high quality bi functional PEGs: Pricey but worth it; also do custom stuff. My wife says they're great. they are an alternative to the now debunk Nektar.
http://www.laysanbio.com/index.php?src=gendocs&link=Products_new&category=Main

▪ polysciences have cheaper PEGS, but I've been told they are an unreliable company unless you have an established relationship with them (I am told)

▪ exoitic thiols company –
Prochimia from poland
http://www.prochimia.com/products.php

Assemblon from Washinton state, USA.
http://assemblon.net/

http://www.quantabiodesign.com/

12. Introductory Image Analysis:

This Section discusses how to perform basic image analysis on stored MFP-3D™ image (.ibw) files. It is presented based on how the author goes about processing image files. Keep in mind, there are not necessarily hard and fast rules / sequences regarding image processing, it depends on the data, and can be a trial and error process. For this reason, the processing techniques are broken into sections, and some examples given.

12.1: Opening Stored images:

✓Click on the 'Browse' button on the *Main* tab of the Master panel; the ARPath dialogue will appear asking where to retrieve the file from (Figure 12.1A).

▪ During image loading, a progress meter will appear (Figure 12.1B), and concomitant smart - mouthed comment (Figure 12.1C).

▪ Once the images have loaded, the Browse window (containing thumbnails of the images/ data; Figure 12.1.1), and the List Panel (contains just the files names; Figure 12.1.2) will appear.

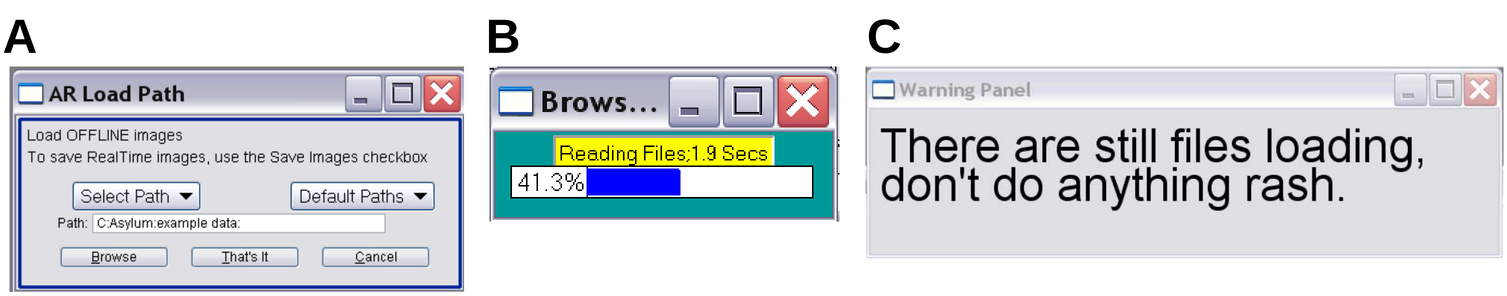


Figure 12.1: Opening stored images: A) Select where stored files are with the ARPathDialogue; B) the progress meter counts down the file loading time; C) concomitant silly message when loading large folders of data.

12.1.1: The Browse Panel-

▪ The Browse panel displays the data as thumbnails (Figure 12.1.1). *This window can be resized to be grabbing the corner and dragging to desired size- the author finds this helps with he screen real estate.*

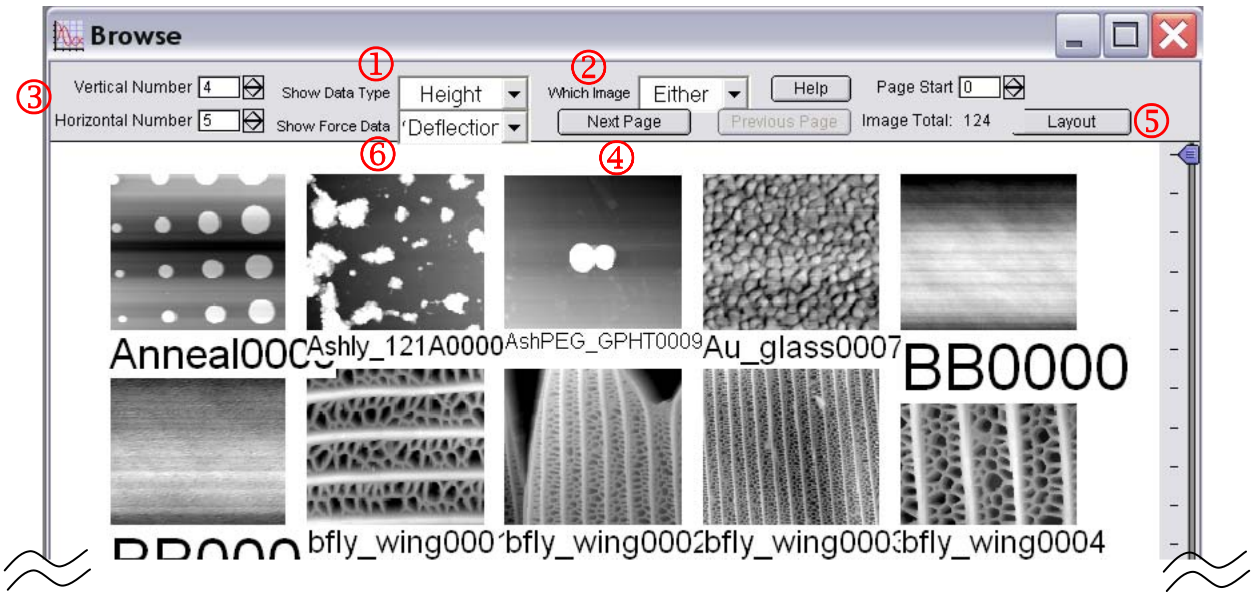
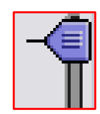


Figure 12.1.1: The Browse Panel allows images to be organized as thumbnails and opened with a mouse click.

① Specific data channels (i.e., Height, Phase, etc.) can be selected in the 'Show Data Type'. This is convenient if looking for specific image data in a folder containing many images- an example would be to choose NapPhase if a MFM image was being searched for- among many non NAP images.

②The Trace or Retrace data can be displayed using the 'Which Image' pull-down menu.

③Thumbnail display array can be selected using the Vertical and Horizontal setvar values.



④ The 'NextPage' button advances to the next page of thumbnails, much like using the Igor scroll bar on the right side of Browse panel

⑤The Layout button will place a snap shot of the Browse panel and dump it into an MFP-3D layout window (see Sections 7.7E; 14.12).

⑥The 'Show Force Data' pull-down menu is useful to show the different deflection data when viewing force-distance curves.

✓ To OPEN AN IMAGE FILE, place the cursor over the thumbnail and double click the mouse button. The image will open a *Display Window* (Figure 12.1.3), as described in Section 12.1.3.

12.1.2: The List Panel-

▪ Images can also be opened in the List Panel (Figure 12.1.2). It consists of two columns- 'Images on Disk' which are the files stored in the folder; and 'Images in Memory' which display the images that have already been opened in the experiment.

✓ To open an image file, double click on the file name. The image will open in something called a Display Window (Figure 12.1.3), as described in Section 12.1.3.

✓ To open multiple images, hold the Shift key while selecting images; double mouse click on selected file names, or click the 'Open Files' button.

▪ Most of the function buttons are self explanatory, so only a few will be discussed- The Help menu offers concise descriptions of these functions.

▪ The 'Refresh List' button updates a list of images in the same path. This is great if images are being acquired while previous images are subsequently being processed /analyzed.

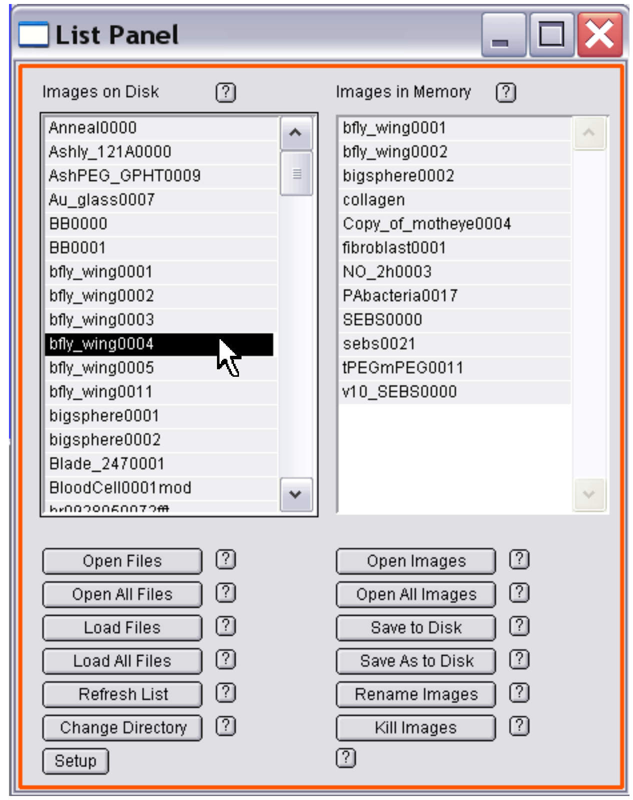


Figure 12.1.2: The List Panel

▪ The 'Change Directory' folder allows different data folders to be employed.

▪ The 'Save to Disk' button allows modifications to be automatically overwritten.

NOTE: this will overwrite the filename with the added modified tabs, but the raw data will still be preserved.

12.1.3: The Display Window:

When an image is recalled, it is displayed in the 'Display Window'. Figure 12.1.3 shows an example of a Display Window image that will be used throughout this section.

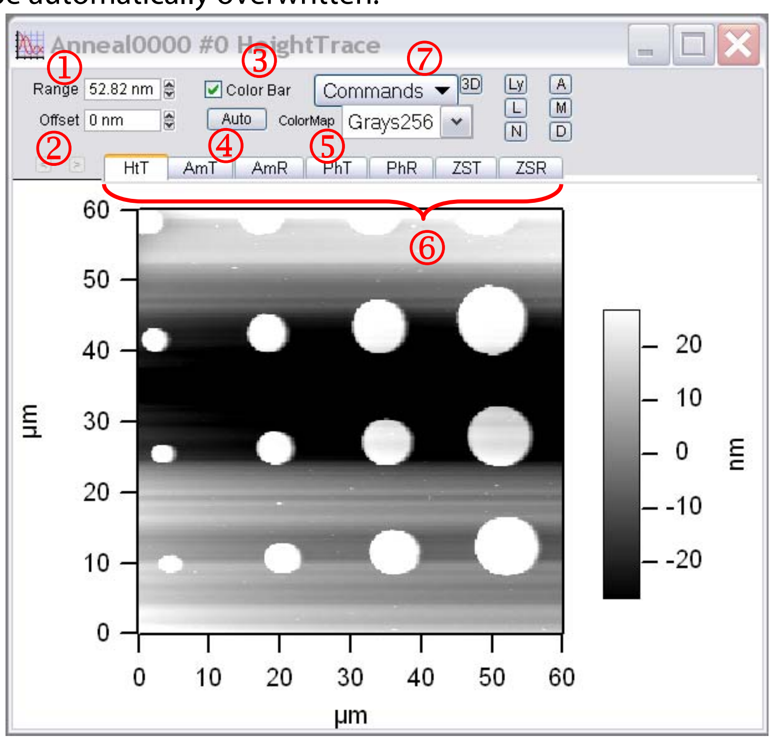


Figure 12.1.3: Display Window; B) Command functions in pull-down menu.

Notice the top of the window has some setvar values, pull-down menus and function buttons:

① **Range**- adjusts Z scale (see Section 7.1).

② **Offset**- adjusts where the center of the Z range is relative to the color table; this allows more flexibility with image contrasts.

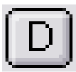
③ **Color Bar** ☑ checkbox allows the Z range color table gradient to be viewed.

④ The '**Auto**' button auto-scales the Z range and offset.

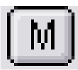
⑤ The '**ColorMap**' pull-down menu offers many

color tables to display the image data with.
⑥ Stored data channels.
ARgyle™ 3D rendering- described in Section 12.

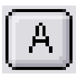
⑦**Command Functions**
The 'Commands' pull-down menu offers a large variety of functions, most of which will be described below. Some of the more frequently used functions are also presented as buttons to the right of the Command pull-down menu-



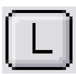
▪ **Display Panel:** this is where images can be more easily managed when there are many open in the software. The author doesn't typically employ this because it would be a far too organized approach.



▪ **Modify Panel:** this is where flattening, planefit, masking, filtering, FFT etc. is performed; *See Section 12.2.*



▪ **Analyze Panel:** This is where roughness measurements, line sections and histograms of pixel data are created/ displayed /generated. See Section 12.5 for in-depth description of this.

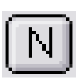


▪ **List panel:** Open images, change directories, save images, rename extract images. See Figure 12.1.2.

▪ **Extract Layer**- this function can remove an image channel tab (*the 'layer'*) out of the display window to be renamed and saved as a smaller file, *OR* do math / further processing. *see Section 12.4.1.*

▪ **Insert Layer**- this function installs a layer back into the display window- *see Sections 12.4.2 for examples.*

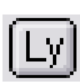
▪ **Delete Layer**- self explanatory.



▪ **Show Note**- This is where every conceivable imaging/ system parameter can be viewed.

▪ **Change User Names**- the author really have no idea what this does; *maybe someday …*

▪ **Save Image**-This will save any modifications to the display window (e.g. Modified data or inserted layers in new tabs). See Section 12.3 for protocols.



▪ **Save then Kill Image**- This saves the image, then closes the file

▪**Export to Layout**- This is a feature that lets electronic notebook style pages be prepared. Described in Sections 7.7E & 14.12.

▪ **Tiff exports:** The number is the resolution of the Tiff, 1, 2, or 4 times the number of pixels in the image. These files can get very large, but are great for graphics that need to be displayed at larger scales (posters).

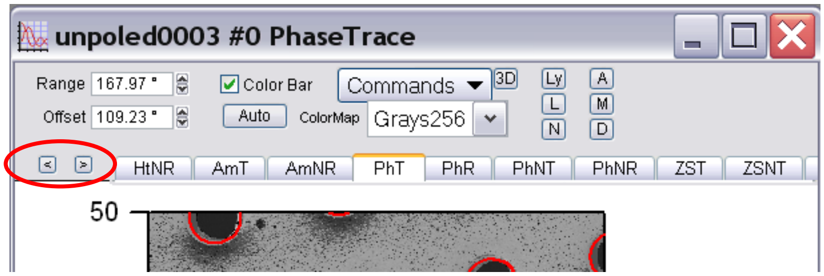
▪ **Set Layout Fields**- This brings up a list of parameters to show when the export to Layout function is executed.

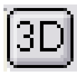
▪ **Crop Image**- this throws away the cropped data if saved (i.e., you won't get your won't get the full image data back)- so make a copy of the raw data before you perform this; *See Section 12.4.5 for protocol.*

▪ **Setup**- selecting this function will bring up the 'DisplayGraphBitControlPanel', which is a control panel that allows selection of which panel shortcut buttons and image channel tabs to be displayed in the Display Window. The author prefers to have all options available out of habit.

▪ **Help**- the help menus are very informative.

▪ For Display Windows with many image channel tabs, small arrow buttons to the left of the image channel tabs allow the user to move left or right through the tabs (see red circle, image right).





ARgyle™ 3D rendering- described in Chapter 12.

12.1.4: AR Thumbnail Viewer-

On the non Igor/ MFP-3D software side, the AR thumbnail viewer is a nice feature if you need to dig through a lot of files looking for something specific in Windows XP file manager.

✓Right click on an image file, and choose Properties.

✓A panel similar to Figure 12.1.4 will appear. The Asylum Research tab will have a larger thumbnail of the stored image (you can choose what channel to display), and the lower portion of the panel has the pertinent scan parameters.

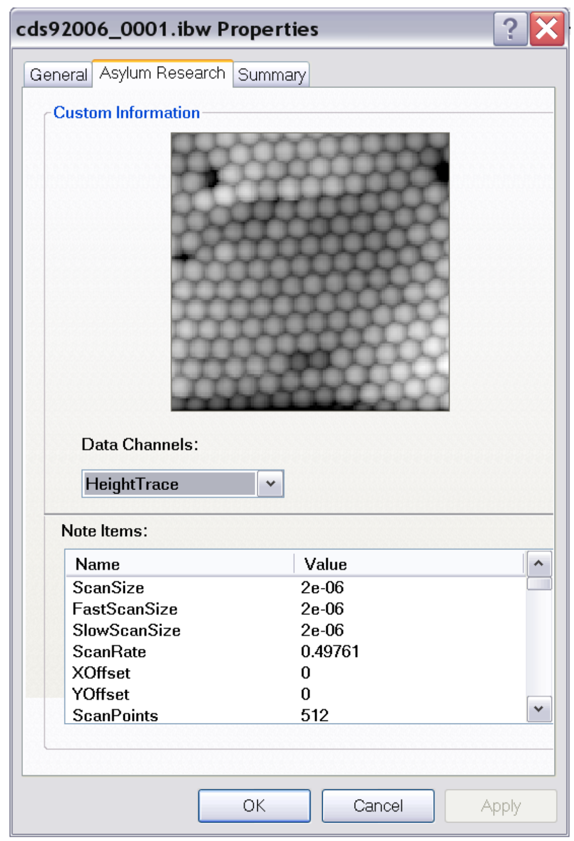


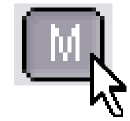
Figure 12.1.4: the AR thumbnail view lets users find pertinent information about stored data in what ever file manager you're using.

12.2: The Modify Panel-

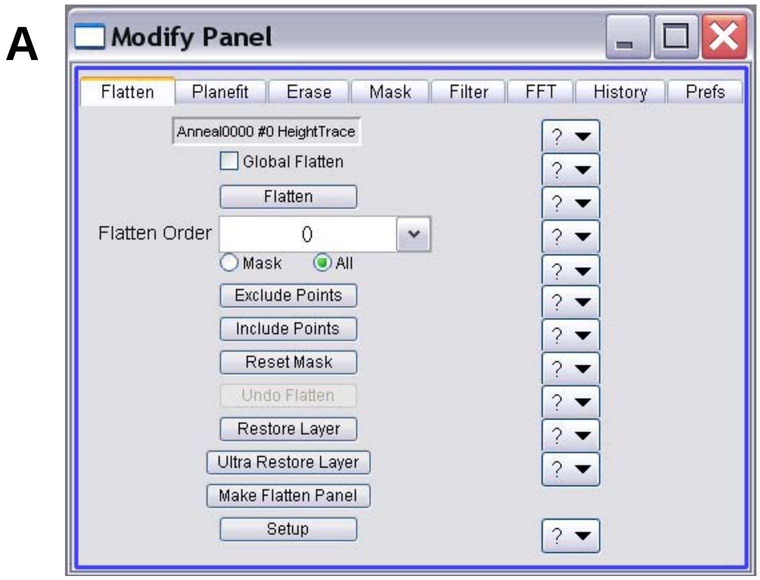
Properly processed images generally involve multiple steps: flattening (to some order), a plane-fit, masking features, proceeded by a final flatten. A brief description of these processes are described below in this Section (12.2).

12.2.1: Flatten Tab:

✓ With a Display Window image open and desired image channel to modify as forward most tab (for example, Height Trace (HtT) in Figure 12.1.3), click the ‘M’ button to open the Modify Panel (Figure 12.2.1.1). This panel contains many of the tools needed for image processing.



✓Just as the default parameters show in Figure 12.2.1.1, start with a Flatten Order of 0 and the ‘All’ Radio button selected.



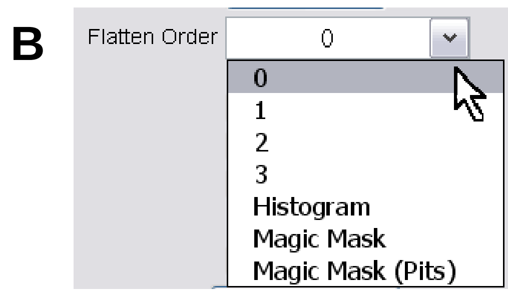


Figure 12.2.1.1: The Flatten tab of the Modify Panel; B) different flattening order options.

✓Click the ‘Flatten’ button; after a brief moment of Igor calculation, the image will be flattened.

▪*Also notice there is a new tab in the Display window: a suffix *0 indicates the image channel has been modified in some way.*

▪ Depending on the complexity of the surface, different flatten orders can be performed on the surface. Figure 12.1.1.2 shows some different examples of the flattening order options in Figure 12.1.1.1B on a moth eye image.

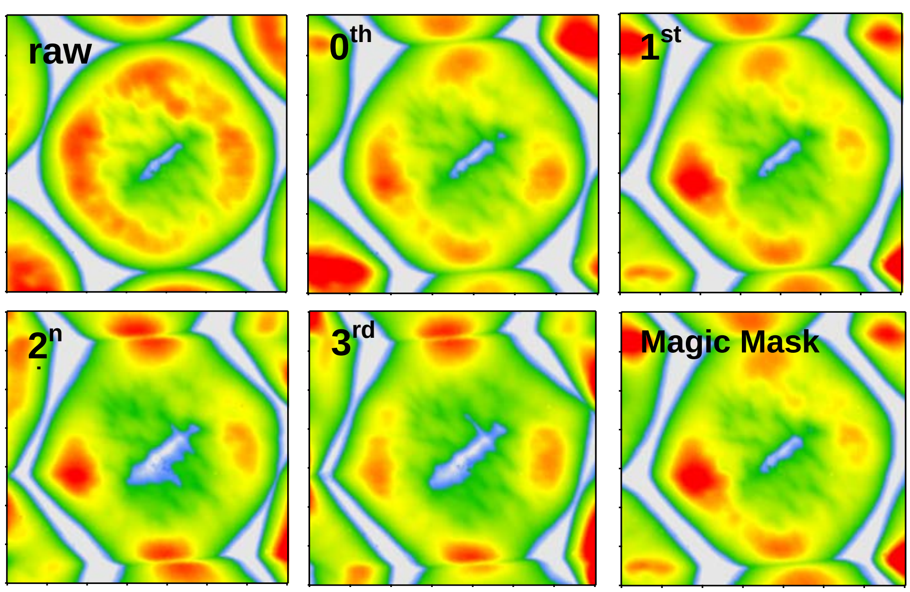


Figure 12.1.1.2: Different Flatten order results on an AC mode image of a moth eye. Notice the subtle differences in the surface features.

✓Click the 'Auto' Button at the top of the Display Window; the image will be autoscaled, allowing a better look at the topography. *Note that in some instances, the autoscale may actually make the image look less appealing*- this is when the Range and/or Offset setvars can/should be adjusted.

▪ Figure 12.2.1.2 shows a series of an AC mode image of a moth eye (35µm, Z scale 2.5µm) flattened with the different flatten order algorithms. *Notice the 'Magic mask' appears to be similar to a first order flatten.* Magic Mask is a new feature, and very convenient, especially for more complex surfaces.

▪ Depending on the data features in the flattened data, their may be flattening artifacts (streaks) around the surface features/ asperities. To remove these features a planefit and image Mask are most likely needed: *see Sections 12.2.2 & 12.2.3*. A sequence of removing such features (via flattening, planefit & masking) can be seen in Figure 12.2.3.4.

12.2.2: Planefit Tab:

✓Go to the *Plane* tab of the Modify panel (Figure 12.2.2.1).

✓Confirm the planefitting order is set to 1 in the pull-down menu. This is the most common plane fit order for data processing. More or less extreme order examples area seen in Figure 12.2.2.2.

✓Click the 'XY' button; this will planefit the image in the X&Y plane with respect to Z.

Alternatively, if the plane fit can also be performed on solely on the X (fast scan axis) or Y planes (slow scan axis).

▪ Figure 12.2.2.2 shows a series of AC mode Z sensor images of a human hair (40 µm; Z: 4µm) Plane fit at 0, 1th, 2nd & 3rd order plane fits.

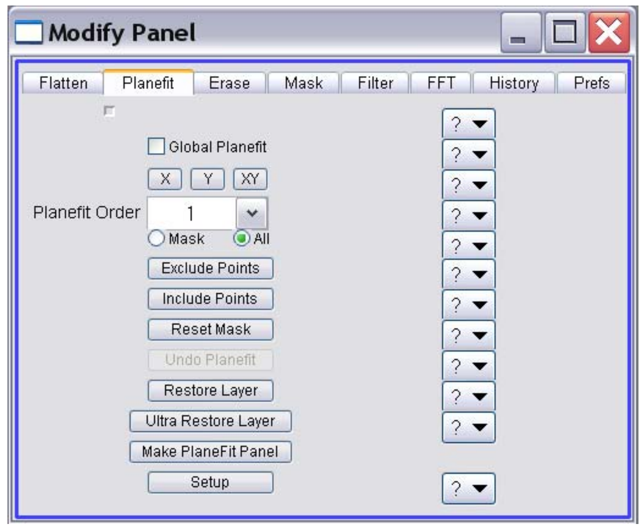


Figure 12.2.2.1: Planefit tab of Modify Panel

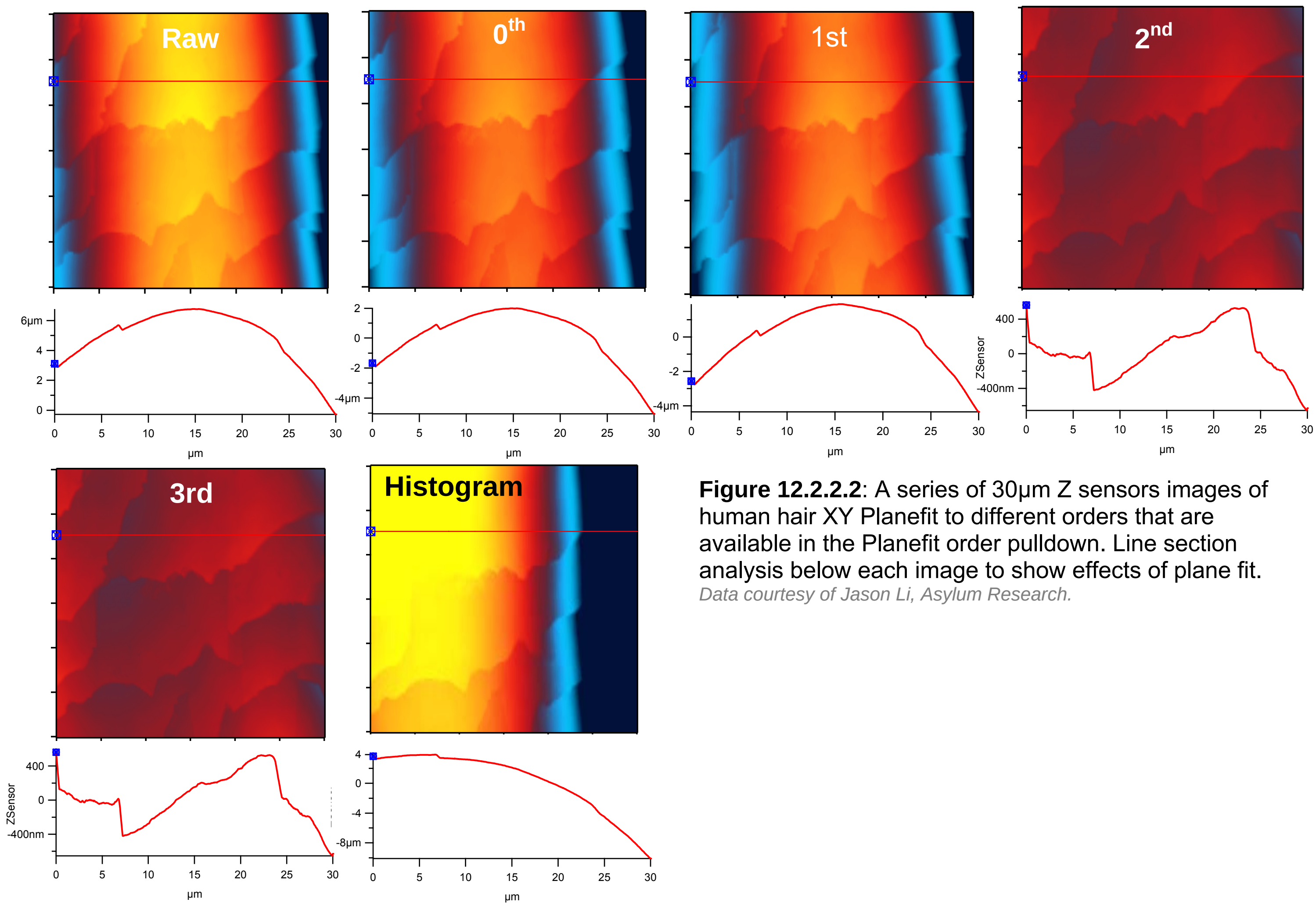


Figure 12.2.2.2: A series of 30µm Z sensors images of human hair XY Planefit to different orders that are available in the Planefit order pulldown. Line section analysis below each image to show effects of plane fit.
Data courtesy of Jason Li, Asylum Research.

12.2.3: Mask Tab:

Image masks save a great deal of time when omitting features from images that would otherwise create flattening artifacts (Figure 12.2.3.4A). Essentially, what the mask does is pick a defined plane / threshold in the Z axis and omit anything above, or below that threshold value.

▪ The mask can also be used to do image processing exclusively within or outside of the mask (see Sections 12.5). *They sometimes require some trial and error Z threshold searching.*

✓Go to the *Mask* tab of the Modify Panel (Figure 12.2.3.1).

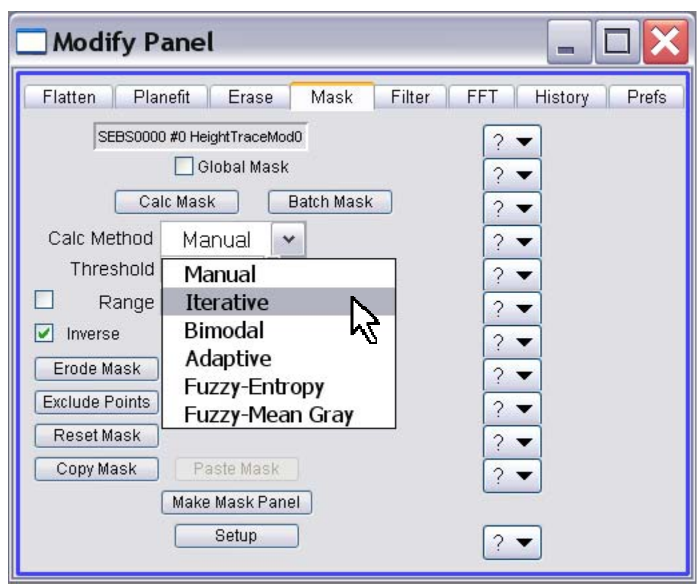


Figure 12.2.3.1: Mask tab of the Modify Panel

✓Confirm that '*Iterative*' is chosen in the 'Calc Method' pull-down menu. This mask type is a good mask to start with to ultimately find the proper threshold value.

▪The author generally starts by activating the Inverse ☑ checkbox - which will effectively exclude the points contained within the mask during the subsequent re-flattening (*see below*), or other calculation.

✓Click the 'Calc Mask' button; Igor will determine a Z range that is generally close to the Z range that is needed to help clean up the image (to human perception; Figure 12.2.3.2B). *Notice the 'Calc Method' has change to Manual- because a Z threshold value has been determined by the software.*

▪ At this point, you may or may not be happy with the Z range the calculation picked- there are many ways to combat this:

❶ Manually enter a threshold range, and re-calculate the mask; the author generally does this to get a precise (*yet subjective*) position of the mask.

❷ Click the 'Erode Mask' button- this will make the mask a little smaller (Figure 12.2.3.2C).

❸ Click the 'Dilate Mask' button- this will make the mask a little larger. (Figure 12.2.3.2D).

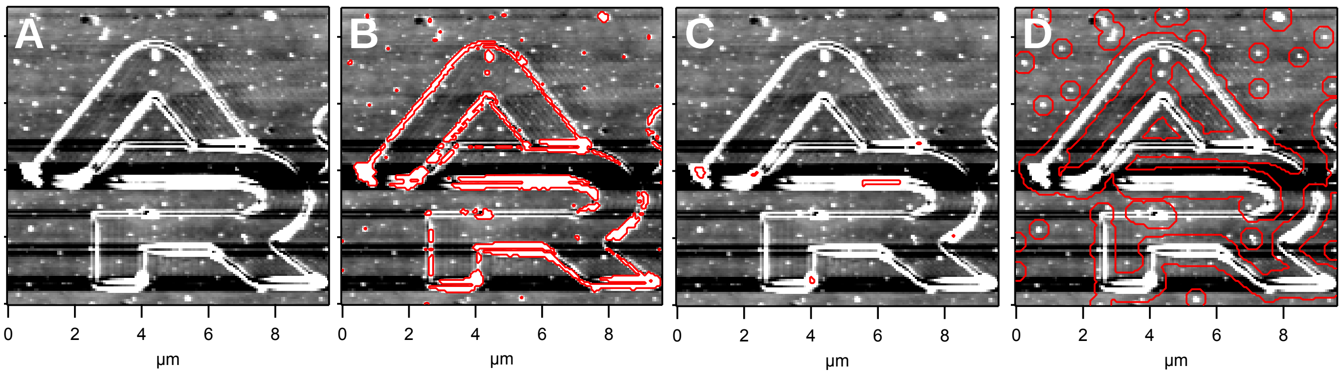


Figure 12.2.3.2: Creating image masks: A) after zero order flatten (*i.e., no mask*); B) after Iterative mask result; C) Panel B) after Erode mask button click; D) Panel B) after Dilate Mask button click.

▪The image can be re-flattened at this point to clean up any flattening artifacts (dark horizontal streaks in Figure 12.2.3.2- See Figure 12.2.3.4 for an example incorporating flattening, plane-fitting and image masks.

Mask Calculation Methods-there are many different mask algorithms to choose from:

-**Manual:** allows the user to enter a value into the Threshold range- this will put the mask at that value above, or below (inverse box ☑ checked)

-**Iterative:** this calculation automatically picks a Z threshold range using an iterative method.

The author doesn't generally use the latter menu selections due to lack of understanding-

-**Bimodal**: Mask calculated based on image histogram is a simple bimodal distribution.

-**Adaptive**: apparently this method evaluates threshold based on the last 8 pixels in each row, using alternating rows. Presumably these last 8 pixels are for the substrate. The author doesn't have much experience with this method.

-**Fuzzy Entropy:** *not really sure what this does*

-**Fuzzy-Mean Gray**: measures the mean gray level in the object and background; seems to do something similar to iterative method

▪ *The author usually sticks with 'Iterative' and 'Manual' calculations.*

Copying Masks-
In some instances, performing two separate iterative mask calculations on two separate image channels in a data file can give different results (for example, the height and phase channels), especially when wanting to overlay two channels in ARgyle (Section 12). In these instances, it's a good idea to copy the mask from one channel, and paste it into another channel.

Another reason to Copy Mask is when trial and error approaches are being employed with plane fit or finding a flattening order in which pasting a Mask saves processing time.

✓Click the 'Reset Mask' button to remove the mask.

▪ *Additionally, Masks can be created using the* ***Exclude Points*** *feature-*

✓Click the 'Exclude Points' button in the *Flatten*, *Planefit*, or *Mask* tabs of the Modify Panel. This will bring up the Igor tool box (Figure 12.2.3.3). Lines, circles and the free hand tool can be used to make shapes on the image.

✓Click the 'Make Mask' Button; this will create the mask (Figure 12.2.3.3C- red lines).

▪ For additional shapes, choose from the Igor tool box; (ctrl + T, upper left box Figure 12.2.3.3A).

✓With the shape drawn, it can be moved while its still blue- use arrow keys for this (Figure 12.2.3.3C).

✓Click the 'Make Mask' Button (Figure 12.2.3.3D).

Image masks can also be used in the Roughness tab of the Analyze Panel to omit data from the roughness measurement (i.e., some spurious asperity that will compromise an otherwise candidate data set). This area is the surface area- it takes into account the XY&Z values in the image data. *See Section 12.5.1 for a brief description of this.*

✓Make the whatever shape is needed- the Igor tool breaks them down into points- (Figure 12.2.3.3B)

▪ Figure 12.2.3.4 shows a typical sequence the author frequently uses to process most images-

1) Start with a zero order flatten and 1st order XY Planefit (Figure 12.2.3.4A).

2) Make an Iterative mask, fine tune with Manual mask setvar if not quite where it needs to be- quite often, the author places the mask on what is perceived to be the substrate (Figure 12.2.3.4B).

3) Reflatten (Figure 12.2.3.4C).

Making masks from histogram

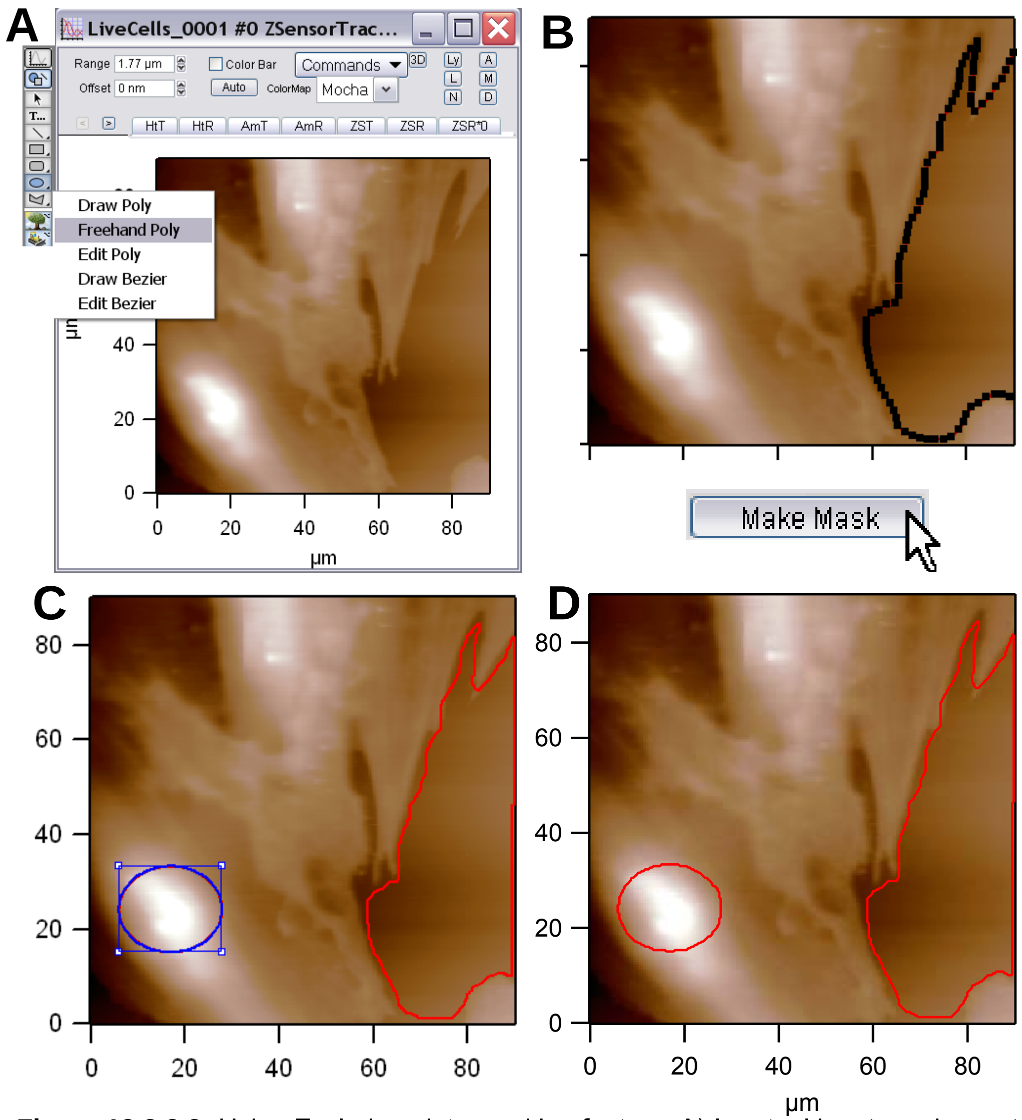


Figure 12.2.3.3: Using Exclude points masking feature: A) Igor tool box to make custom shape; B) free hand tool used to make shape on image; C) Addition ellipse shape made- blue shape indicated it can be moved on image with arrow keys; D) finished mask. *Data courtesy of Keith Jones, Asylum Research; sample courtesy J. Schlenoff, FSU Chemistry.*

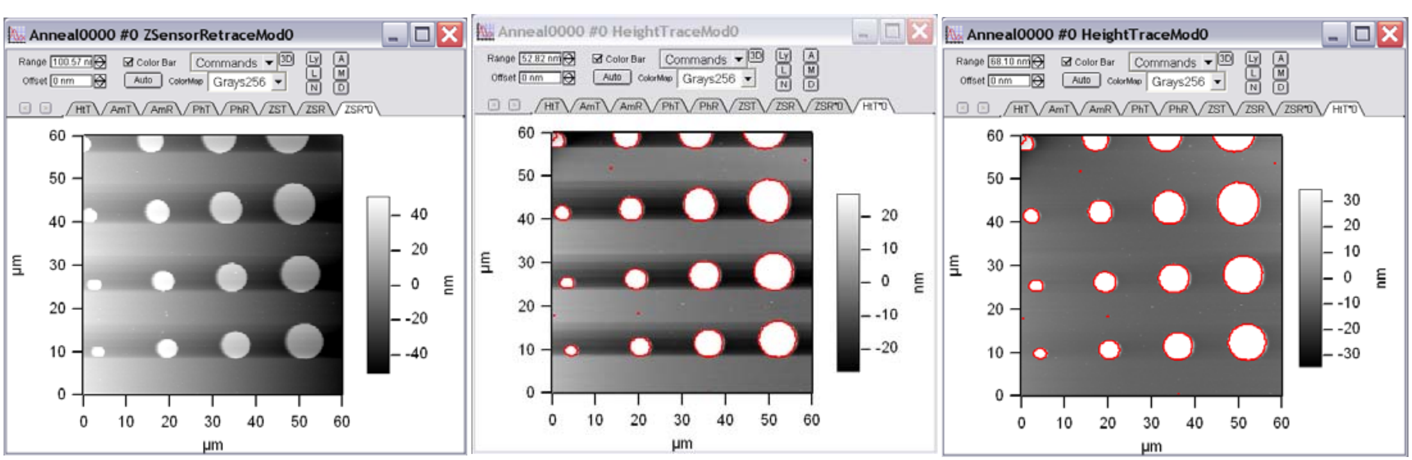


Figure 12.2.3.4: Typical processing sequence of a Height image with surface features of raw data found in Figure 12.1.3: A) Zero order Flattening; B) First order XY PlaneFit; & Iterative Mask calculation; C) Subsequent zero Order Re-flattening; Mask is still present.

12.2.4: Erase Tab:

Erasing aberrant scan lines is easily done with the MFP-3D™ software. Note that erasing too many lines for a publication can work against you- Experienced SPMers expect an aberrant noise line here and there in publication images. Please use this feature with discretion.

✓ Expand small area around line(s) to be erased.

✓ Go to the *Erase* tab of the Modify Panel.

✓ Click the 'Draw Lines' button.

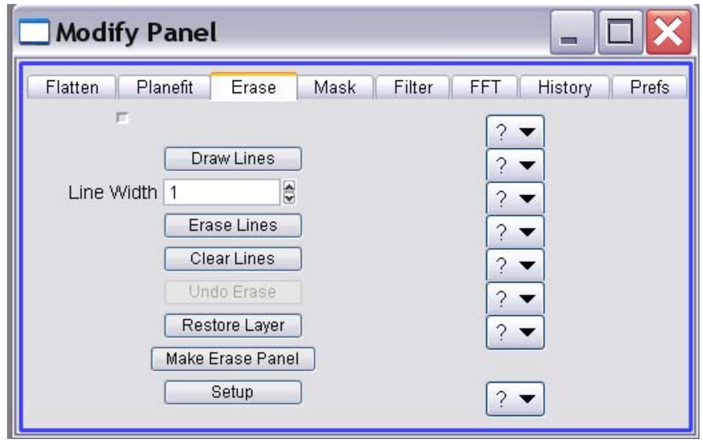


Figure 12.2.4.1: The Erase (scan lines) tab of the Modify Panel.

✓ While holding the left mouse cursor down, place the pointing finger cursor (Figure 12.2.4.1C) over the scan line you wish to remove, and release. *Notice the line goes from red while positioning, and blue when set*. In this example, one didn't seem enough, so it was increased to two using the 'Line Width' setvar window. If placement of line is dissatisfactory, click the 'Clear Lines' button to remove and repeat attempt.

✓ Click 'Erase Line' button.

✓With the cursor over the image, click Ctrl + A to restore to original size to assess results.

▪ Figure 12.2.4.2 shows a very noticeable aberrant scan line in the ARgyle 3D rendering (Figure 12.2.4.2A). To erase scan lines precisely, the author finds it easier to expand on a small area(s) containing the aberrant scan line (Figure 12.2.4.2B), giving more control over placement or cursor before erasing it (Figure 12.2.4.2C).

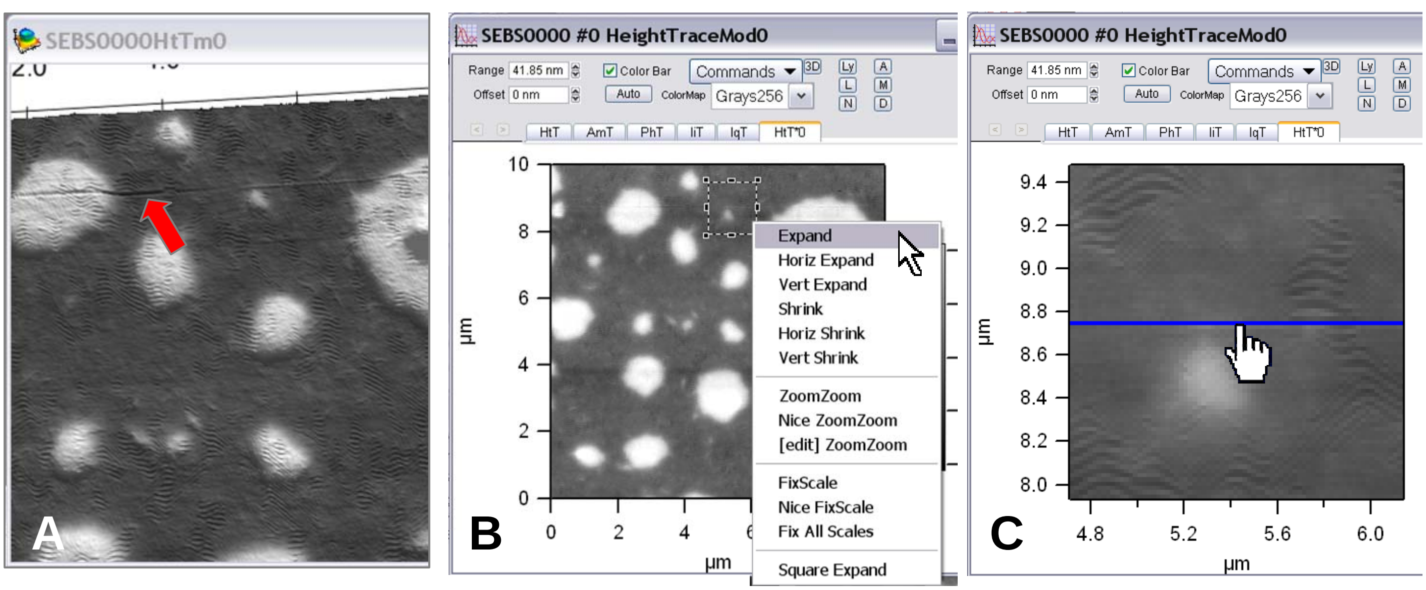


Figure 12.2.4.2: ARgyle 3D rendering showing highlighting aberrant scan line; B) Expand on area where scan line is to be erased; C) Place 'Draw line' cursor on scan line and erase.

▪ To increase the number of scan lines to erase, increase the 'Line Width' setvar value.

12.2.5 Filter Tab

This tab performs various images filtering. The author doesn't claim to have a great knowledge of image processing and filtering algorithms, but just experimenting with some of the parameters yielded the examples described below.

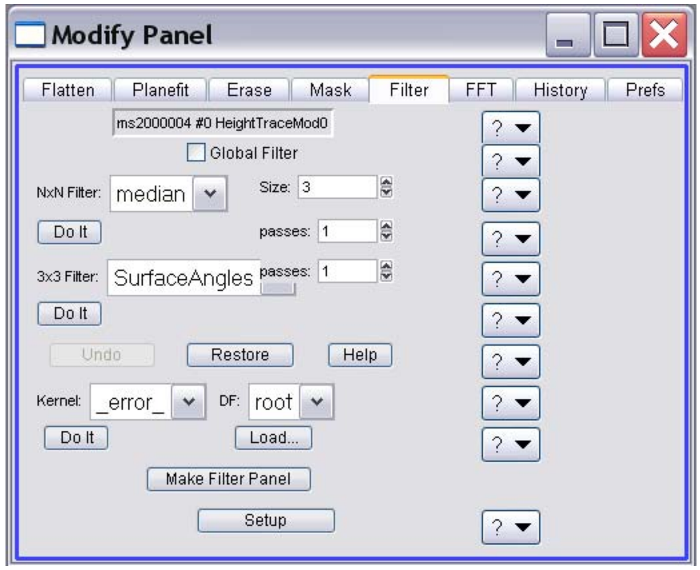
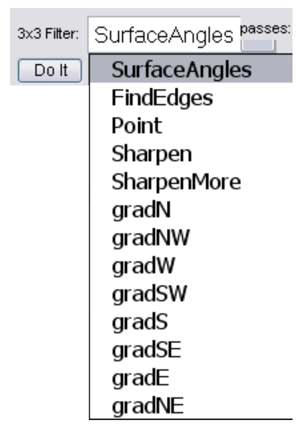


Figure 12.2.5.1: Filter tab offers various matrix filter with respective image filter types and user defined processing intereations.

The two filters that are mainly used are the NxN and 3x3 matirx filter widths. At each pixel of the image, they look at the nearest neighbors of that pixel, and apply an operation on those neighboring pixels to calculate the new pixel. A description of these matrix filters can be defined better in the software help menus.

NxN filter pull-down: this matrix filter has various filter method types including: Median, Average, Gauss, min, max, NaNZapmedian (whatever that is…).

The **Size** setvar defines the size of the NxN matrix; the larger the number, the longer the calculation time. The author looks at it similar to a smoothing function.

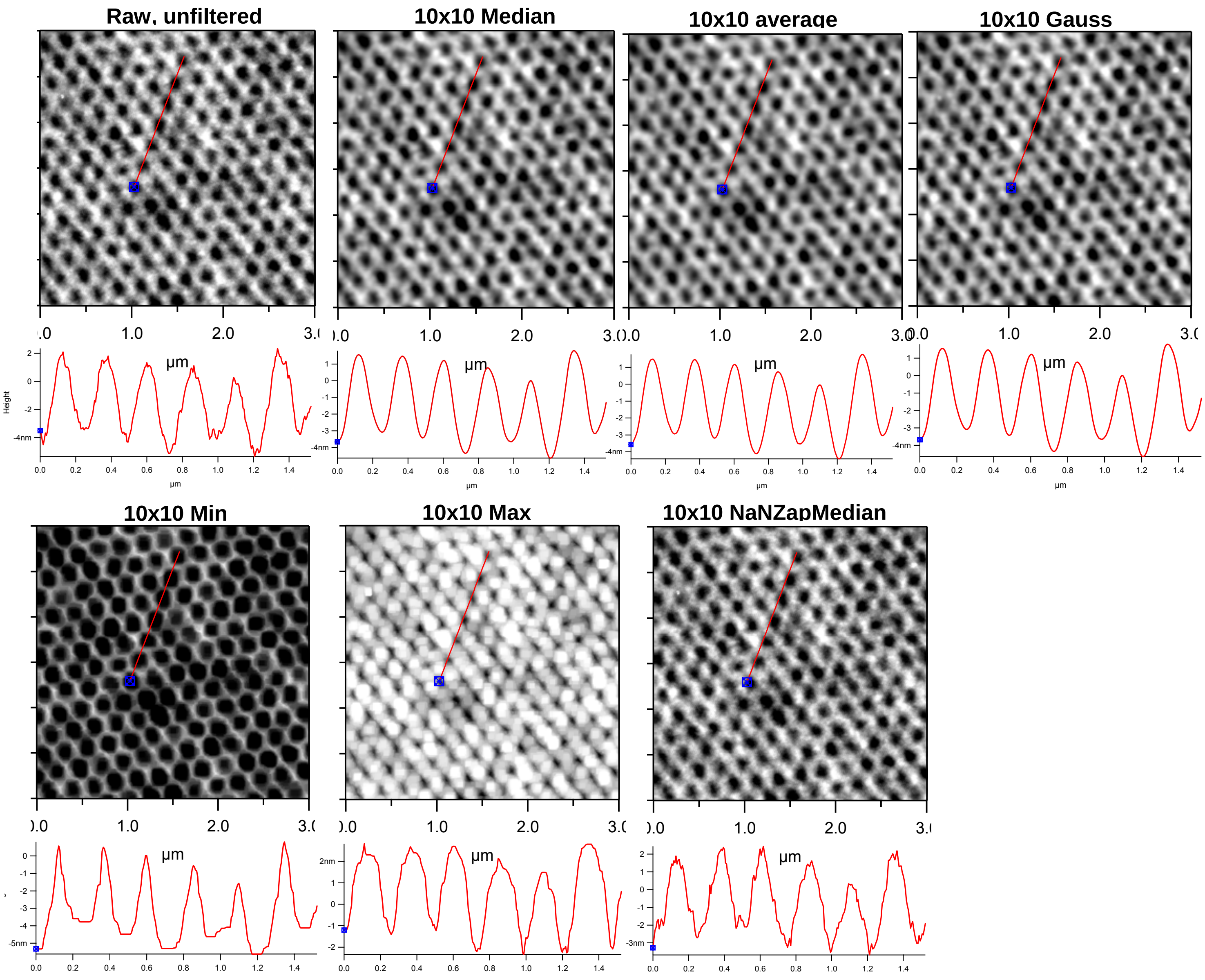


The **Passes** setvar defines the number of iterations that filtering process undergoes.

The **3x3 Filter** pull-down (seems to) performs a 3x3 matrix filter on the image; filter types here include: Surface edges, Find edges, point, sharpen, sharpen more, and the gradient filters in each direction (N,S,E,W).

▪ Figure 12.2.5.2 shows some examples of what the filter type methods do- the author choose to use a 10x10 matirx filter so the changes are (more or less) obvious relative to the raw unfiltered data. Respective line sections below images also show differences between filter processing.

The author recommends experimenting with these parameters if image processing is needed on your data, but cannot offer much practical advice due to lack of experience filtering images.



12.2.5.2: Examples of NxN filter method types: Matrix is 10x10; z scale 7nm.

12.2.6 FFT Tab

The author cannot competently describe this at this time.

12.2.7 History Tab

This tab will show what modifications have been done to the image that is being processed (Figure 12.2.7).

▪If the image have been killed and reopened, the history window will not show what modifications were made.

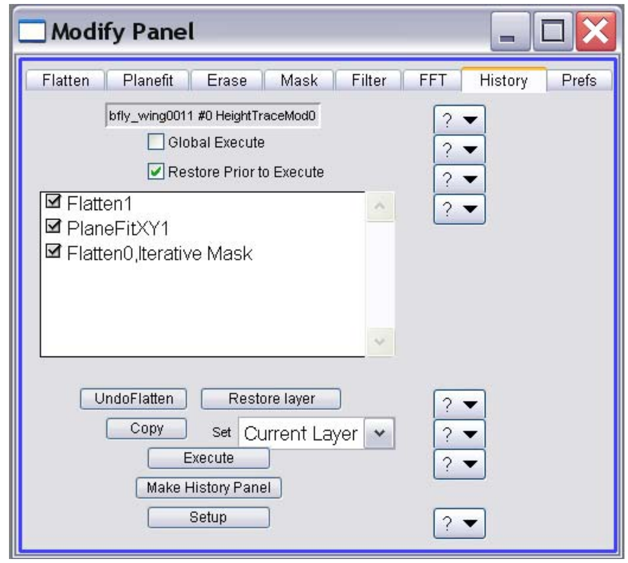


Figure 12.2.7: The History tab of the Modify Panel- This example shows the modifications that have occurred.

12.3: Saving Modifications to Display Window

Any image modification can be saved by going into the Commands pull-down menu, and select 'Save Image', or 'Save then Kill Images'. This will save the modifications, which will be present when the image is killed and reopened. Any additional changes made and saved will overwrite the modified data (* tabs), but the raw data will be preserved.

12.4: Misc. Operations: Below are some useful operations for image processing.

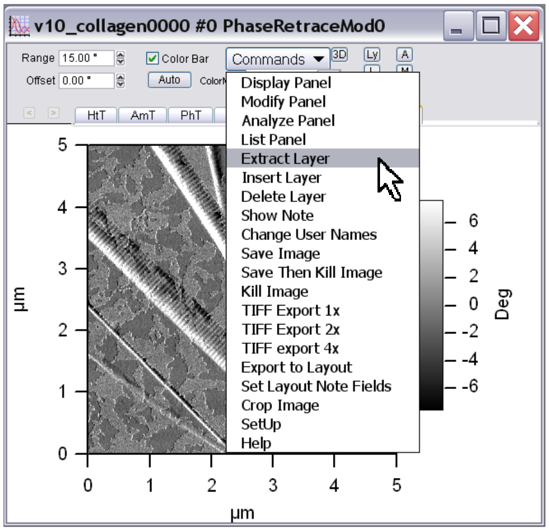
12.4.1: Extracting layers:

Layers (i.e., channel tabs) can be extracted from the display window into their own file containing just that image. Math can then be done on these individual layers, or is a way to package individual image channel tabs to reduce file size for emailing an ibw.

✓With the desired channel as the forward most tab, select 'Extract Layer' in the Command pull-down menu; this will create a (single tab) image in Memory called 'Layer Data'.

✓To view this file, click the 'Open Images' button.

▪ This new image can be renamed and saved, or have math performed on it and reinserted back into the display window.



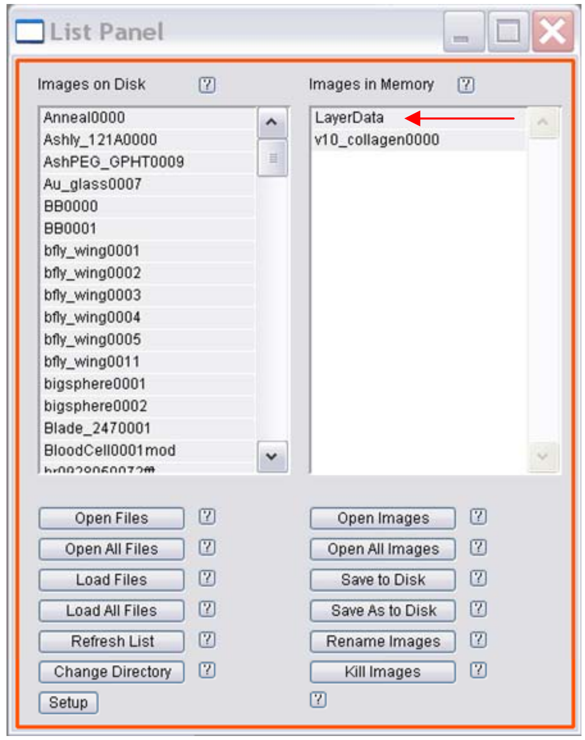


Figure 12.4.1: A) Extracting a layer from the display window; B) the new extracted layer is dumped into the 'Images in Memory' in the List panel as 'Layer Data' (orange arrow).

12.4.2: Inserting layers:

Once an image has had some math or some other function performed on it, it can be reinserted into the Display Window it came from, or another display window. An example of the latter is perhaps a force volume map image layer was created, and you wanted to insert it into an AFM image of the same area- an example of this can be seen in Figure 12.12 in Chapter 12 (Argyle 3D Rendering).

✓ If the layer is called something other than LayerData, change it back so it can be inserted (Figure 12.4.2A).

✓ Select 'Insert Layer' from the Commands pull-down menu.

✓ The Insert Layer dialogue appears, allowing the modification suffix* of the tab should be changed (Figure 12.4.2B).

✓ Click the 'Do It' button.

▪ The layer will be inserted into the display window (Figure 12.4.2C).

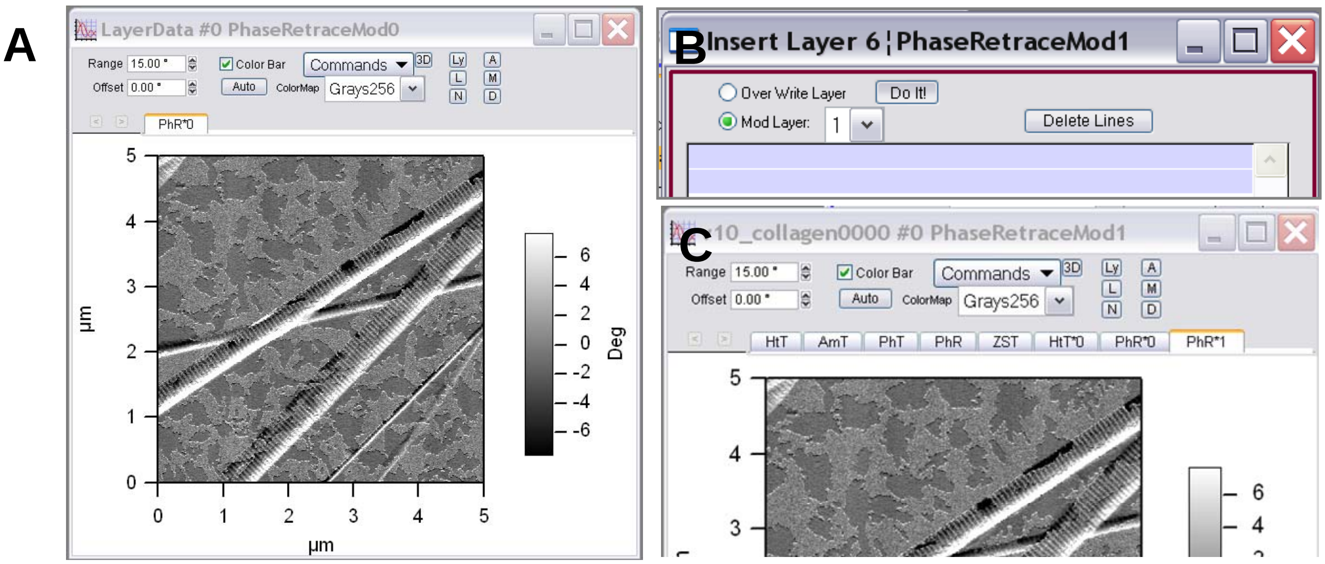


Figure 12.4.2: Inserting layers; A) modified image renamed to 'LayerData'; B) Insert Layer panel allows modification suffix to be changed; C) Inserted layer.

12.4.3: Subtracting images:

Want to get Height images that appear super sharp like a JPK, or some Veeco marketing crap? It's easy- what you have to do is subtract the amplitude image from the height image. *That being said, **use this with discretion**- If you present this among your peers, the ethical thing to do is state that it's a subtracted image-*

▪ In this example (Figure 12.4.3), the amplitude of an AC mode image of pseudomonas aeruginosa is going to be subtracted from the Height image.

✓In the display window of the image you wish to perform the subtraction on, select 'Extract Layer' from the commands pull-down menu. The 'Images in Memory' column of the List panel will now have 'LayerData' as a choice. *In this example, the modified amplitude image was extracted first.*

✓Click the 'Rename Images' button- the New Name dialogue will appear- rename the file to what ever you want
-OR-
In the command line, rename that extracted layer to what ever you want
example -*type:* Rename layerdata PAAmt

✓Do the same for the other image (in this example the modified Height trace), but you don't have to rename it if you don't want. If you do rename second extracted layer, use this name instead of layerdata in next step

✓In the command line, subtract one channel from another;
type: Layerdata = Layerdata -PAAmT
Alternatively, you can type: layerdata -=PAAmt
(Does the same operation)

✓Then type: Newimage /F(imagename) - to create the subtracted image (Figure 12.6.3C).

✓Re-insert the Layer into the Display Window so it can be viewed as a modified tab, allowing masking, section analysis or ARgyle to be performed on this new image tab. *see Section 12.4.2.*

▪ Figure 12.4.3 shows the sequence of subtracting a modified Amplitude form a modified Height image-

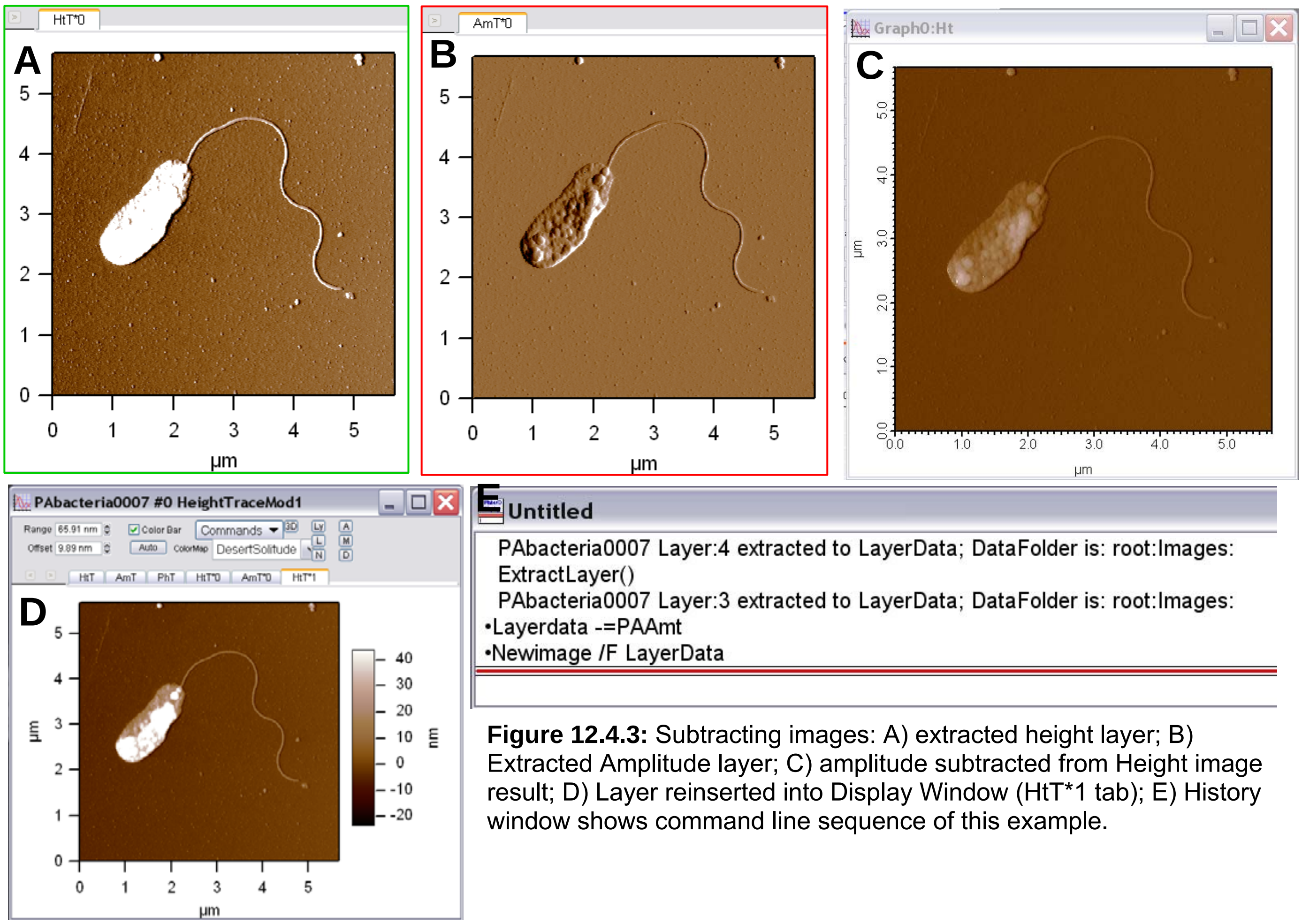


Figure 12.4.3: Subtracting images: A) extracted height layer; B) Extracted Amplitude layer; C) amplitude subtracted from Height image result; D) Layer reinserted into Display Window (HtT*1 tab); E) History window shows command line sequence of this example.

12.4.4: Rotating images:

In some rare instances, an image must be rotated.

✓ Extract the layer of the image desired to be rotated.

✓ In the command line type: duplicate layerdata, filename *-i.e., rename that layer*

✓ In the command line type: layerdata = filename [q][# of image pixels-p]

▪ In the example shown in Figure 12.4.2, the filename was PA bac, and it was a 640 x 640 image resolution.

At this point, the author has only be taught how to rotate 90 degree CCW.

▪ For an additional example, see Section 14.23.

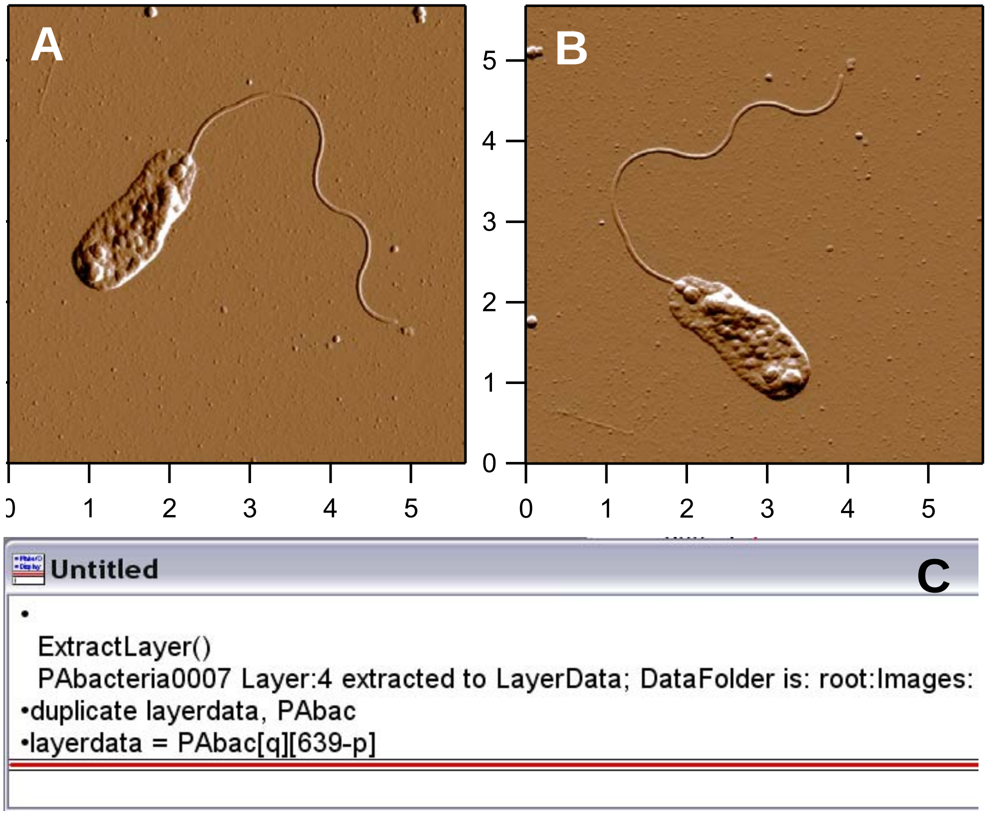


Figure 12.4.2: Rotating a stored image; A) Extract desired layer to rotate; B) With a couple of command line executions, the image is rotated.

12.4.5: Cropping Images:

Cropping images is straight forward- basic image cropping will be discussed first, then precision cropping

✓Copy the .ibw file before cropping an image- this process will cut the image data, which will be lost- so its best to crop the copied file.

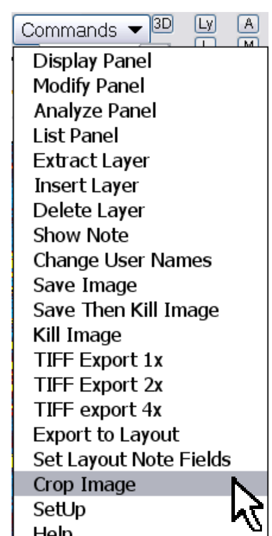
✓Expand or 'Square Expand' on the area you want to crop.
NOTE: Square expand only works for offline data- not real time imaging.

✓Go to commands pull-down and select 'Crop Image'.

✓ Go to commands pull-down and select 'Save Image'.

▪*Notice the scale is not a nice 'round number'* (Figure 12.4.5B)

✓Extract layer; this fixes the scale (Figure 12.4.5C).



Precision cropping-
There is a way to crop more precisely-

✓Open the Modify Axis Panel and manually enter the range under the 'Axis Range' tab to suit need.

✓Crop image.

✓Save Image.

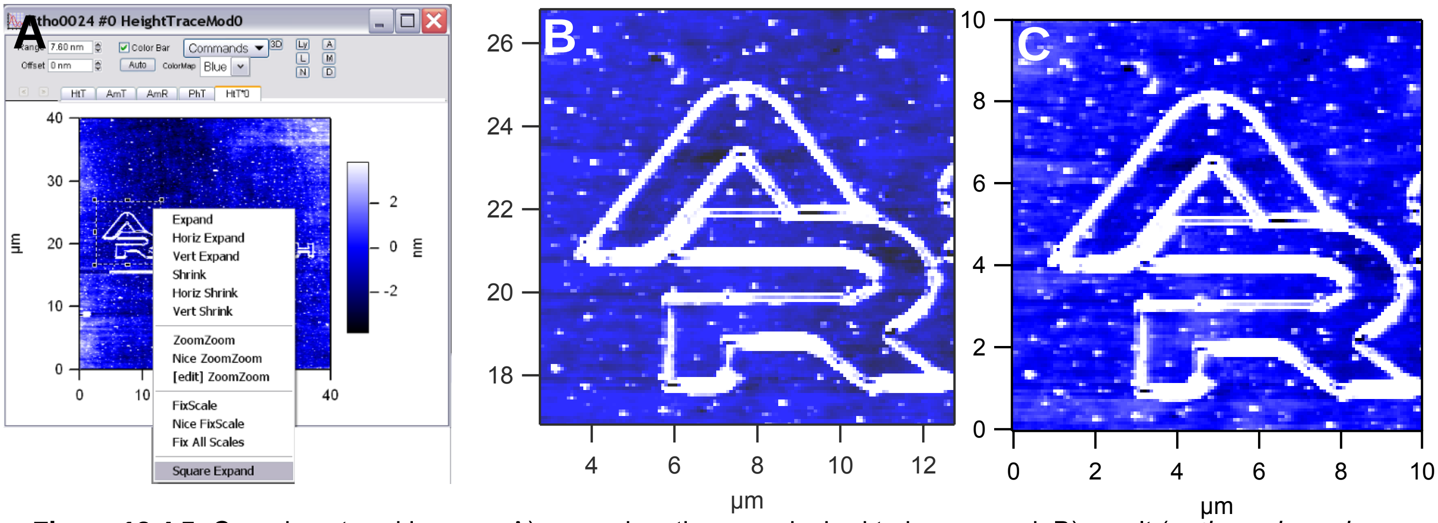


Figure 12.4.5: Cropping stored images: A) expand on the area desired to be cropped; B) result (*notice axis scale relates to where it was expanded from in raw data*); C) extract layer to rescale image axis.

Notice the resolution of the cropped image decreases because of the data loss- cropping from large pixel images suffer less data loss.

12.5: Analyze Panel

The Analyze panel is where roughness, area calculations, line sections & histograms of image pixels can be viewed.

There are various ways to open the panel-
❶ Click the 'A' button in the Display Window
❷ Go to MFP IP → Analyze Panel.
The Analyze panel will appear (Figure 12.5.1.1).

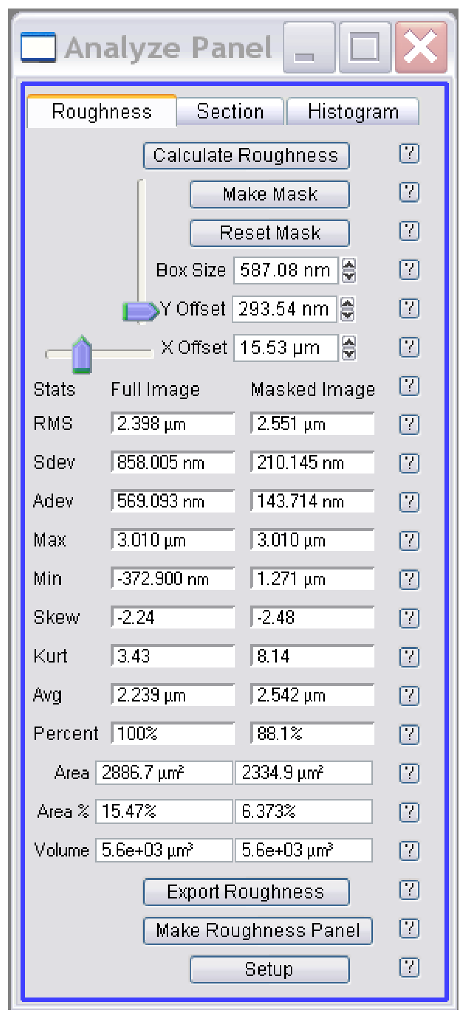


Figure 12.5.1.1: Roughness tab in the Analyze Panel.

12.5.1: Roughness Analysis

✓ Open the Roughness tab on the Analyze Panel.

▪ The most recent image will have its 'Stats' (presented in the Roughness tab) displayed. *See help button for descriptions of Stats.*

▪ If the image is masked, then the 'Masked Image' column will have the Stats for the mask.

NOTE: If inverse ☑ check in Mask tab of Modify Panel has a direct effect over what is being masked (thus the calculation) in the image.

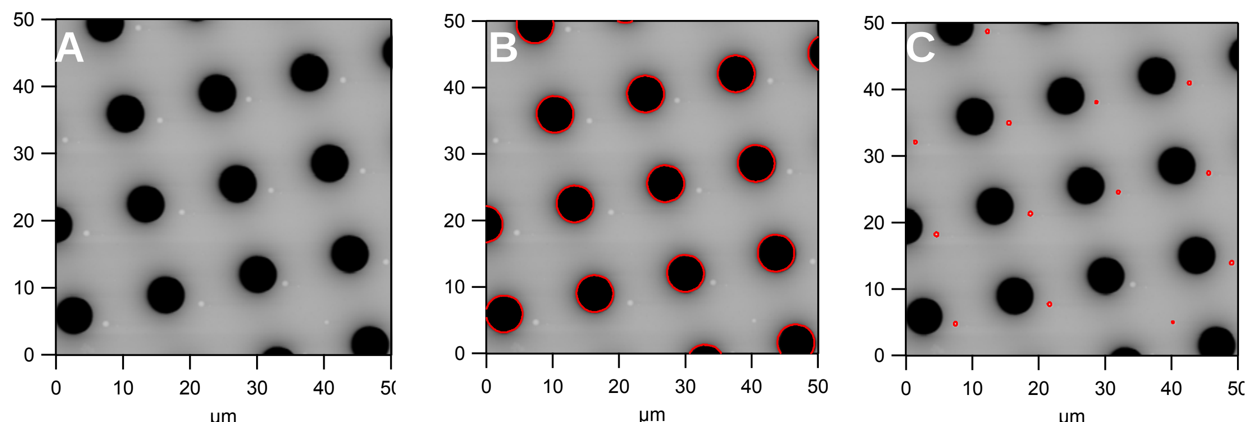
▪ If the Inverse ☑ in the *Mask* tab of the Modify is NOT activated, then space <u>inside</u> the mask is what's contained by the mask.

▪ If the Inverse ☑ is activated, then space <u>outside</u> the mask is what's contained by the mask.

▪ An example of image roughness measurements relative to surface features contained by the mask is shown in Figure 12.5.1.2. Panel (A) shows an image of a elastomeric mold that contains no image mask- Notice the 'Masked Image' column of the *Roughness* tab shows 'Nan N' which effectively is Igor speak for not a real number (i.e., no data) in the dimension of meters; Panel B shows the result of an 'iterative mask' with inverse checkbox NOT activated (see Figure12.2.3.1)- the values in the 'Masked Image' column represent the areas outside the mask (i.e., the lighter areas of the image). Finally, in Panel C), the masked was manually set to include the smaller features of the mold above the plane of its main surface- these 'Stats' numbers represent the image with inverse checkbox activated.

The way the author figures out what area is inside or outside the mask relative to the status of the inverse checkbox activation in the *Mask* tab is to look at the 'Percent' stat. If the data in question is too close to tell, it may be a good idea to go back to compare some data that is more intuitive- like a calibration grid, to get a good sense of what how the calculation works.

.

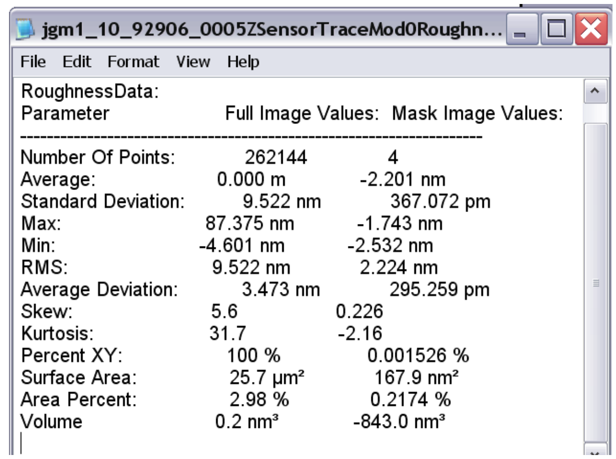


Stats	A: Full Image	A: Masked Image	B: Full Image	B: Masked Image	C: Full Image	C: Masked Image
RMS	2.398 µm	nan m	2.398 µm	2.551 µm	2.398 µm	2.945 µm
Sdev	858.005 nm	nan m	858.005 nm	210.145 nm	858.005 nm	27.694 nm
Adev	569.093 nm	nan m	569.093 nm	143.714 nm	569.093 nm	23.584 nm
Max	3.010 µm	nan m	3.010 µm	3.010 µm	3.010 µm	3.010 µm
Min	-372.900 nm	nan m	-372.900 nm	1.271 µm	-372.900 nm	2.900 µm
Skew	-2.24	nan	-2.24	-2.48	-2.24	0.285
Kurt	3.43	nan	3.43	8.14	3.43	-0.962
Avg	2.239 µm	nan m	2.239 µm	2.542 µm	2.239 µm	2.945 µm
Percent	100%	0.0%	100%	88.1%	100%	0.1%
Area	2886.7 µm²	nan µm²	2886.7 µm²	2334.9 µm²	2886.7 µm²	2.4 µm²
Area %	15.47%	nan%	15.47%	6.373%	15.47%	8.572%
Volume	5.6e+03 µm³	nan Ym³	5.6e+03 µm³	5.6e+03 µm³	5.6e+03 µm³	8.6 µm³

Figure 12.5.1.2: Roughness panel Stats: A) unmasked image; B) iterative mask results; C) manually adjusted mask to mask smaller features at top of pattern.

FYI: AREA is the actual area (i.e. Z position per XY is factored in; not a projected area value).

▪ Click the 'Export Roughness' button to create a text file of the Stats, and dump it wherever the stored image is saved. An example of exported data can be seen in Figure 12.5.1.3.



RoughnessData:

Parameter	Full Image Values:	Mask Image Values:
Number Of Points:	262144	4
Average:	0.000 m	-2.201 nm
Standard Deviation:	9.522 nm	367.072 pm
Max:	87.375 nm	-1.743 nm
Min:	-4.601 nm	-2.532 nm
RMS:	9.522 nm	2.224 nm
Average Deviation:	3.473 nm	295.259 pm
Skew:	5.6	0.226
Kurtosis:	31.7	-2.16
Percent XY:	100 %	0.001526 %
Surface Area:	25.7 µm²	167.9 nm²
Area Percent:	2.98 %	0.2174 %
Volume	0.2 nm³	-843.0 nm³

Figure 12.5.1.3: An exported Stats text file.

12.5.2: Section Analysis:

The *Section* tab (Figure 12.5.2.1) is where to perform line sections- lines can be of user defined lengths, full vectors across the image, free hand, or averages of scan lines. The lower portion of the panel will show the Stats on the Igor cursors (⊗,⊠), relative to each other in three dimensions.

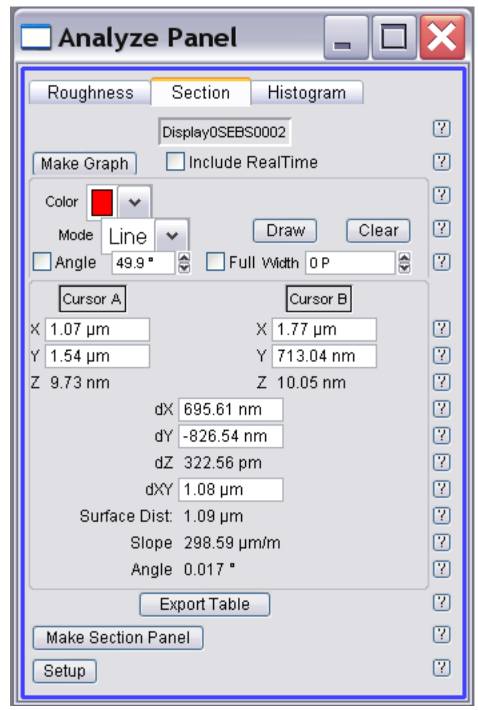


Figure 12.5.2.1: the Section tab of the Analyze Panel.

12.5.2A: Basic Line Sections:

✓ Open the Analyze Panel by clicking the 'A' button in the Display window; or MFP IP → Analyze Panel.

✓ Open the *Section* tab.

▪ The Mode pull-down menu offers vector type lines or free hand lines to be drawn . The first example will be line; a free hand example is shown in Figure 12.5.2.2.

✓ Select 'Line' from the Mode pull-down menu.

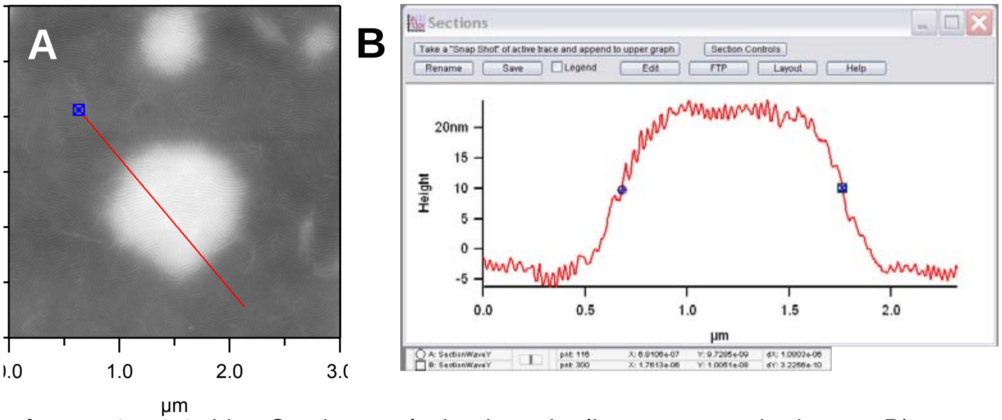
✓Click the 'Draw' button.

✓ Pull cursor over to image feature to draw a line on the feature of interest in the image (Figure 12.5.2.2A).

▪ Select Full Width checkbox ☑ if it is desired for the line section to traverse to the edge of scan areas along the line vector drawn.

▪ Multiple scan lines can be averaged to eliminate noise or give more statistical relevance to the sectioned line (see Figure 12.5.2.6).

▪ Once the line is drawn, a Section plot similar to Figure 12.5.2.2B will appear. The Igor cursors (Ctrl + I (⊗,⊠)) can be placed on the curve to measure points on the line (*notice blue cursors on the features in image correspond to cursors on the line section* Figure 12.7.2.2B); and their respective cursor location Stats are displayed in the lower half of the Analyze panel (Figure 12.5.2.2C).



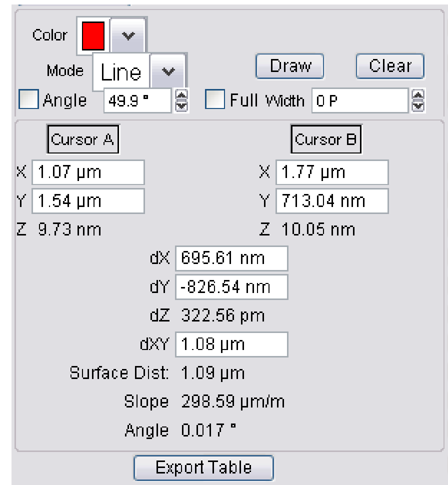
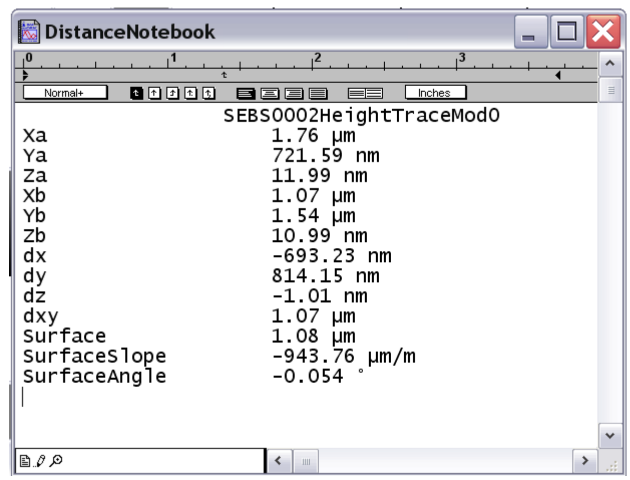


Figure 12.5.2.2: Line Section analysis: draw the line vector on the image; B) corresponding line vector; C) Section tab shows Stats of ⊗,☒ cursors relative to each other in three dimensions. *(SEBS triblock polymer sample courtesy of the Kramer lab).*

✓Click the 'Export Table' button to get these cursor stats. These can be saved as a text file, or dumped into various types of software for further organization (Figure 12.5.2.3).


DistanceNotebook

	SEBS0002HeightTraceMod0
Xa	1.76 µm
Ya	721.59 nm
Za	11.99 nm
Xb	1.07 µm
Yb	1.54 µm
Zb	10.99 nm
dx	-693.23 nm
dy	814.15 nm
dz	-1.01 nm
dxy	1.07 µm
Surface	1.08 µm
SurfaceSlope	-943.76 µm/m
SurfaceAngle	-0.054 °

Figure 12.5.2.3: Exported cursor data Stats.

12.5.2B: Leveling Line Sections:

▪ If the image happens to possess some tilt, it won't be intuitive to get a proper height from the cursors. There are two ways to get around this:

❶ Perform a 1st order XY plane fit on the image. Most times that helps get around this. *See section 12.2.2 for further description.*

❷ Hold Crtl key over curve to be 'leveled'; a ⇕ cursor will appear; right or left mouse click over line, and move to make image flat. From here, the Igor Crtl + I cursors can be put on to measure a more accurate height differentials. *See Figure 12.5.2.4 for an example.*

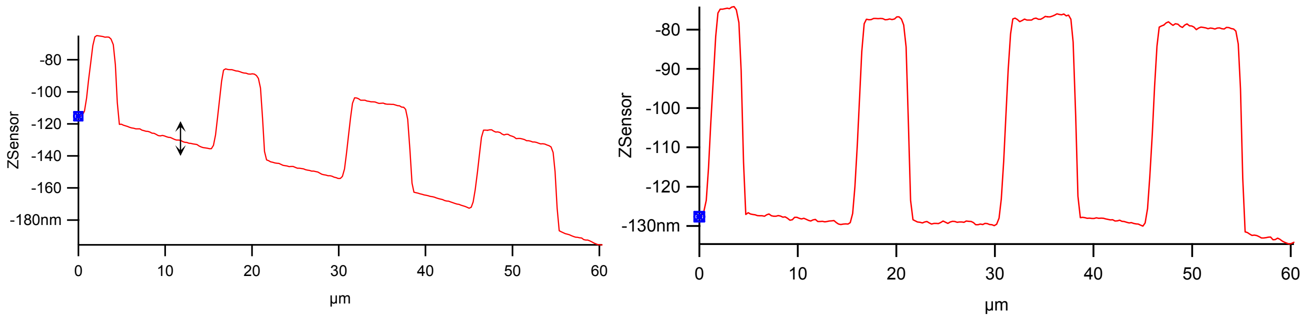


Figure 12.5.2.4: Leveling line section waves: A) over the sloped line section, hold Ctrl button and left mouse over wave- a ⇕ cursor will appear; move cursor up or down level wave; B) Result allows quick height differentials to be measured more accurately- *Notice Y axis rescaled in B).*

12.5.2C: Adjust the Angle of the Section Line-

There are multiple ways to adjust line section angles:

❶ Click the 'Clear' button in the Analyze panel and redraw a new line over feature of interest.

❷ Grab one of the end Igor cursors on the section line in the image by left click & hold while dragging cursor to new point (orange arrow, Figure 12.5.2.5A).

❸ Use the ☑ Angle check box to manually adjust with the Angle setvar window.

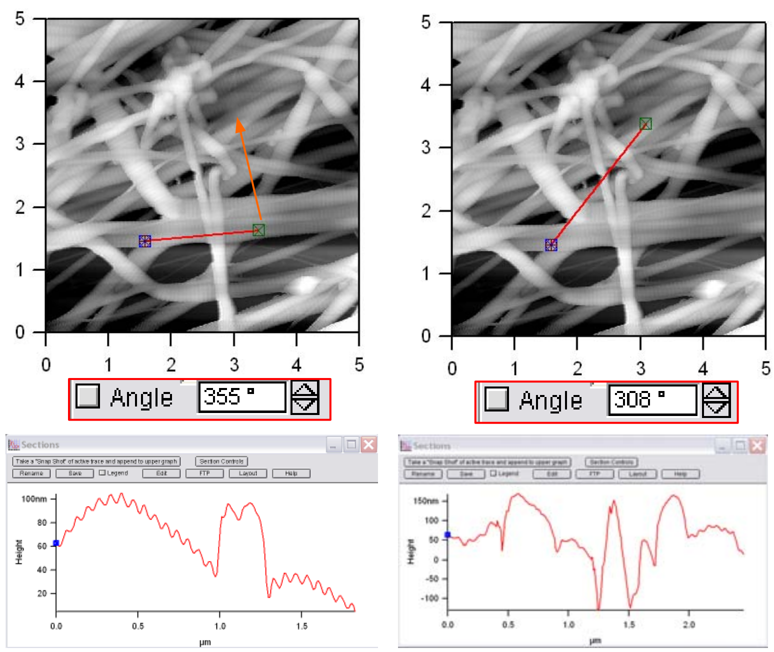


Figure 12.5.2.5: Adjusting line section angles: A) drag one of the Igor cursors to a new area; B) result.

12.5.2D: Averaging multiple section lines:

✓Enter the desired amount of scan lines to be averaged in the Width setvar window in the Analyze panel. The line will be displayed with solid center indicating center, and dotted lines represent the averaging scan line width (Figure12.5.2.6- a 25 line average in a 512 x 512 image resolution)

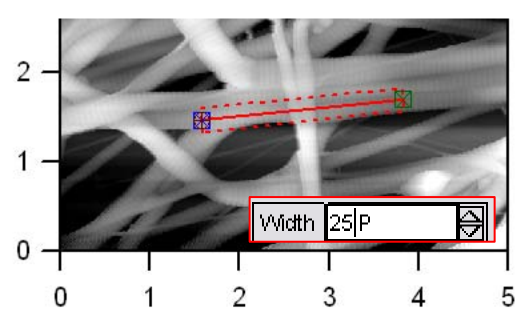


Figure 12.5.2.6: Averaging multiple scan lines.

12.5.2F: 'Free Hand' lines:

Free hand line section can be made in the offline section analysis:

✓Select 'Free Hand' from the Mode pull-down menu (Figure 12.5.2.7A).

✓Click the 'Draw' Button.

✓Draw the cursor path through the surface features desired to be incorporated into the Free Hand line section; (Figure 12.5.2.7B).

▪ Once the mouse button is let go, the line becomes a series of square points (Figure 12.5.2.7C).

✓Move the mouse cursor back into the image, and double click- this initiates the section to be calculated.

▪ Notice the line changes back to a thin red line (Figure 12.5.2.7D), and the line section appears in the 'Sections' plot (Figure 12.5.2.7E).

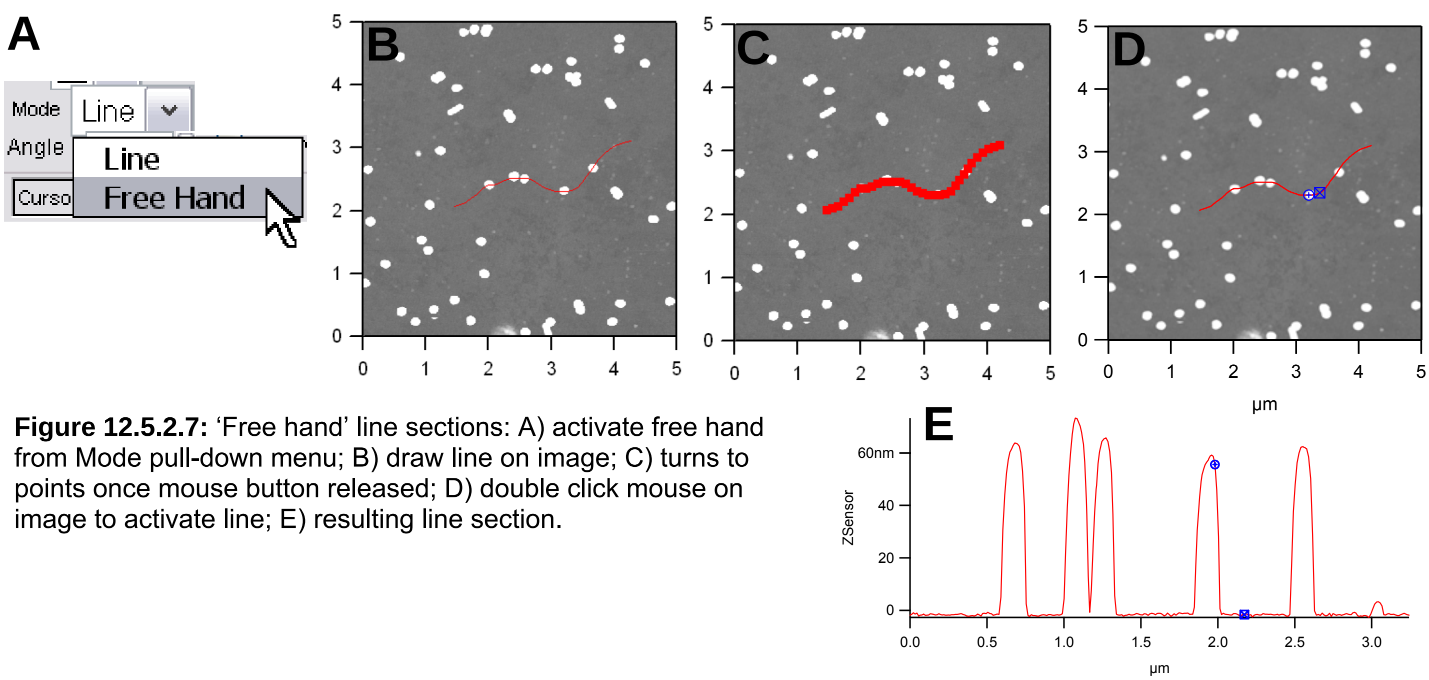


Figure 12.5.2.7: 'Free hand' line sections: A) activate free hand from Mode pull-down menu; B) draw line on image; C) turns to points once mouse button released; D) double click mouse on image to activate line; E) resulting line section.

12.5.2G: Plotting multiple line sections displayed on one section plot-

✓Create a section line as described above.

✓Click the 'Take a "Snap Snot" of active trace and append to upper graph' Button.

✓Create additional section line.

✓Click the 'Take a "Snap Snot" of active trace and append to upper graph' Button.

▪ Figure 12.5.2.8 shows an example of this.

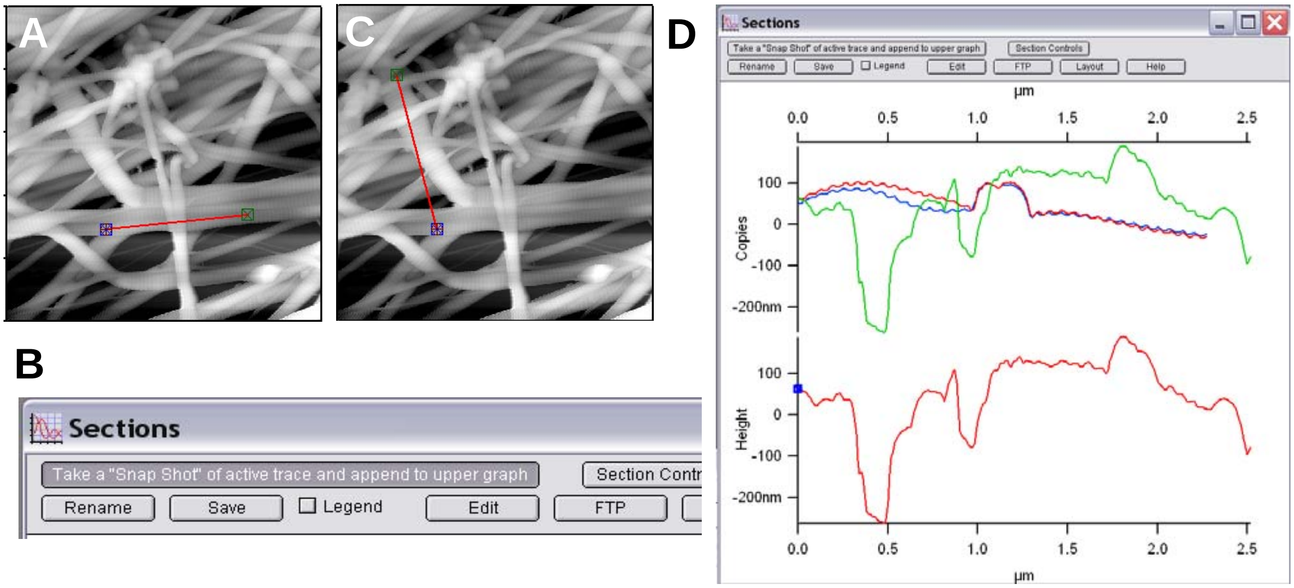


Figure 12.5.2.8: Plotting multiple section on one section plot: A) create first section plot; B) click "Snap Shot" button in Sections window; C) Create additional Section plot; B) click "Snap Shot" button in Sections window again; D) resulting multiple sections plot(s). Sample: Collagen in air.

12.5.2H: Exporting line sections as XY data:

✓ Click the 'Edit' button in the Section plot- this will bring up a table with a Y value, relative to the points in the Igor Wave (*see Igor 'Getting Started' manual to understand the definition of a wave*), and the points where the blue cursors are- kind of meaningless for use with another spreadsheet.

What the author would then do upon this inquiry is:

✓ Go to Windows → New Table…:

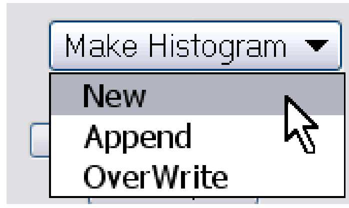
✓ Select SectionWaveX & SectionWaveY;

✓ Click 'Do It'- this will create an X,Y delimited table from which can be cut and pasted.
None of the Asylum Research inmates will verbally condone the use of programs other than Igor Pro.

12.5.3: Histogram tab

The histogram tab (Figure 12.5.3.1) allows image pixels to be counted and presented- this is analogous to 'Bearing' analysis found in other commercial AFM software.

✓ With an image open, select 'New' from the Make Histogram pull-down menu.



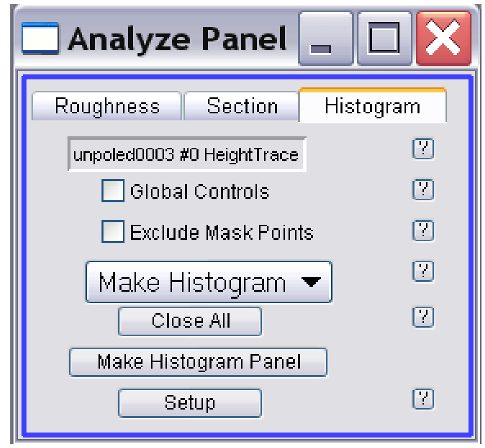


Figure 12.5.3.1: Histogram tab of the Analyze Panel.

▪ The Histogram Graph (Figure 12.5.3.2) will appear with pixel count (Y) relative to Z scale (X). This panel has a fair amount of features-

▪Figure 12.5.3.3 shows two histograms excluding masked area (A), and including Masked area (B). *Notice the pixels counts are much different.*

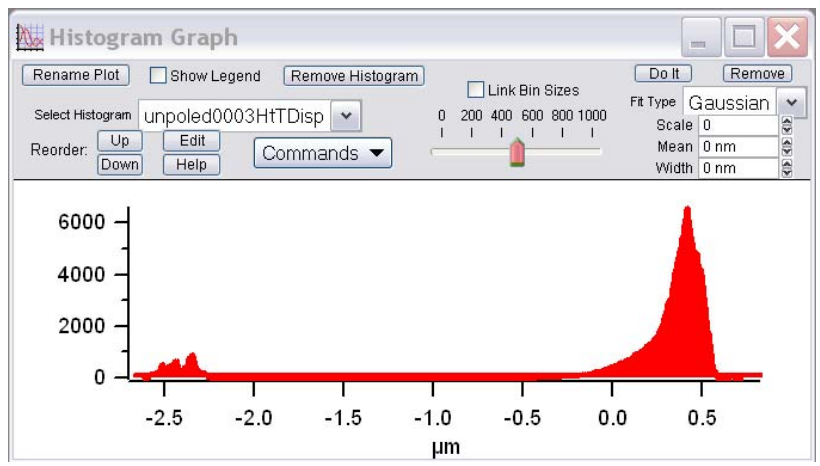


Figure 12.5.3.2: Histogram Graph-

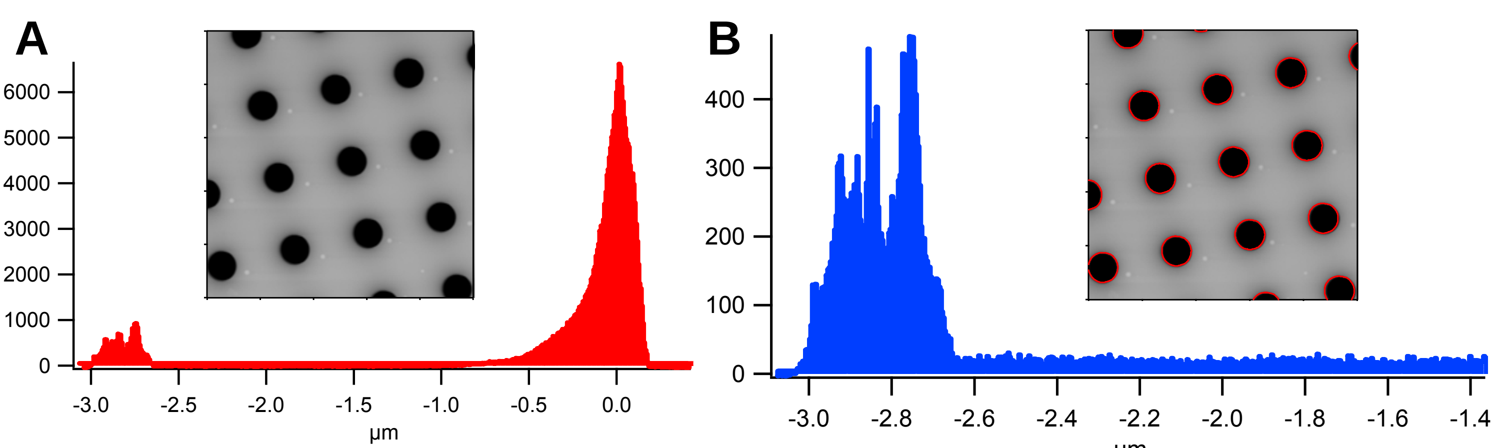


Figure 12.5.3.3: Creating pixel histograms from the image in Figure 12.5.2: A) unmasked image; B) masked image.

▪ Using the same image, the Histogram subtab can also make masks from the data using one of the features from the Command pull-down.

Procedure-
Install the Igor cursors on and area of the histogram that may be of interest to determine where on the image these pixels reside (Figure 12.5.3.4A)

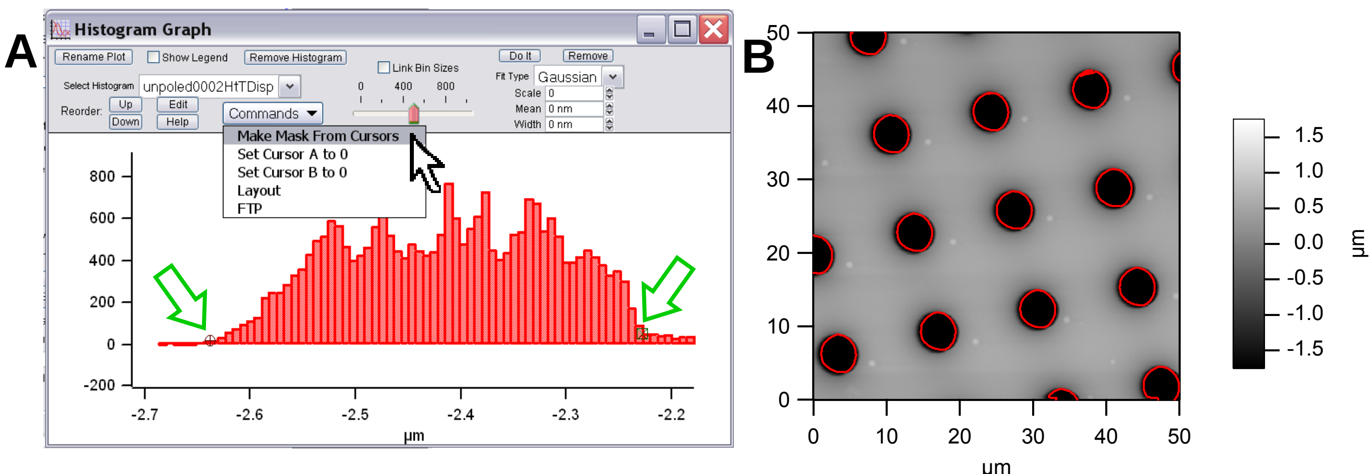


Figure 12.5.3.4: installing the igor cursors on the histogram to define the a mask on the corresponding image: A) expand left side of histogram in Figure 11.5.3.3A, install Igor cursors, then slect 'Make Mask from Cursors; B) resulting Mask threshold on image.

Other options under the Commands pull-down in the Histogram window (Figure 12.5.3.4A):

Set cursor A to 0 (cursor A is the Igor circle cursor)
Set cursor B to 0 (cursor B is the Igor square cursor)
Layout- sends histogram to an Igor layout (*which are awesome!*)
FTP- this sends the data to AR's incoming ftp site- The author is not aware how often this must occur.

12.6: Particle analysis

This particles2.ipf program was written by Stefan Vinzelberg (Atomic Force F&E GmbH-an AR affiliate; long time Asylum Research technical representative in Europe) as a way to determine volume of particles, but it works well to determine area, volume or perimeter of *masked* regions in an image. Stefan describes it as an extension of Igor's particle analysis protocol. Stefan has posted it to the AR forum, and can be found at:

http://asylumforum.com/forum/showthread.php?p=1224#post1224

The particle definition is based on a mask that must be generated from image analysis *(see Section 12.2.3)*. Use the mask tool in the modify panel to define the particles. The mask must completely cover the particles of interest.

Significant errors can arise from a bad definition of the surface. In order to ensure accuracy of the data my procedure uses the surrounding of each particle (defined as all pixels that lie adjacent to the particle boundary) and calculates its average height. This serves as a "local" reference plane for the volume. The integration is then simply done by summing up all the "little volumes" under each pixel. The data is exported to a table that contains also all other particle statistics from the particle analysis feature.

Instructions:

✓Go to File→ Open Procedure, particles2.ipf;

✓Compile the ipf; then minimize the window.

✓Open the image file you want to do particle analysis on; flatten and plane fit if necessary

✓Mask it to the desired threshold- the mask must completely cover the particle(s) of interest

✓Go to the MFP IP menu, and choose 'Particles'; a new window appears (Figure 12.6.1).

✓Set up the panel such that the desired parameters will be calculated.

Panel parameters:

Min. Pixels: determines the minimum number of pixels that are detected as a particle; helps exclude very small objects due to image 'noise'

Mask: Pull down menu to choose what image channel provides the mask that identifies the particles. *Note: image must be masked first.* If multiple images are open, select the proper image.

☑ **Volume** checkbox: if this is checked BEFORE initiating 'Do It', two additional parameters are calculated- volume (of particle) and base height (average height level of the area right around the particle). The base height is a reference height for the volume calculation.

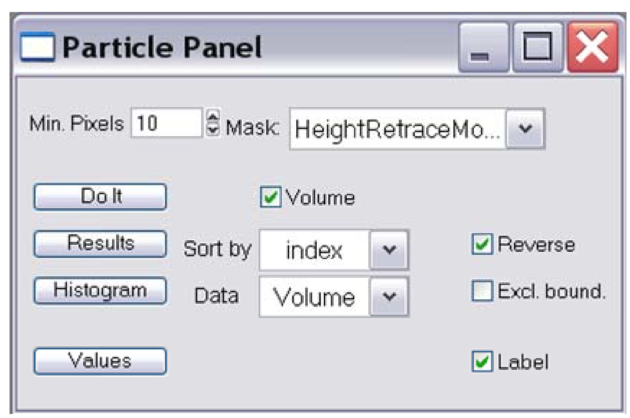
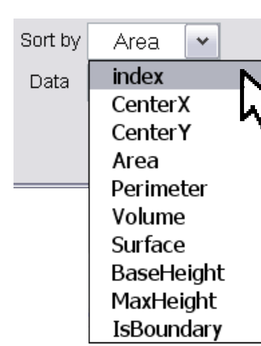


Figure 12.6.1: Particle analysis panel:

Sort by pull down: what ever value you are interested in, this will be listed in the 'Results' table from largest to smallest. For example, Figure 12.6.2 used index as the sort by; the results table lists the index from largest to smallest.



Data pull down: this pull down will be the X axis for the histogram.

☑ **Reverse** checkbox: this will reverse the order in the table for the 'Sort by' and 'Data' pull down selections- instead of largest to smallest, it will make it smallest to largest.

☑ **Excl. bound.** If this checkbox is checked, all boundary particles will be excluded from the histogram

'Do It' button: does the calculation on the data in the displayed data channel using the selected mask to identify the particles; tells you how many particles it finds shown as a log text in the history window.

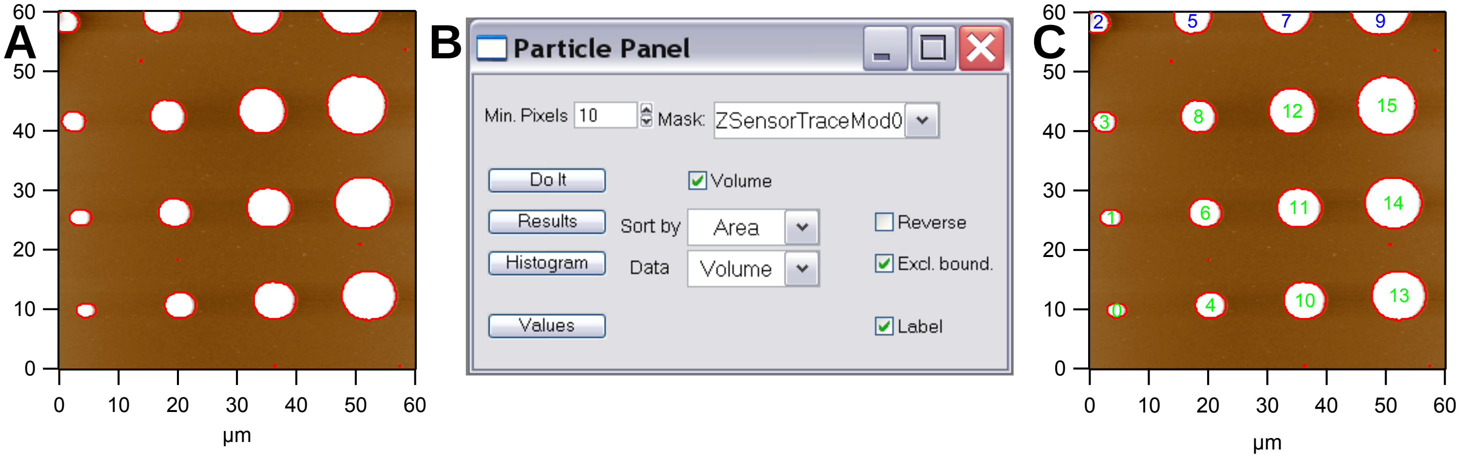
'Results' button: this function generates a table showing parameters: index, center coordinates, area (in m^2) of each particle, and a flag called IsBoundary (which is set to 1 if the particle touches the image boundary and therefore may be incomplete, set as 0 if not touching). All these parameters can be plotted in the histogram function.

'Histogram' button: Use Histogram Data pull down menu to choose what data is used to generate a histogram. Click Histogram button to give counts (y –axis) of what ever parameters (x-axis) you choose. *Note: Surface is not yet implemented* as of 7/2006.
'Values' button: opens a table where the analysis values for a single particle are displayed. You can click on the respective particle in the image (the corresponding label will increase its size) and the table will be updated with its values.

✓ Click the 'Do It' button- Igor will crunch the numbers and report how many particles were found, including how many happened to be on the boundary of the scan range- reported in the History window.

▪ An example is seen in Figure 12.6.2: The sample is some sort of lithographically prepared circles of varying diameter. After the image is processed (planefit, flattened), a mask threshold was fit to surround each pattern (Figure 12.6.2A). With the parameters set in panel B (specifically the 'Sort by' pull-down set to *Area* and the 'Data' pull down set to 'Volume', the results are seen in Figure 12.6.2C&D. Notice the indexes are labeled according to the 'Sort by' and 'Data' pull down selections: the largest index value is for the largest area/volume; the smallest index labels the smallest area/ volume. Also notice any masked feature at the perimeter of the scan range is labeled with a blue index (Figure 12.6.2C), indicating the program is aware the feature is not fully contained in the image. This can also be seen in the results table as a '1' under the IsBoundary; fully contained features are represented as a '0' (Figure 12.6.2D). Figure 12.6.2E shows the resulting histogram, with the X axis as volume (because that is what the 'Data' pull down is set to.

Also, the particles will be labeled with blue indexes on the masked image (Figure 12.6.2C).



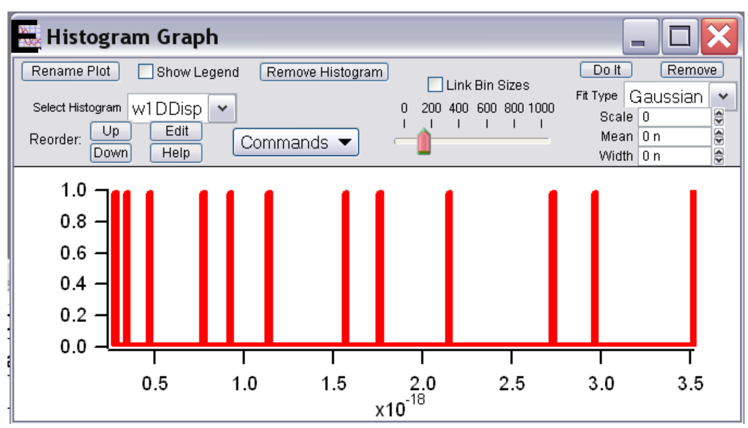
D

PAResults:ParticleResults.ld

R0 C0 | 2

Row	ParticleResults[][0	ParticleResults[][1	ParticleResults[][2	ParticleResults[][3	ParticleResults[][4	ParticleResults[][5	ParticleResults[][6	ParticleResults[][7	ParticleResults[][8	ParticleResults[][9
	index	CenterX	CenterY	Area	Perimeter	Volume	Surface	BaseHeight	MaxHeight	IsBoundary
0	2	4.56998e-06	9.81136e-06	6.42215e-12	9.31765e-06	2.5413e-19		-2.91261e-09	5.20031e-08	0
1	7	3.61104e-06	2.54118e-05	8.13841e-12	1.04471e-05	3.3397e-19		-2.81615e-09	5.13097e-08	0
2	15	1.45711e-06	5.83076e-05	1.06298e-11	9.69412e-06	5.34185e-21		-5.65812e-09	3.83812e-08	1
3	11	2.47477e-06	4.15408e-05	1.09066e-11	1.21412e-05	4.55107e-19		-1.98572e-09	5.09579e-08	0
4	3	2.04039e-05	1.06858e-05	1.81592e-11	1.55765e-05	7.7043e-19		-4.38113e-09	5.10517e-08	0
5	13	1.73749e-05	5.84867e-05	1.97647e-11	1.98118e-05	8.81274e-19		-3.22435e-09	5.08533e-08	1
6	5	1.95009e-05	2.62495e-05	2.10381e-11	1.69882e-05	9.11694e-19		-4.43073e-09	5.04896e-08	0
7	14	3.32226e-05	5.84944e-05	2.48581e-11	2.4e-05	1.12871e-18		-3.70116e-09	5.1056e-08	1
8	9	1.83484e-05	4.24648e-05	2.58547e-11	1.87294e-05	1.1291e-18		-4.83114e-09	5.34599e-08	0
9	12	4.91181e-05	5.84763e-05	2.94533e-11	2.76235e-05	1.33768e-18		-4.45252e-09	5.11575e-08	1
10	1	3.6314e-05	1.14845e-05	3.59308e-11	2.22588e-05	1.56583e-18		-4.38158e-09	4.95939e-08	0
11	6	3.53603e-05	2.70985e-05	3.97509e-11	2.34353e-05	1.75788e-18		-4.71409e-09	5.21362e-08	0
12	10	3.42033e-05	4.3395e-05	4.80554e-11	2.59294e-05	2.14393e-18		-5.18419e-09	5.19621e-08	0
13	0	5.22282e-05	1.23048e-05	6.05675e-11	2.89882e-05	2.71283e-18		-3.56321e-09	5.09804e-08	0
14	4	5.12765e-05	2.79189e-05	6.57163e-11	3.01647e-05	2.9588e-18		-3.53688e-09	5.55609e-08	0
15	8	5.01295e-05	4.43184e-05	7.77301e-11	3.29412e-05	3.52587e-18		-3.68939e-09	5.13325e-08	0

Figure 12.6.2: Particle analysis example: A) masked image; B) Panel parameters; C) labeled masked image; D) results table- Area column being listed from smallest to largest; E) histogram showing volume distribution.



▪ Another example can be seen in Figure 12.6.3- a NSL sample from the Van Duyne lab. This example shows a better particle distribution in the histogram, relative to the example shown in Figure 12.6.2.

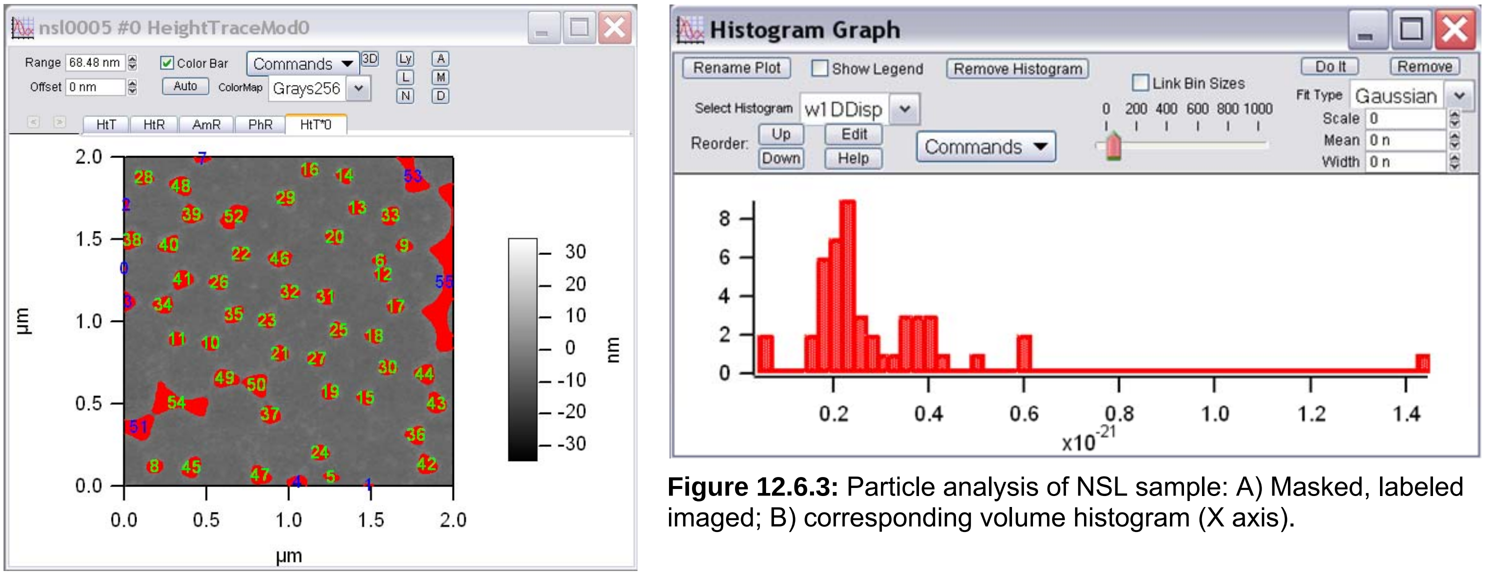
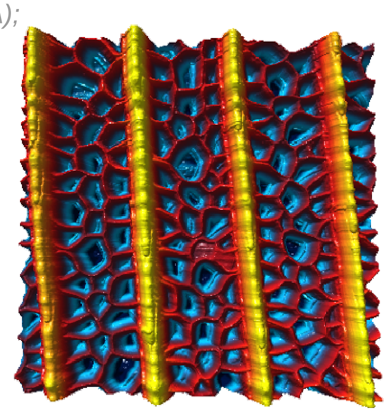


Figure 12.6.3: Particle analysis of NSL sample: A) Masked, labeled imaged; B) corresponding volume histogram (X axis).

13. ARgyle™
3D Image Rendering



ARgyle™ is the MFP-3D's 3D image rendering software that uses open GL source code. It can produce some remarkable AFM data representations in real-time or with offline analysis with custom colors & specular lighting. It also allows multiple data channel overlay as a fourth color dimension to correlate with surface topography.

▪ There are two ways to get started using ARgyle™ depending on if you are imaging in real time or doing offline analysis. Turning on Real Time ARgyle™ will be presented first; then basic ARgyle protocol for image adjustment discussed in Section 13.2 applies whether viewing a real time image collection or an offline image.

13.1: Activating Real time ARgyle™:

✓In the main toolbar of Igor, Go to MFP IP → 3D surface plots. The Master ArGL Panel will come up (Figure 13.1). It contains 7 tabs that are all useful in producing a fine presentation.

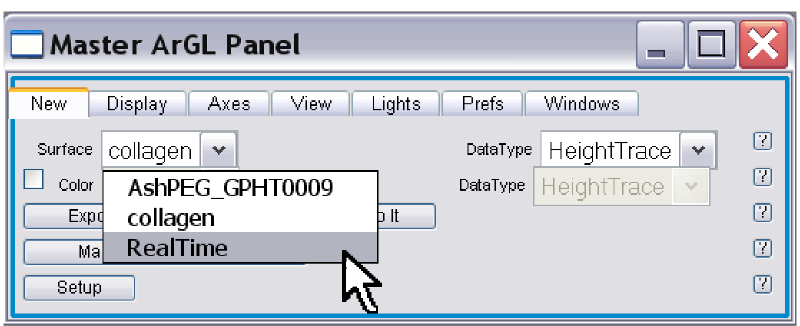


Figure 13.1: A) New Tab of Master ArGL Panel; B) choosing the real time channel to display as an ARgyle™ image.

✓In the *New* tab, select RealTime from the Surface pull-down menu (Figure 13.1).

✓From the data type pull-down menu, select the channel you wish to display (Figure 13.2).

For multiple channel overlay:

✓Click the color check box ☑ to the right of the surface pull down menu.

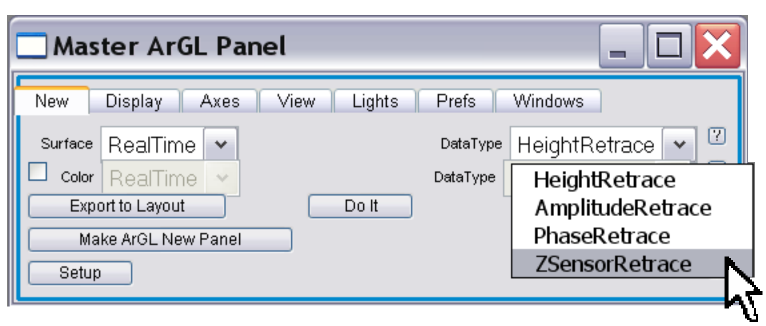


Figure 13.2: Choosing data channel to display in ARgyle.

✓Select what channel you want to color the topography with. *Channels that are open in the Master Channel tab will be available in the Data pull-down menu.* See Section 13.5 for further description.

Useful ARgyle Hotkeys:
Crtl + left mouse button zooms the size of the image.
Shift + mouse does moves the image within the ARgyle window.

13.2: Activating ARgyle™ offline:

For more about processing images in the MFP-3D™ software, see Section 12.

✓Click the 3D button at the top of the display window tab that has the (processed) data that you wish to display in ARgyle™ (Figure 13.3). This will bring up the Master ArGL Panel (Figure 13.1A) and the ARgyle™ 3D rendered image.

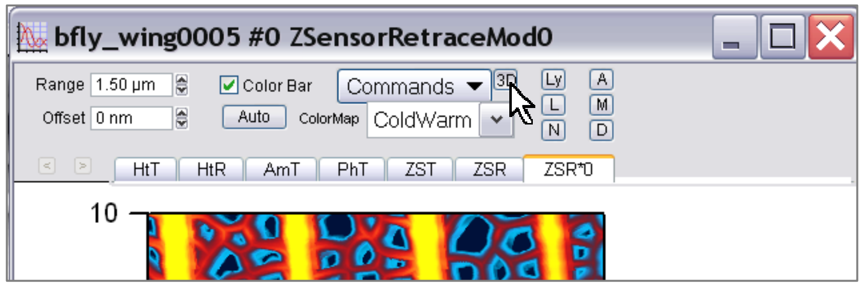


Figure 13.3: selecting 3D button calls up ARgyle windows.

If this is the first activation of the 3D image in a new MFP-3D template, it will have certain default parameters (i.e. a Zoom factor of 1 & Z scale of 0.33 in Zoom tab) that may appear off-scale (in Z) if the surface features are large relative to the XY scan area, or close to scale (in Z) if the feature are large; if you have already processed a 3D window in the current experiment, the new ARgyle image will have the same rotation, zoom, etc as the previous; it will have the same Z data scale as the 2D image in the display window.

13.3: Adjusting ARgyle parameters:

The author usually follows the same protocol to get a handle on the off-scale default parameter:

✓ Click on the 3D rendered image, left mouse click and rotate it to an orientation you are happy with. The author usually rotates it to a top down view.

✓ In the *Prefs* tab, choose the background color you prefer.

✓ In the *View* tab, select 'None' from the Clip pull-down menu; choose Zoom from the View Mode pull-down menu.

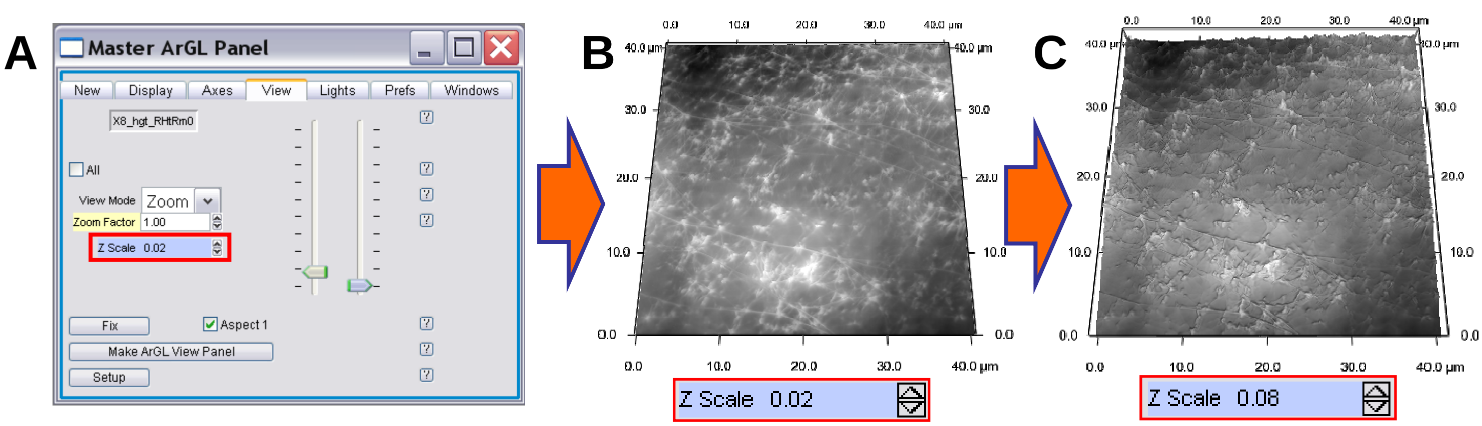


Figure 13.4: Re-scaling the Z axis in Argyle images.

✓Check the 'Aspect 1' checkbox ☑ (Figure 13.4A), this will scale the Z data to the XY data (Figure 13.4B). If this isn't enough Z scaling, adjust the Z scale parameter to larger value (Figure 13.4C). *A Z scale of 0 will effectively create a 2D image.*

▪ Activating the 'All' checkbox in the upper left corner of most tabs, applies the ARgyle parameter settings to all open images.

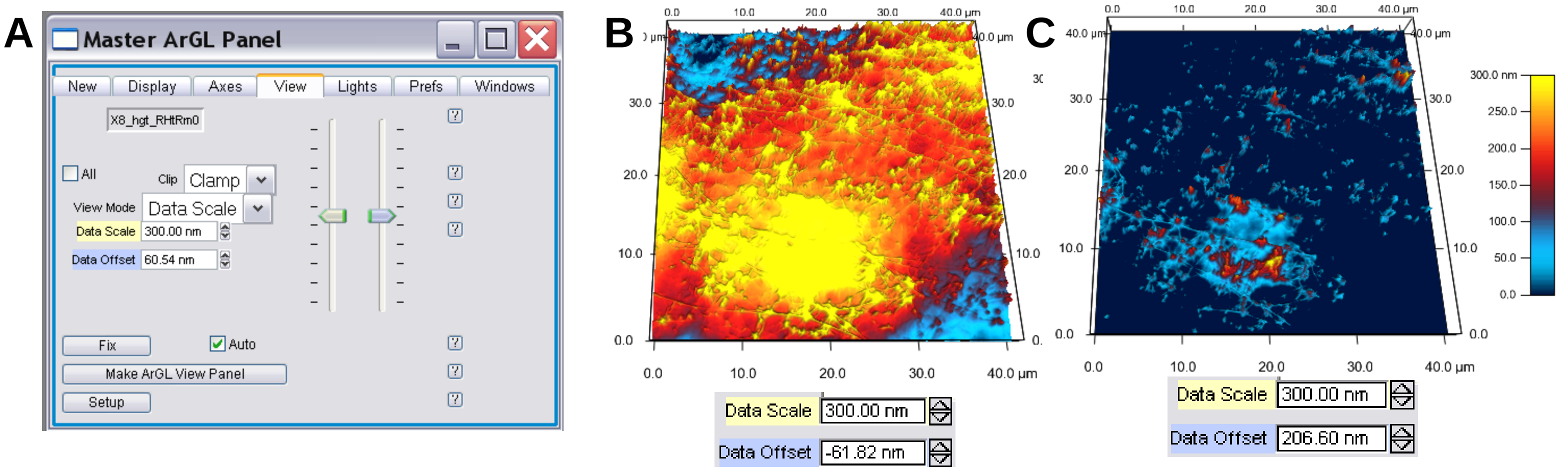


Figure 13.5: Examples of adjusting the Data offset in an ARgyle image: B) -61.nm ; C) 206.6 nm. Color 'Cold Warm'.

✓In the *View* tab, select 'Data Scale' from the View Mode pull-down menu. Here you can adjust the Z scale of the data just as you can in the display window – use left vertical slider bar, or data scale 'setvar'. The 'Data Offset' – essentially where the plane of the image sits within the color scale (color bar). Figure 13.5 B & C shows an example of changing the Data Offset.

COLOR TABLES-

✓In the *Display* tab, choose an appealing color from the ColorMap pull-down menu; Notice you can invert the Z scale or color. Custom colors tables can also be generated by choosing 'Define Custom'. The author has little mastery of this process, but it's fun to try.

✓ Adjust other values such as the Data scale values, light rotation, zoom and offset. Background colors are found under the '*Prefs*' tab.

✓ To add Specular lighting, go to the *Lights* tab, click the shiny check box ☑ (Figure 13.6). The percent of specular light can also be defined in the 'Factor' setvar.

▪ *Lighting (either normal or specular) can be adjusted by holding the right mouse over the 3D rendered image, and moving it around the image until an appealing angle is achieved. Adjust light to hide scan lines for better presentation.*

▪The light rotation and pitch setvar values can be viewed in the Light Rotation sub-tab of the View tab

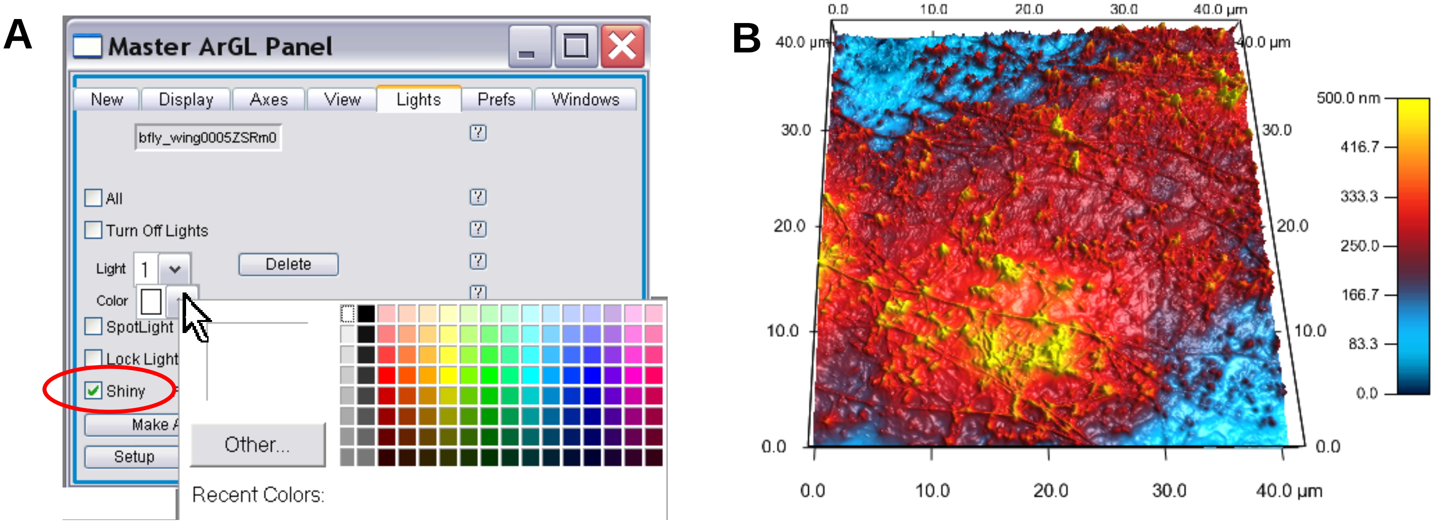


Figure 13.6: Adding Specular lighting to the ARgyle image in the Lights tab. A) Click 'Shiny' checkbox ☑; Color of Specular lighting can be selected (in Color pull down menu), as well as 'Percent' intensity; B) Specular light can be seen as sheen in image- allows visualization of small feature shadows on the surface not readily apparent without light.

▪To render ARgyle™ image as an anaglyph (for use with 3D glasses), the back ground of the image **must be black** (choose in Prefs tab), or an error message will come up; then in command line-
✓ *To turn ON Anaglyph in ARgyle window type:* Argl_WriteValue("","anaglyph",1)
✓ *To turn OFF Anaglyph in ARgyle window type:* Argl_WriteValue("","anaglyph",0)

13.4: Other ARgyle™ features:

▪The image can be set to spin automatically.

✓ Select Spin from the View Mode pull down menu in the View tab (Figure 13.7).

▪ ARgyle™ image axes, and ticks can be adjusted in the *Axes* tab.

▪ Axis fonts, font sizes and background colors are can be adjusted in the *Prefs* tab.

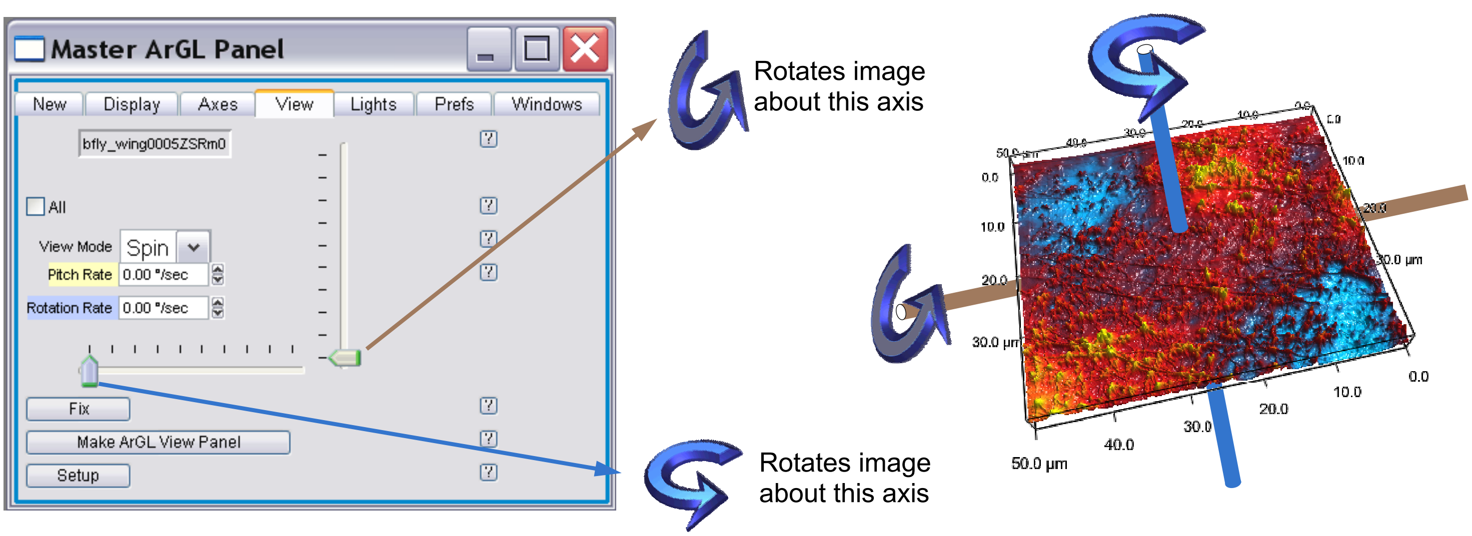


Figure 13.7: Setting automatic spin of ARgyle image: Lower slide bar spins about the image plane; slider bar sets the speed; Vertical bar spins

Clip Mode: The author finds this function difficult to describe- this is typically set to 'None' during use, adjusting the Zoom scale and /or data scale instead. That being said, this feature can come in handy for presentation. It's best to experiment with it to figure out what it does, but here's the author's attempt to visually describe the functions.

▪ Figure 13.8 shows an image of a nanosphere lithography (NSL) pattern with an exaggerated Z scale. Panel A) shows the 'Clip' pull-down set to 'None'; Panel B) shows the 'Clip' pull-down set to 'Clip'- the higher asperity features are clipped set to some limit such that the ARgyle image appears to have holes where the NSL asperities are; Panel C) shows the 'Clip' pull-down set to 'Clamp'- the higher asperity features are clamped set to some limit such that the ARgyle image appears to have plateaus where the NSL asperities are.

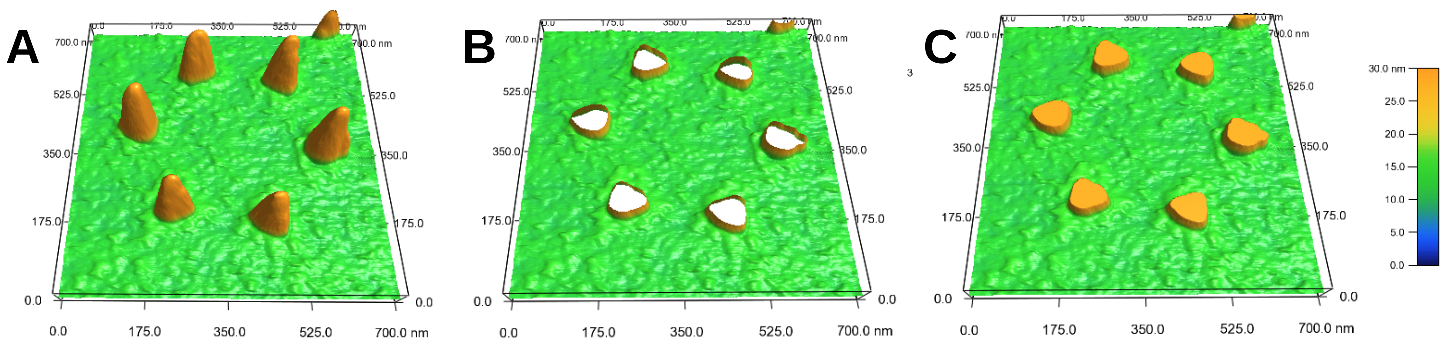


Figure 13.8: Clip mode function: A) none: B) clipped; C) clamped; *Nanosphere lithography sample courtesy R. Van Duyne, Northwestern University.*

Windows tab: This tab just manages the ARgyle™ windows when they are up. The author typically doesn't use this tab because it would be too organized.

▪ Movies can be made with ARgyle™ image. Contact Support@AsylumResearch for this instruction file. Essentially this program takes defined ARgyle™ angles, rotations and zoom factors and strings these values together to make a continuous movie that seamlessly changes the user defined selections. Contact Support@AsylumResearch for this instruction file.

13.5: Multiple Channel Overlay:

Overlaying channels in ARgyle™ is as simple as a couple mouse clicks-

✓In the New tab of the Master ArGL Panel, choose the image and image channel you want the topography to be displayed (i.e. the image that will have 3D rendering to it).

✓Click the color checkbox ☑, and then choose the same image file, and the other data channel that you want to over lay (Figure 13.9). Make sure to keep the same direction in the overlay for proper registration (i.e. Trace with Trace).

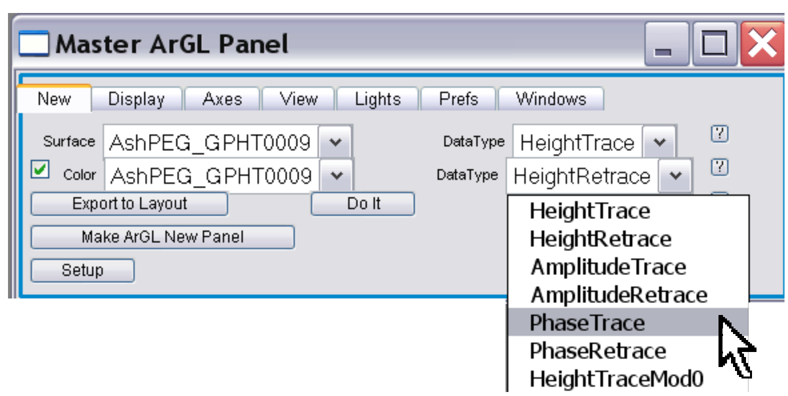
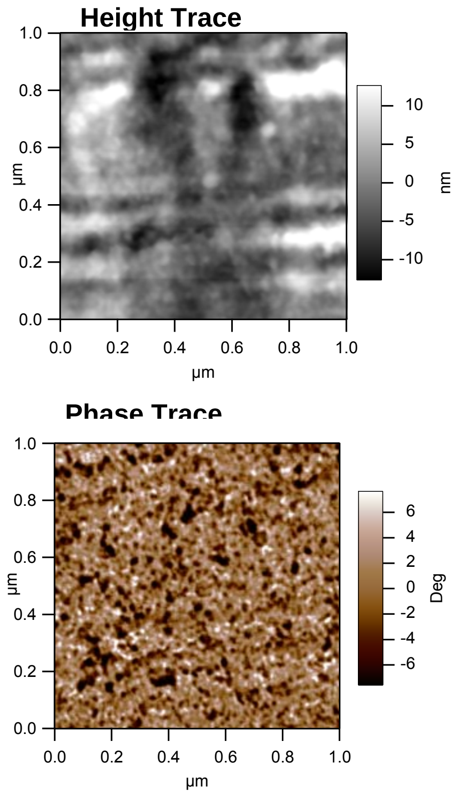


Figure 13.9: Overlaying image channels in ARgyle.

▪Figure 13.10 shows an example of overlaying the phase channel onto topography. The sample is a polymer material imaged in air with AC mode- notice the interesting phase data doesn't necessarily correlate with the topography data.



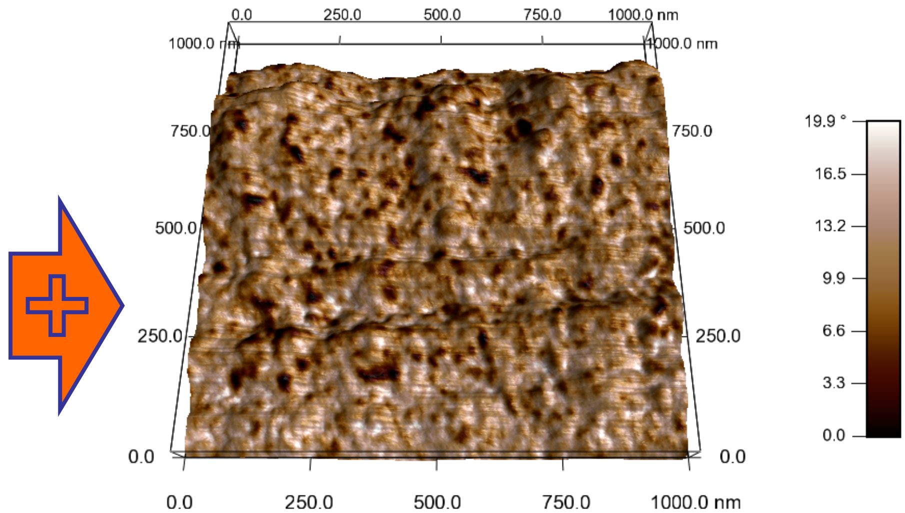


Figure 13.10: Example of Phase Channel (color) overlay on height channel (topography). Color 'Desert Solitude'

▪ The old school way to accomplish channel overlay is to use the command lines: *If your MFP-3D has older software on it….*

✓ Process your two channels to your liking (i.e flatten, mask, pick colors). Create an ARgyle image for **each** image channel. *One of the channels needs to be grayscale (height), while the other is some color (phase). Adjust the z scale to 0 for the color channel*

✓ In the command line, *type*: argl_bindmesh("filename/suffix","filename/suffix")
Its best to type exactly what the window is called in the title bar- if something is wrong, Igor will let you know.

13.6: Exporting Images-

The are many ways to export images from the ARgyle window:

❶Make the 3D image window the forward-most image (active window), Copy to clipboard, and dump into another program (Paint, Photoshop, PowerPoint, etc.)

-OR-

❷Go to the Edit menu of the Main Igor tool bar; select 'Export Graphics…' select type of file you want to export it as (TIFFs are larger files than JPEGs, but they are lossless)

-OR-

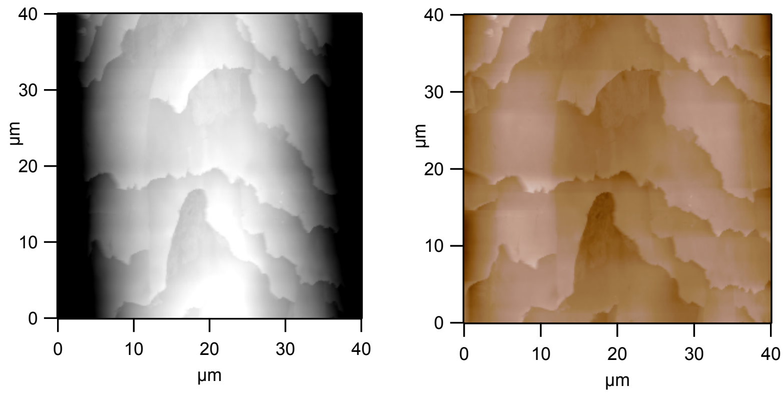
❸Click 'Export to Layout' button in New tab of the Master ArGL Panel. *Learn more about Igor Layout windows in Section 14- Misc. Procedures.*

❹ Command line export: export a 2x, 3x, 4x by typing argl_export2("", "outfile path", 0, 0, cx, cy) into the command line. The max of cx and cy are 4k x 4k.

13.7: *Misc. Operations-*

Scott MacLaren (UIUC- Materials Research Lab) taught me this trick: Say you want to do have a 3D rendering of some material that has smaller features some larger curvature of radius (i.e., large spheres or hair are great examples). Problem is that the finer details topography features don't get colored well in ARgyle due to the extreme changes in Z scale of the material.

☞ What you want to do is flatten a height image channel with a second order flatten to take out large scale the curvature of the material, and then overlay onto a non-flattened (or zero order flattened) Z sensor or copied height channel.



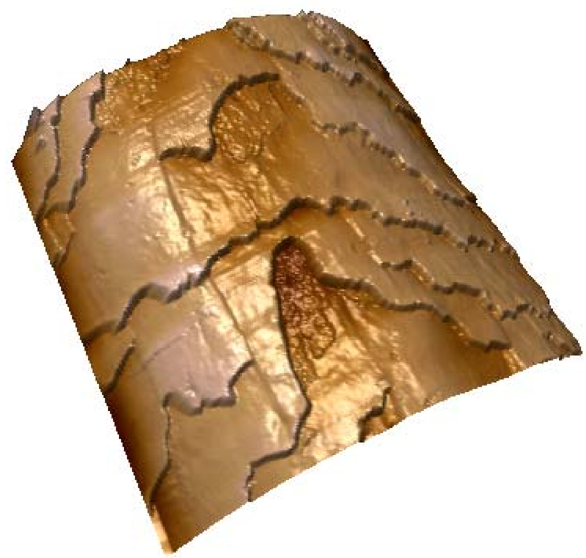


Figure 13.11: Using ARgyle to accentuate smaller surface features onto materials with large radii of curvature: A) Non flattened Z sensor image, painted with B) 2nd order flattened height channel; resulting in C) 4D rendering of overlaid images.

Using ARgyle, overlay the 2nd ordered flatted onto the other, such that the 2nd order flattened is the colored layer- it will bring out the smaller features much better than without. Figure 13.11 shows an example of this technique.

Cell Images-

Another trick to display cells is to paint the error signal over the height. For example, amplitude painted onto Height/ Z sensor; or Deflection onto Height/ Z sensor.

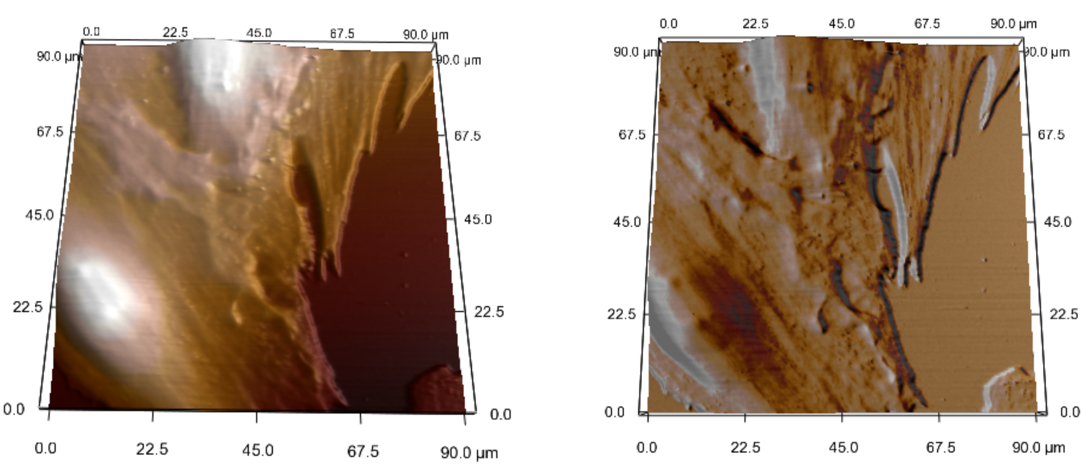


Figure 13.12: A) Fibroblasts on glass; B) Amplitude painted onto Z sensor channel, with some blue colored specular lighting added.

Force Map on AFM channel-

Force maps can also be overlaid onto another AFM channel. Here's an example of adding a force array overlay onto a lateral image, shown in Figure 13.13. The sample is a micro contact printed mercapto undecanoic acid (-COOH terminus), back filled with dodecanethiol (-CH_3 terminus). The Au coated SiN_x tip had the acid thiol on it is well, and was acquired in a pH 4 buffer standard. The example was done in moderate haste to show this can be done, and seems to correlate reasonably well.

The array map was analyzed in the *Analysis* tab of the Force Display Panel. *See Section 11.2. for further description.*

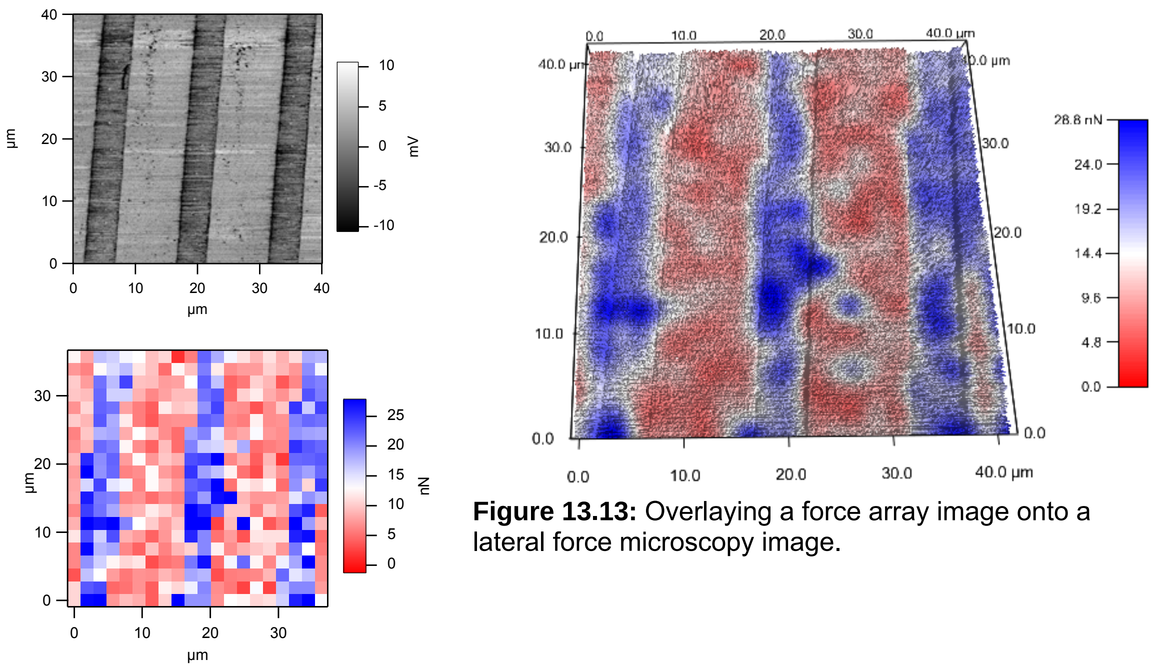


Figure 13.13: Overlaying a force array image onto a lateral force microscopy image.

16. Noise Test Measurements

WARNING: This chapter does not suggest (in any way, shape, or form) that users explore the 3D Test Panel tabs other than what is described within. This chapter was designed merely to check the noise health of an MFP-3D system- (i.e., if it has been moved or some environmental change has increased the noise). Users should NOT change any parameters in the Info Blocks, which will usually change system calibrations for the worse. If unsatisfactory noise persists in the system, DO NOT take the repair onto yourself: contact AR support to remedy the situation. The MFP-3D is a high precision instrument, and has gone through extensive post production testing and calibration- under normal circumstances there will be no need for re-calibration.

NOISE MEASUREMENTS: The MFP-3D software has a testing section that can show how much noise the system is experiencing both internally (instrument) and externally (environment). Noise tests for the X, Y & Z LVDT closed loop sensors (at different input voltages) can be measured, and the 5 MHz A/D converter (ADC) on the photodetector (referred to as the 'Fast' ADC) can also be monitored. By placing the tip on the surface (with known InvOLS value- *see Section 9*) with gains OFF, the noise coupled into the tip from the surface below can be measured over a typical imaging bandwidth (0.1Hz to 1kHz) to diagnose what kinds of noise frequencies are present. Custom inputs can also be adapted to this software.

This chapter will describe basic noise tests for system set up, or general diagnosis. If this chapter does not describe what your MFP3D is doing, please contact support@asylumresearch.com.

Asylum Research has established specific factory specifications for the noise on the MFP-3D. Table 15.1 lists these below. Sections 16.1 – 16.3 describes how to measure these.

Table 16.1: Use freshly cleaved mica or graphite surface for 'Fast Engaged' test

	'Fast' ADC	X	Y	Z
Engaged	< 60 pm	NA	NA	NA
Free Air	< 30 pm	< 600 pm	< 600 pm	< 300 pm
Free Air ARC2*	< 30 pm	< 500 pm	< 500 pm	< 200 pm

Typical InvOLS values used are between 20 and 80 nm/V
ARC2 controllers shipping after July 2009 have these specs*

16.1: X,Y,Z LVDT Closed Loop Sensor Noise measurements:

✓ In the Main Menu, Go to Programming → Load Test Procedures (Figure 16.1.1). This creates a new pull down menu item called 'testing' → test panel (Figure 16.1.1B, inset).

This will bring up the 3D Test Panel (Figure 16.1.2). This panel consists of seven tabs for various measurements, many of which will not be described herein.

✓Open the *Noise* tab (Figure 16.1.2).

▪ With the tip disengaged, the noise of the closed loop sensors can be monitored at any value between -10 V and 150V. Only one axis (sensor) can be tested at a time, and must be deselected before selecting another axis. Values below 600 pm meet AR's factory specifications for the X & Y LVDTs, while values below 300 pm meet specs for the Z LVDT (Table 16.1).

✓ To measure if the LVDT noise is within factory specification, activate the desired axis 'Input' ☑ checkbox. In Figure 16.1.2, the X axis input is selected. *AR Factory specifications are collected at 1 kHz bandwidth.*

▪ Typically, the bandwidth and offset/lever default values are suitable. A piezo voltage of -10V means the it's starting from its fully retracted end of travel.

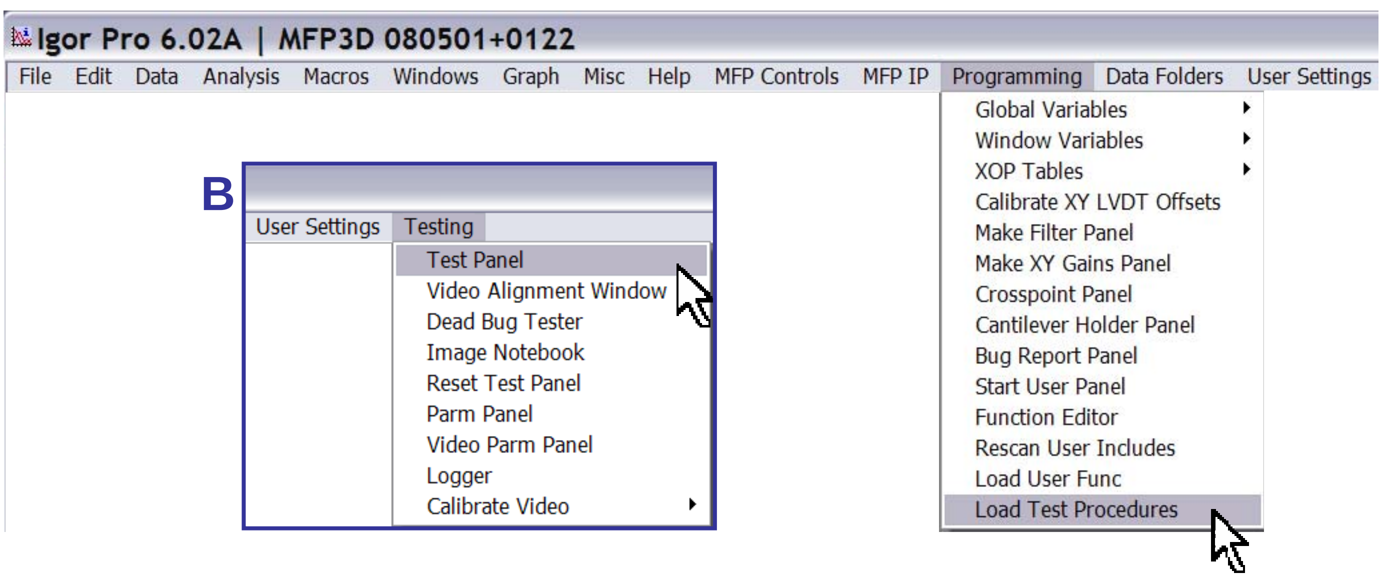


Figure 16.1.1: opening the test software gives hidden 'Testing' main menu; B) inset shows where to select 3D Test Panel.

✓ Notice the low pass filter bandwidth can be selected, as well as the offset piezo voltage to check noise at edges or center regions of piezo travel.

✓ Click the 'Start' button. The X (*Y or Z*) Sensors Noise panel will come up with two sets of axes (Amplitude vs. Frequency; and Amplitude vs. Time).

▪ The Resolution and Averaged number of spectra can be adjusted in the respective setvars under the 'Start' button (Figure 16.1.2).

▪ As the spectra are being collected and averaged, the counter will indicate which count it's on.

✓ To continually collect spectra, ensure the 'Stop ☑' checkbox is unchecked in 3D Test Panel.

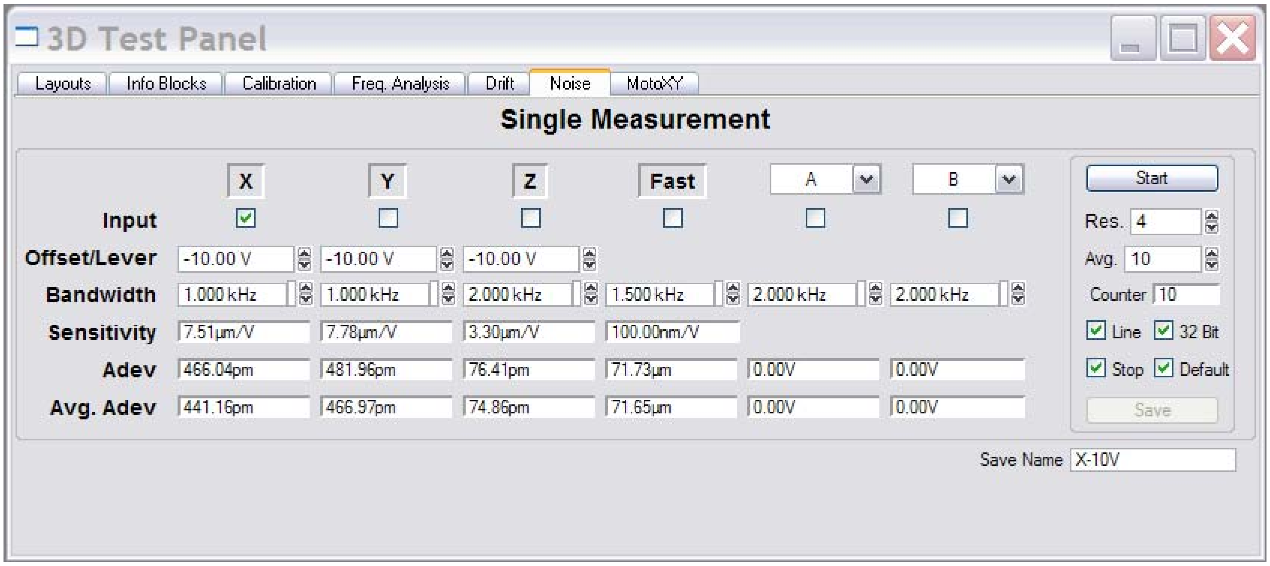


Figure 16.1.2: *Noise* tab in the 3D test Panel: monitor LVDT and Fast ADC noise here.

▪ Figure 16.1.3 shows an example of a typical X LVDT sensor noise spectrum. The lower plot (amplitude *vs.* frequency) is the amplitude spectrum over the typical AFM imaging bandwidth. The spectra are averaged and displayed in real time. The blue curve is the integration of the black noise spectrum. You can also see the roll off of the 1kHz low pass filter. The upper plot is a temporal plot that is updated in real time, but not averaged. The average deviation of the noise can be seen in the *Noise* tab (Avg. Adev).

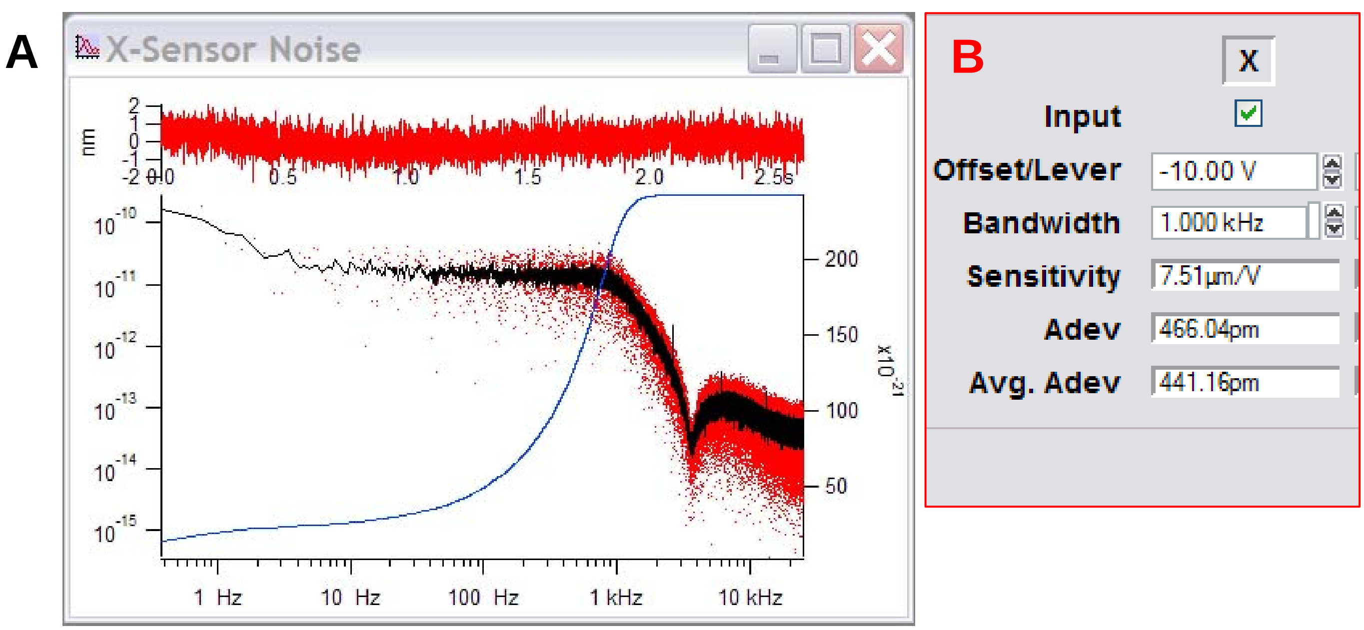


Figure 16.1.3: Typical X,Y LVDT Sensor noise plots. A) Typical Amplitude *vs.* Frequency (average of ten curves) plot of X or Y LVDT closed loop sensor; B) Measurement settings and result (Avg. Dev.) of X sensor used in this example.

Noise test TROUBLESHOOTING:

If the power spectrum doesn't look something like Figure 16.1.3, something may be wrong. Either the scanner is rubbing somewhere, or perhaps the LVDTs have been compromised (aqueous media can compromise them). It's typically a very robust system.

▪ An example of a poor sensor signal is in Figure 16.1.4. In this particular example, the X scanner was rubbing at the end of fully retracted part of the scan- *a representative scanner hysteresis plot can be seen in Figure 16.3.3.*

▪Also notice the length scale in Figure 16.1.4: it's in *zm (i.e., x10^{-21})*- doesn't make sense because that's way too small, so there must be something not correct with the system. In this particular example, since it was constrained at the fully retracted end, adjusting the piezo range to +10 V to +150V gave a healthy noise spectrum similar to Figure 16.1.3. With some mechanical adjustment and help from AR support, the piezo was moved back into proper position.

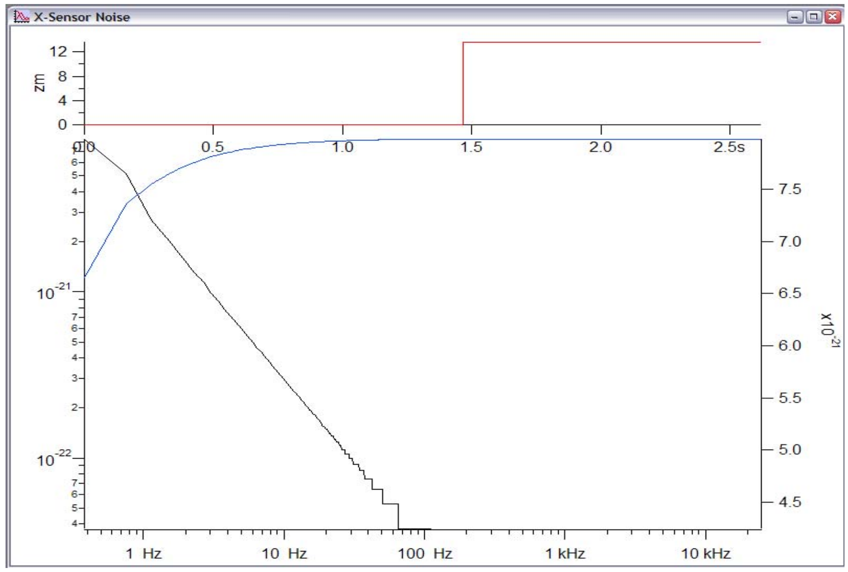


Figure 16.1.4: Improperly functioning LVDT sensor noise plot.

16.2: *Scanner calibration*: The health of the scanners can be monitored in the Calibration tab of the 3D Test Panel (Figure 16.3.1). *Here, the scanners won't be calibrated, but the amount of piezo hysteresis that the closed loop sensors monitored can be viewed.*

✓ Choose the scanner from the pull-down menu, make sure it is in the 'Drive' Action, and X (Y or Z) and setvars in range of –10 V to 160 V.

✓ Click 'Start' button. A graph similar to Figure 16.3.2 will be displayed if everything is working properly. A nominal range of ~ 14 V and a hysteresis value range of 4 to 5 % or less is typical (*not sure on AR's specs on the percentage*).

▪ Choosing smaller ranges for the respective piezo to move results in smaller hysteresis values (generally).

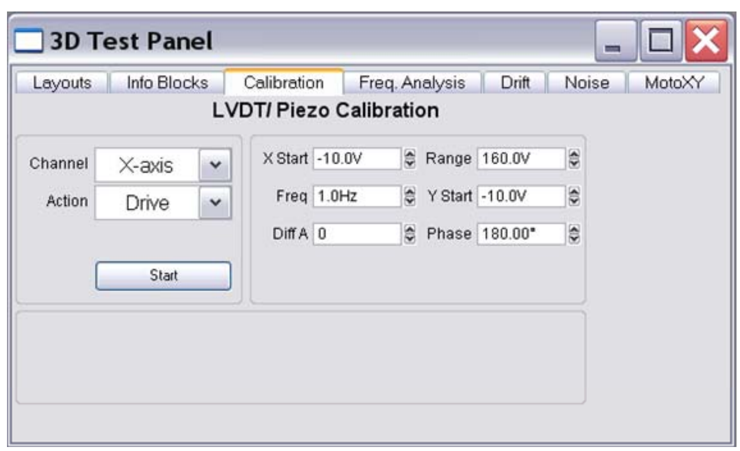


Figure 16.2.1: The Calibration tab allows noise tests on the LVDT sensors as the piezo is actuated through a voltage range.

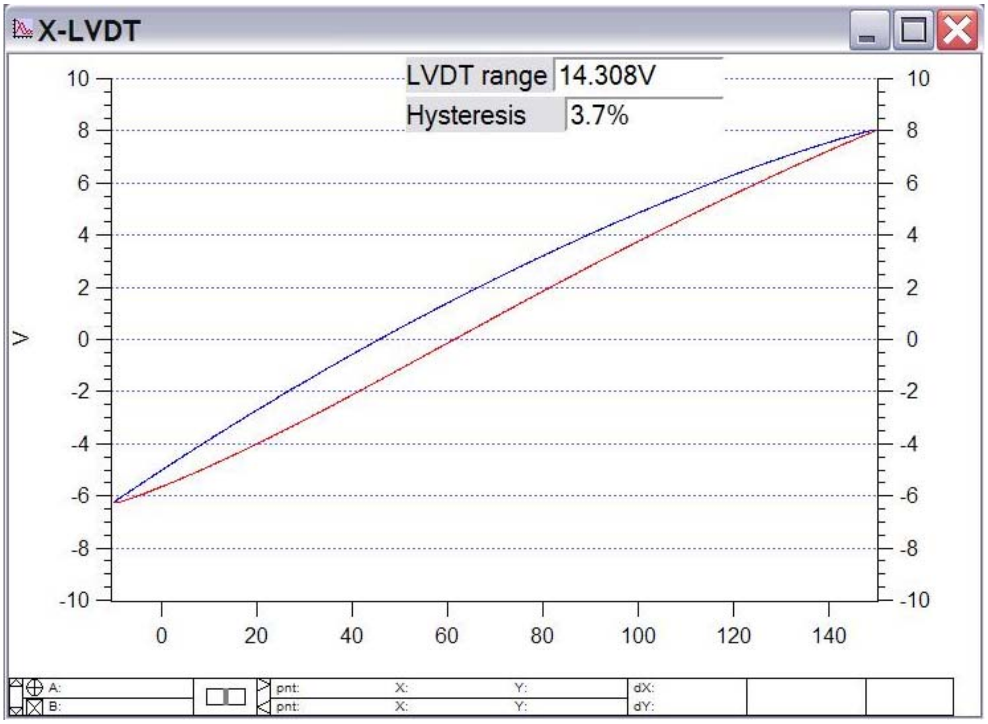


Figure 16.2.2: typical LVDT (X or Y) scanner hysteresis plot.

▪ If the hysteresis plot bottoms out/ rails at the bottom or top (red circle, Figure 16.2.3), the flexure assembly needs to be adjusted. At this point, the author recommends calling or emailing AR support. *Please treat these individuals with the same professionalism that they will treat you with.*

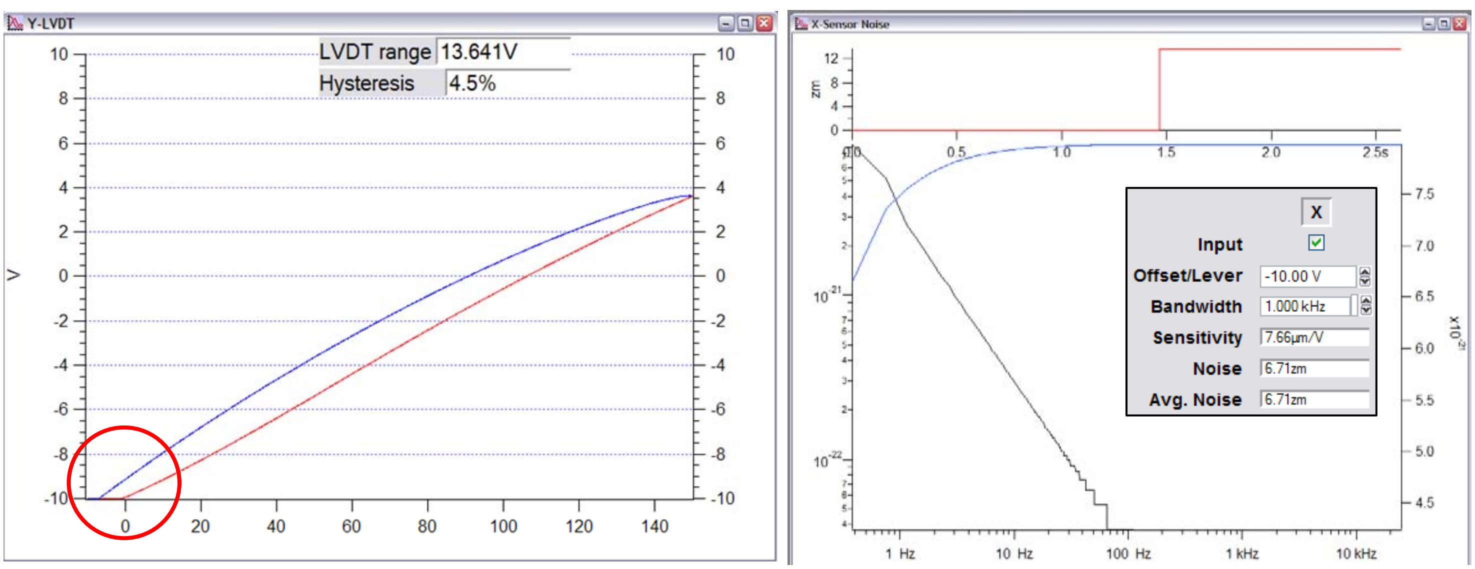


Figure 16.2.3: Piezo example where it is constrained due to improper mechanical position in the scanner housing; B) representative Sensor noise for a situation like this.

▪ Figure 16.2.3 shows the piezo being constrained at the end of its travel- Panel A shows the LVDT sensor hysteresis loop: Notice the voltage 'railing' on the lower left. What has occurred is this piezo wasn't properly centered in its housing, causing it not to be able to move in the 0V to -10V bias range applied to this particular piezo, resulting in a improper LVDT noise measurement. Panel B shows the subsequent noise measurement of this LVDT sensor- notice the noise is 6.71zm (inset), which is an unreasonable distance dimension. Incidentally, in this example, the Offset/Lever voltage was changed from -10 to a value higher (let's say 0V because this is what the author actually did), the LVDT noise fell into factory spec, according to the values in Table 16.1.

▪ When done testing, go to Programming → Remove Test Procedures; *or don't.*

16.3: 'Fast Engaged' ADC Noise:

The 'Fast' ADC is assigned to the photodector by default for obvious reasons in most scanning applications. The 'Fast' ADC noise is what AR engineers use to monitor how much noise is being coupled into the system from its environment when the tip is on the surface with feedback loop gains OFF over the typical imaging bandwidth (<1kHz). This is a good test to do every once in awhile to check the noise health of the MFP3D system, or if the instrument has been moved in the lab and set up trouble shooting is needed.

✓Determine the InvOLS of the cantilever **on freshly cleaved mica or graphite** (something atomically flat, and infinitely hard- clean glass slides work too). *See Section 9.1).*

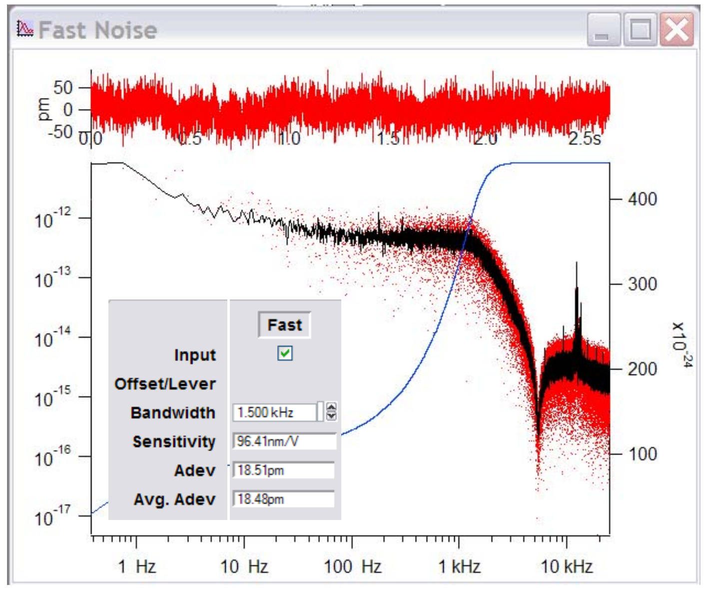


Figure 16.3.1: A typical 'Fast' Free Air noise spectrum; Inset- Avg.Adev. values for tip/ system used in example.

Make sure the fiber light isn't on- the cooling fan in the light can couple noise (~60Hz) down the fiber optic cable into the system.

▪ AR spec tests use a 100 µm Si_3N_4 triangular cantilever, or AC 160 (*k*~40N/m; f_o~335kHz). The value will automatically be updated in the 'Sensitivity' setvar value in the Panel. These noise numbers are sensitive to InvOLS, which in turn is sensitive to the 'Sum' on individual cantilevers. Don't be sloppy with your InvOLS calibration when performing noise tests. The lower the InvOLS value, the lower the 'Fast' Engaged value.

✓Check the box below the Fast ☑ column (Figure 16.1.2).

The 'Fast' ADC noise is measured two ways:
❶ Free air (disengaged): The free air will give you a sense of how noisy the system is. *Factory specification is 30pm or less.*

❷ *Engaged:* with the tip on the surface & gains off, this measurement is going to give a real sense of how noisy the MFP's environment over the typical imaging bandwidth of 0.1Hz to 1kHz, because this fast ADC can monitor what frequencies are being coupled into the tip (i.e., seismic, acoustical, mechanical, electrical, etc.). *Factory specification is 60pm or less.*

Free air: this checks the noise of the system; i.e., optical lever (Brownian) noise with tip withdrawn.

✓Confirm the tip is withdrawn from the surface.

✓(optional) Enter 70 V in 'offset/lever'. This will put piezo in center of its range.

✓Hit the '**Start**' button; A noise plot comes up which shows the sampled noise (Figure 16.3.1). The fast input free air value should be less than **30 pm** to meet AR's specs (Table 16.1).

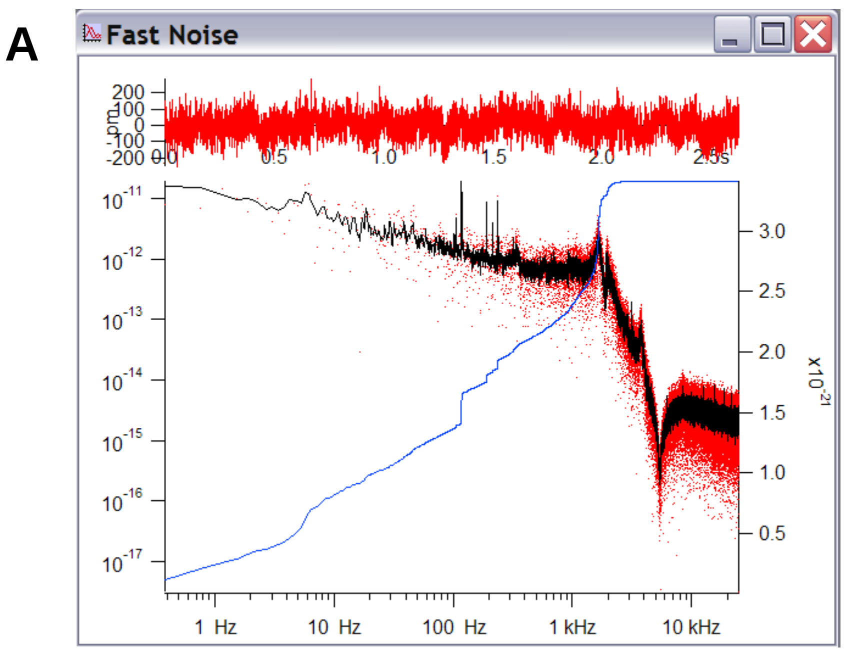
Fast Engaged: This measurement monitors the noise at the tip-sample interface over the typical imaging bandwidth of 0.1Hz to 1kHz.

✓Engage tip in contact mode.

✓Confirm bandwidth is set to 1kHz.

✓**Adjust Integral gain to 0.01** (to deactivate feedback loop). *This step is important!* The integrity of this measurement means nothing if the gains can respond to the frequencies being coupled into the tip.

✓Click the '**Start**' button; This will be an inherently noisier system (than Fast free air) because the tip is in contact with the substrate, allowing environmental frequencies to couple into the system. The 'Fast input' (the 5 MHz ADC) engage value should be less than **60 pm** to meet AR's specs Table 16.1).



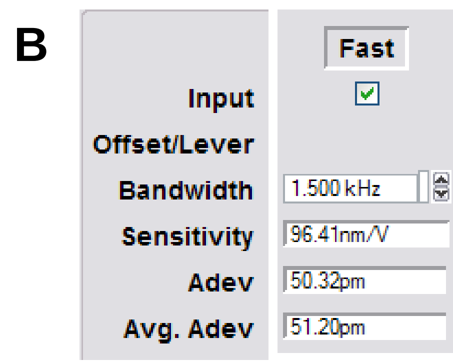


Figure 16.3.2: Typical 'Fast' engaged noise spectrum. Integration plot (blue) show largest contributors in the 0.1 Hz to 1kHz bandwidth; B) screen shot of 3D Noise Panel Avg. Adev. of measurement.

▪ Figure 16.3.2 shows a typical noise spectrum of a system that meets AR spec. The upper plot is time vs. Amplitude , updated at whatever interval is designated (i.e., not averaged). The low spectrum is Frequency vs. Amplitude, which is an average of all the spectra taken during that measuring period. The blue line represents the integration of the black power spectrum plot.

▪ Figure 16.3.3A is an example of one of an MFP-3D's Fast input 'Engaged' noise that is very noisy. Figure 16.3.3B is an expansion of the 1Hz to ~1.2kHz range done in Igor. Regular noise with periodicity can be clearly seen in the time plot (upper temporal, red). That works out to be around 32 Hz noise, which can be seen as a broad peak (*and strong noise contributor*) in the frequency plot (black lines Figure 16.3.3B)- this suggests some mechanical external noise. In our case, I suspect it's caused by some air handling equipment outside the old lab's window. The spikes and peaks in the frequency plot show various types of external noise.

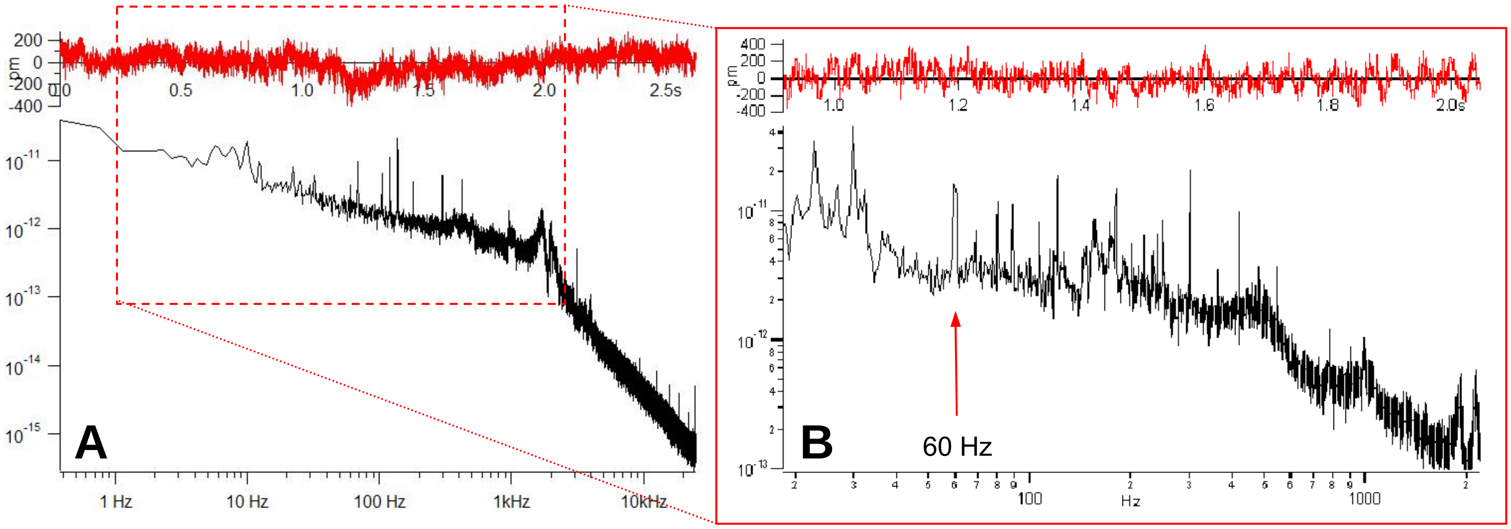


Figure 16.3.3: A) Typical fast engaged noise test meets spec; B) Fast Engaged spectrum showing seismic, acoustic, mechanical noise in lower frequencies (red box). *Screen shot obtained before integration plot was added to software.*

▪ Another example of a system in a spectrum with a lot of lower frequency noise (Figure 16.3.4): the ~2Hz contribution can be seen in the spectrum (large integration contribution), and in the time domain, the larger sine wave is ~2Hz. Counting peaks on the small sine wave superimposed upon that is ~24Hz- this is confirmed on the spectrum and other large integration contribution at ~24Hz.

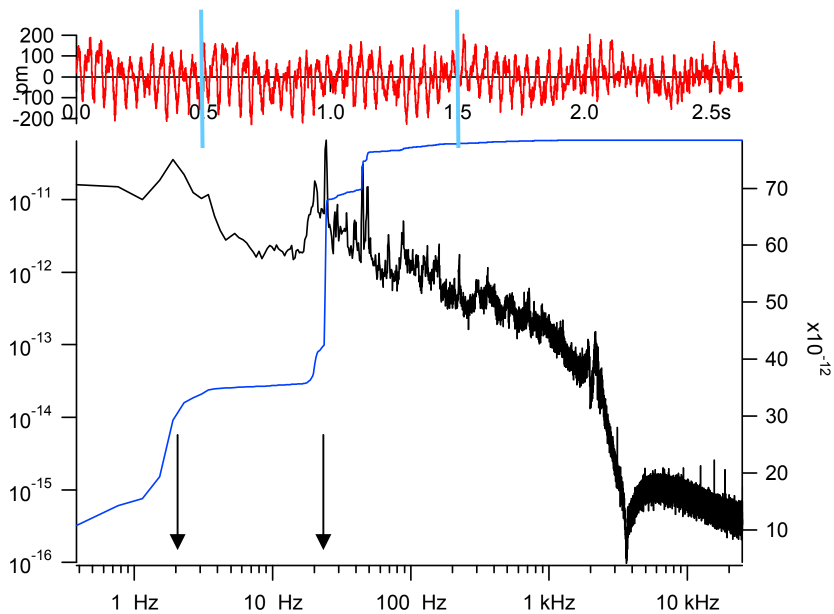


Figure 16.3.4: Example of noisy low frequency Fast Engaged spectrum.

16.4: 'Waterfall' Plots

A waterfall plot is the king daddy of all 'Fast' engaged measurements: it can log the frequency changes over longer lengths of time than the fast engaged measurement described in Section 16.3. It plots the 'Fast' engaged Average Deviation noise *vs.* time, and also plots this noise as pixels over a 1Hz to 1kHz frequency range over the temporal length scale.

▪ The advantage of running a waterfall plot is to see what occurs over time- say over the course of a day where the building noise can be noisy during the day, and more quiet at night; or if something freakish happens somewhere along the way.

To obtain a water fall plot, follow the following procedure:
Note: it is assumed that a freshly cleaved piece of mica is the substrate, and the cantilever's InvOLS is calibrated.

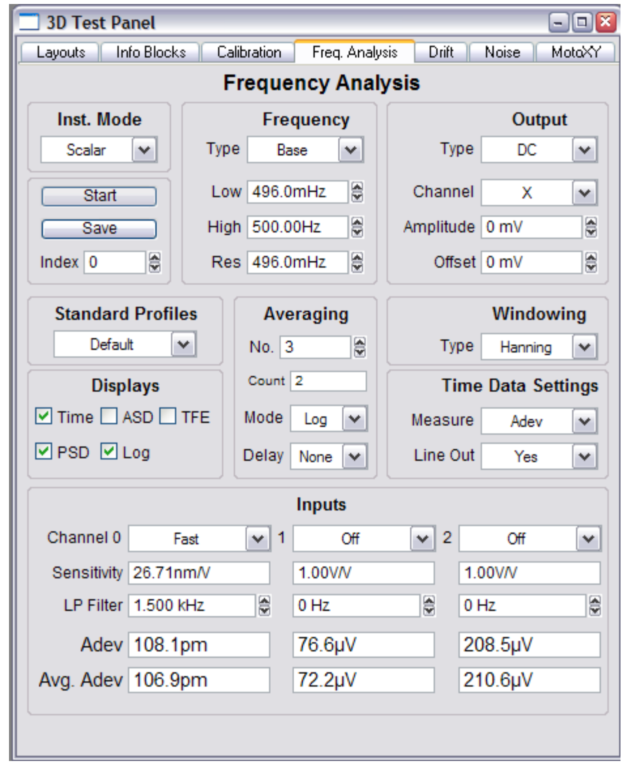


Figure 16.4.1: Frequency analysis tab parameters for waterfall plots. Follow these parameters for log setup.

✓Open the 'Freq. Analysis' tab in the 3D Test Panel.

✓Choose the Time, PSD and Log Displays- these will be the noise plots that will give the important information

✓Set the Low Frequency setvar to 500mHz; Set the High frequency setvar to 500Hz. The resolution setvar will update to be the same as the 'Low' setvar.

✓Under averaging, set the Mode pull-down to 'Log'- this will produce the image seen in the Log display.

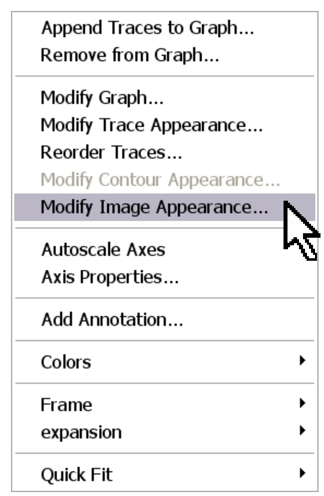
✓Also under averaging, set the number setvar to 3 or 4- this will take that many noise measurements, average them and display- because of this expect a slight delay.

✓ Under the Inputs- set Channel 0 to Fast (for fast engaged). Other inputs can be used to measure noise, but that is a more custom experiment, and this procedure should allow one to figure that out with little trouble.

✓ Confirm that the tip is on the surface, and the Integral gain at 0.01.

✓ Click the '**Start**' button. All three Display plots will start to display their respective data.

▪ The Log plot will be displayed in grayscale by default. Changing the color table can aid the eye in subtle temporal frequency range changes. To do this, left click on the pixilated portion, select 'Modify Trace Appearance' to get this Igor dialogue.



Over the user defined period of time, the time and PSD plots will update per averaged measurement, while the Log plot will show the temporal frequency data. An example can be seen in Figure 16.4.2- note this figure has had its frequency range manually attenuated (recall the raw data collected 500mHz to 500Hz- but for this noise chasing application, only about 50Hz were sufficient based on previous PSD plots (data not shown). The color table was also change to planet earth to accentuate the areas of excessive noise to more easily correlate with frequency.

The upper plot is the Fast Engaged PSD data vs. time. The lower plot shows the waterfall plot- features that the author would like to point out area the blue and white pixels- which are the higher frequency contributors found in the PSD plots (see Figure 16.4.3). Unfortunately, the author isn't sure how to install a z scale color table bar- just know that yellow and green are lower frequency contributors, while blue and white are the largest contributors.

Back to the plot- look at the blue& white horizontal steaks- specifically, 44, 29, 25 and 2 Hz were the major contributor frequencies, and these could also be seen as the culprits in the integration plot of the PSD *vs.*

frequency plot (Figure 16.4.3). Additionally, over time, the 18 to 20 Hz was bleeding in: notice at the left, these frequencies were quelled because the author made some adjustments.

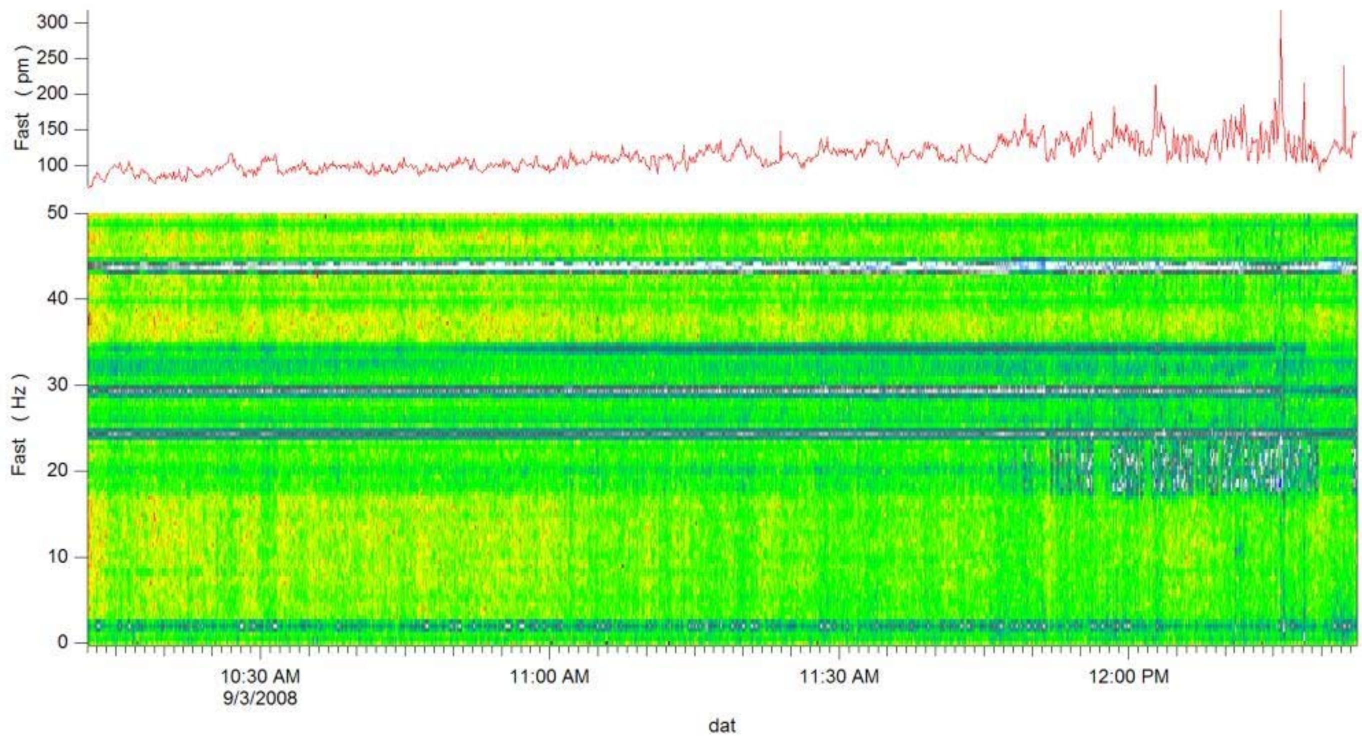


Figure 16.4.2: Waterfall plot- Fast engaged frequency bandwidth plotted temporally (lower plot) along with Fast Engaged Average Deviation noise plotted temporally (upper plot).

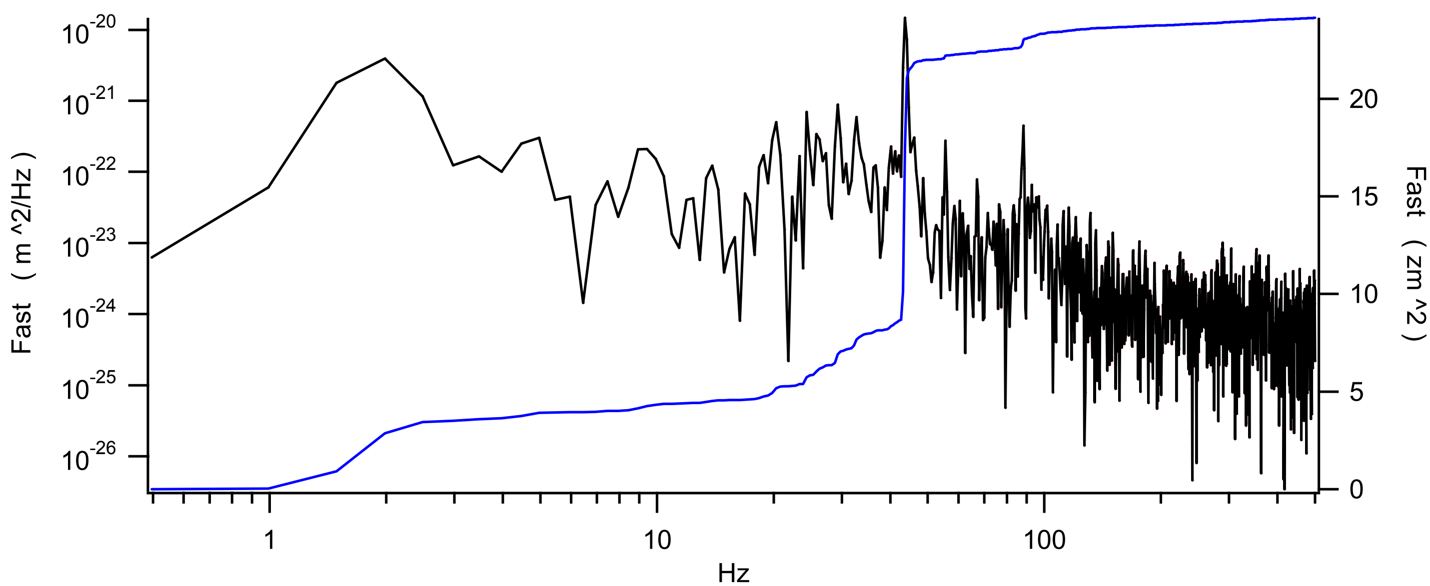


Figure 16.4.3: Corresponding Fast Engaged noise for Log plot in Figure 16.4.2. Note that this is only one power spectrum (i.e., not averaged).

16.5: Saving Noise Data:

It's a good idea to save the noise data, be it fast engaged or hysteresis data. There are essentially two ways to save the data:

❶ File→ save graph copy; designate the path, etc. This saves the plot as a pxp file and can be called up later on. Tricky part is that they cannot really be called up (easily) with the MFP software open.

❷ The other, better way is to click the 'Save' button in the any of the 3D Test Panels when the measurement is complete. This dumps the graph into the Igor Data Browser, allowing it to be called up later, or even multiple plots easily overlaid. This is useful for comparing noise environments/ situations. The AR engineers use this software extensively to prepare reports for the testing of system components after being built, or repair, or installation. This section is devoted to showing how to do this-

The example used in this section is using various Fast Engaged noise spectrums from changing out different rubber pads under the hood legs in efforts to try to get the best performance out of a system in a very noisy lab (same lab as in Section 14.4).

▪ Four different PSD noise spectrums were saved after each time different durometer rubber pads were changed.

To view these PSD spectrums, open the Data Browser (data→ Data Browser). This will open the Igor Data Browser (Figure 16.5.1).

This data gets dumped into the root→ Test Results→ Noise Path. Each PSD and Time domain plots get saved as subfolders in this folder. Figure 16.5.1 shows the expanded folder system.

✓ To view a single plot, highlight the file, and right mouse click. This display a Igor Menu.

✓ Select 'Display'. This will create a plot with linear axes (to view as log axis, the Igor 'Modify Axis' panel has to be set up).

✓ To *overlay multiple* spectra, Crtl+ left click on the files of interest (Figure 16.5.1).

✓ Right mouse click and select 'Display'. This will create a plot with all the selected curves- only problem is that they are all the same color, but can easily be changed in the Igor 'Modify Trace Appearance' Panel (double click on curve).

Another problem is there is no legend: to do this,

✓ Right click in some dead space on the plot and right mouse click.

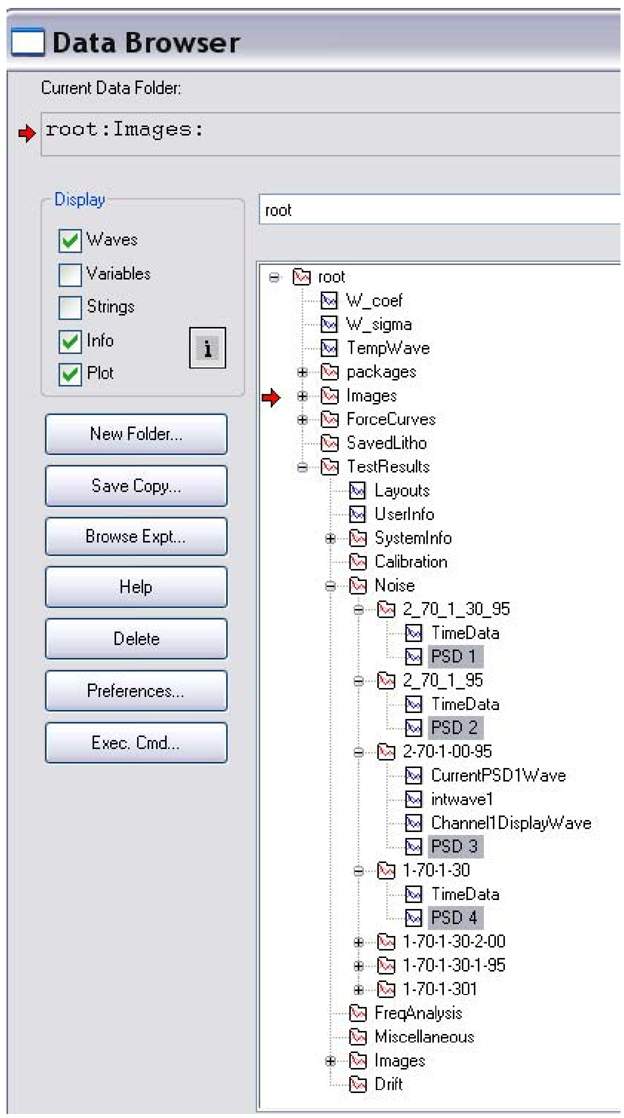


Figure 16.5.1: Saved Noise measurement plots from the 3D Test Panel are found in the Igor Data Browser.

✓ Select 'Add Annotation'- this will bring up the Igor 'Add Annotation' Dialogue.

✓ Select 'Legend' from the annotation pull-down. This will install the legend in the plot.

Figure 16.5.2 shows the four PSD spectra from Figure 16.5.1, properly annotated. Notice the overlay clearly shows how varying the rubber durometer sandwiches under the hood legs affects the various frequency ranges – the PSD4 has less low frequency contributions (2Hz), but much higher mid range (25 & 44 Hz); while PSD 2 has large low frequency contribution, but lower mid range contributions. These comparisons are very useful in troubleshooting noise.

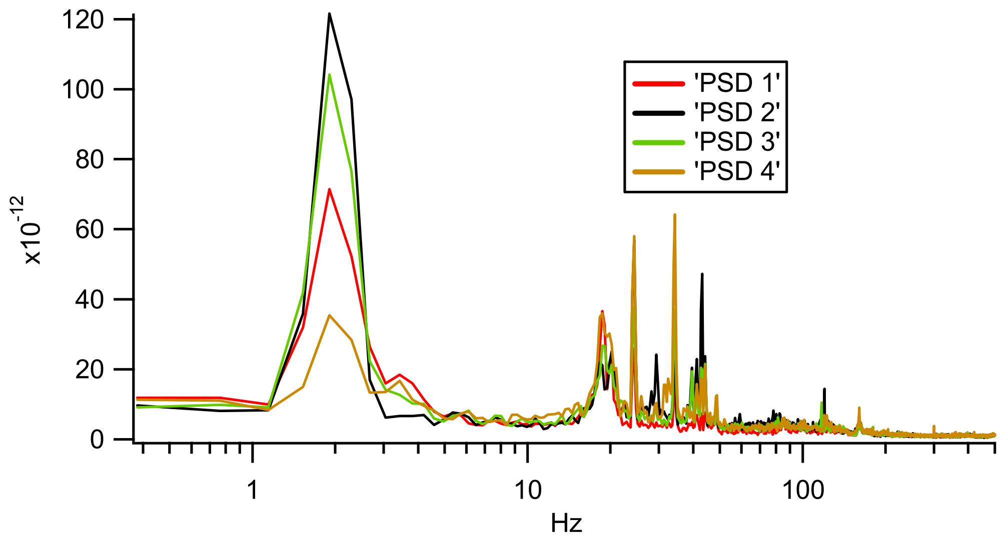


Figure 16.5.2: Overlay of various PSD spectra showing the affects of changing the MFP3D system environment.

16.6: Trouble Shooting Noise:

In most labs, rarely does a system come in under factory specifications the first time out from instrument reassembly. There are a few things to keep in mind when determining how to quell the sources of noise.

The controller has a frequency converter (and audio out) built into it to allow the user to listen to noise in the system. Building noises suchs as pedestrians in nearby hallways, pumps, doors, elevators, electrical humming can be heard in the human hearing range. Even adjusting the monster cable can cause audible frequency changes that can aid in system noise reduction. Things to listen for are periodicity of the noise, where the noise might be originating from and subtle differences between adjustments (*see more in Section 16.6 & 16.7*.

Some questions to ask if the system is not in spec:
-Is the table stable on, or isolation enabled? Look for the Red LED light to be on (Figure 16.7.1).

-*Are there large sources of noise in the room? Including/combining, but not limited to:*
▪**Seismic** (high lab floor number; subway / busy road / hallway nearby;); usually seen as low frequency noise, non periodic, variable amplitude values.

▪**Electrical**- (various motors, pumps, transformers, table gains too high (*see Section 16.7*); usually seen as multiples of 60Hz somehow (N. America), and sharp spikes in the noise spectrum.
▪**Mechanical**- (floor noise coupling in through acoustic chamber; cable clamp/ acoustic hood clamp NOT secured; HVAC units nearby; fume hood fans; elevator motors, fiber light fan on?); usually seen as broad, lower frequency noise in the spectrum.
▪**Acoustic**- (sonicators, pumps, music, parties, fume hoods, labmates that don't have 'AFM voices').

-*Do any of the cables have excessive torque or tension in them or their connections?* (this can mess up ground).

-*Excessively high InvOLS values can increase the value*: typically the Olympus cantilevers used to obtain the specification values in Table 16.1, generally give InvOLS values between 20 and 80 nm/V. Cantilevers with larger InvOLS (>100 nm/V), will give a 'Fast' Engaged Avg. ADev. value that is out of spec (60pm), but would be in spec with one of the Olympus levers.

-*Can the source noise be tracked with using the headphones for the Fast Engaged measurement?* The headphones can be very useful for tracking noise.

▪ Figure 16.6.1 shows an example of a noisy 'Fast' engaged spectrum and how it was improved: It shows an example with a lot of low frequency noise: look at as the large broad peak on left in the Amplitude vs. Frequency plot, the analogous steep integration line (blue) indicating that ~2Hz is a vary large contributor of noise.

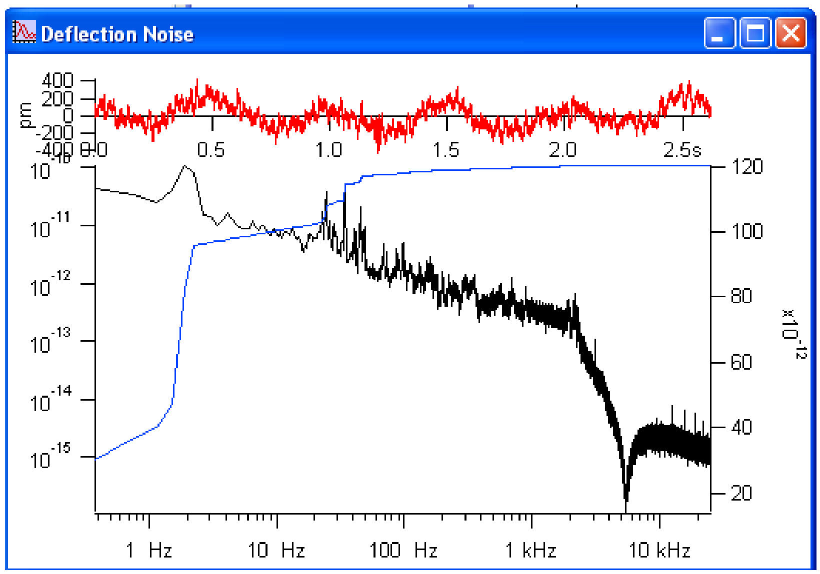


Figure 16.6.1: Example of out f spec system with low frequency noise.

▪ Also notice the noise time scale (red plot)- the periodicity of the is ~2Hz. This particular system was on an eight floor of a bldg- where the entire bldg likely swayed at a frequency of ~2Hz; this system noise was dramatically quelled by using various rubbers and polymers underneath the acoustic hood legs (see Section 16.8).

HOW TO FIX IT?

(hopefully)

The author can't solve all your problems, perhaps none at all. But there are some adjustments one can do to get the system operating with less noise.
Seismic- rubber under table/ hood legs (see Section 16.7), table stable servo gains adjustment (see Section 16.7).
Acoustic- If you are using an acoustic enclosure, close the front door, or eliminate any holes that could allow noise to enter. It's amazing how a small hole can increase the decibels in the hood.
Mechanical- properly clamped cable, rubber feet under hood/ table legs (*see Section 16.8*).

Figure 16.6.2: The cable clamps can offer protection against vibrations traveling down the monster cable, and seal up the acoustic enclosure.

Electrical- *There are a couple things that can easily add noise:*

-Improperly coupled cable into its socket- if the ground shields around the outside of those connectors don't have good contact, noise can get in.

-System cables/ wires crossing each other- this may be suspicion, but the author tries to keep things clean behind the PC and controller, especially the USB (PC to controller) cable; Also, the controller USB should be in a bank by itself on the back of the PC (keep its neighbor empty)

-Perhaps the system should have a ground wire from acoustic hood to wall socket- this could be especially useful for those doing electrical techniques (Figure 16.6.3).

Figure 16.6.3: ground the acoustic hood to wall socket with piece of copper wire.

16.7: Active Vibration table adjustments:

16.7.1: The active dampening tables (JRS/ Herzan) table stable is a very easy system to use. The basic use and some things the author uses to help get a system are described here:

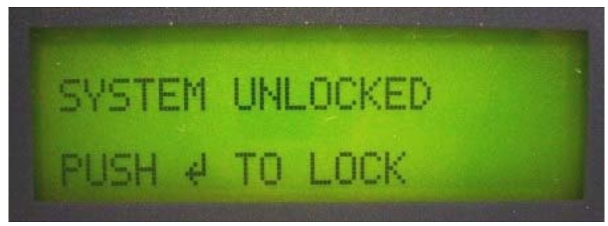
Is the active dampening of the table turned on? The isolation on (ISOL. ON) LED should be illumination. If not, hit the 'E' button.

The default gains are set at 235- this is just at the edge of the servos 'ringing'.

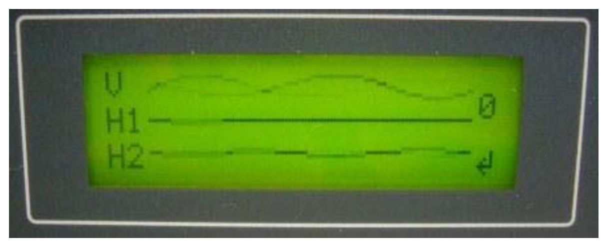
Figure 16.7.1: Front panel of Table Stable active dampening systems.

The menu can be changed by clicking the arrow buttons on the face plate. The select, press the Enter sign ↵.

✓If the table is to be moved, **you must lock the servos** or you can damage the table. Look for the menu that says SYSTEM UNLOCKED; press the Enter sign ↵; power table down; move it.



This window will give you an idea of how hard the table is working. (V is the Z axis, H1, and H2 are the horizontal planes).



NOTE: If you are working in a multiple user facility that is supervised by someone, you should ask them if you are allowed to do this next part. They could have spent a great deal of time optimizing this.

16.7.2A: Manual gains on active dampening tables:

Many of these active dampening systems have a second menu that allows the user to manually change the servo gains. To do this with the Herzan (JRS) Table Stable, power the table down (it doesn't have to be locked).

Figure 16.7.2: Scan range box in AR Video Panel: A) setting tip location; B),C), D)- different scan areas and XY offsets (indicated below CCD image.

✓ While holding the Enter ↵ key down, turn power on (Figure 16.7.2A).

✓ Shuttle thru the menus until you see the Manual Gain menu, similar to the image in 16.6.2B.

✓ Using the ↑↓ arrow keys, enter the desired gain value. This will update the gain in real time.

✓ Make sure Isolation is enabled- Now the LED will blink, indicating it's under manual gain control. *ACTIVE is equal to 000 when isolation is not enabled* (Figure 16.7.2).

▪Normally, when trying to tweak the tables, the author will have the tip on the surface with the gains off (Fast Engaged) while adjusting these gain values, letting the table adjust for a few seconds, then monitor the noise value in the 3D Test Panel. It can be a trial and error process.

As you reduce the gain, you are decreasing the bandwidth that is isolated. So if there are 120 Hz floor vibrations, the 120 Hz peak in the On-Surface noise will get worse when the gain is reduced. However, usually the biggest problem for us is noise peaks between 15 Hz and 60 Hz. Often these are caused by three-phase motors in the air-conditioning equipment. At a gain of 215 or 220, these peaks should be isolated well. If we see noise above 100 Hz, then it is often from acoustic coupling (through the air) rather than vibrational coupling (from the floor) so the acoustic enclosure is what stops it. The one case where we see vibrational coupling above 100 Hz is noise from a transformer. This is strongest at 2x the powerline frequency, i.e. 100 Hz or 120 Hz depending on location. *(text courtesy of Deron Walters, Ph.D.; Asylum Research)*

16.7.2B: Passive dampening (Minus k) tables:

Usually these are trickier to adjust due to torque on table top from microscope cables. The tables work on a negative spring system, and can be effective isolators, but also a little less user friendly.

Some of the questions to ask when tracking down poor performance issues (and try to correct if maladjusted) are:

-*Is the weight on the table distributed properly?* It's good to have the mass centered on the table. This helps the leveling process.

-*Is the table top torqued at all?* This can cause rubbing on the side hull / skirt and kills the efficacy of dampening.

-*Is the load adjustment centered for the load that will be on it during the scanning?* Use the crank at the bottom to adjust the load.

▪Adjusting the stiffness of the spring is also tricky. Essentially you want the table top to oscillate at less than a hertz to be at all effective.

16.8: Vibration dampening pads under table legs:

By using different 'soft' materials under the hood legs, different frequencies can be dampened in floors with excessive seismic or mechanical noise.

▪AR ships these Shore 00 premounted polyurethane pads (glued to a steel plate) with each new system- they can be pretty effective when doubling up and placing under the legs of the acoustic hood (See Figure 16.7.1 left side). They are available thru McMaster Carr.

Some notes on these Shore 00 pads:
The soft polymer pads do a great job of isolating the typical ventilation noise at 25-30 Hz ("midrange") but they create a rocking mode of the whole hood at 1-3 Hz. This is normally OK because in a basement, but it's bad if you are on an upper floors where more horizontal acceleration at low frequencies occurs. Because the hood is top-heavy, acceleration of the floor has a big lever arm to drive the rocking modes. These can tend to increase low frequency noise if too many are stacked in hopes of additive dampening.

▪ We have also been experimenting with different rubbers and polymers to offer better vibration dampening. The 1" thick neoprene rubber pads on the right side of Figure 16.8.1 are a 70 A durometer and 30 A durometer, as well as a amber polyurethane 95 A durometer polymer sheet.

Figure 16.8.1: Different rubber pads used to quell floor vibration coupling into the MFP3D.

Some notes on pads under the hood legs-
-This process of quelling noise can be challenging- often it is trial and error.

▪ Too much softer materials can amplify the lower frequency noise.

▪ Too many pads under each leg can increase the center of mass, also contributing to the lower frequency noise if the building is noisy (i.e., higher floors can do this).

▪The hood leg feet are probably better off being in contact with a hard material, like the steel plate that comes with the Shore 00 pads described above.

▪ *Never put rubber pads under the table stable- it won't know what to do, constantly over correcting.*

17. Quick Reference

Tip loading: gently slide tip under tongue, such that tip is in middle of clear trapezoidal window; tighten screw finger tight with screwdriver. *Do not overtighten!* (see Chapter 2)

SLD Alignment: use camera or IR card to rough align SLD on cantilever; optimize signal by maximized Sum voltage in the S&D meter. *Do not over torque LDX&Y or PD thumbwheels!*

Set point determination in Contact mode: after SLD (laser) alignment, adjust PD to slightly negative voltage value; choose set point MORE POSITIVE than this for gentle engagement. In contact mode, higher set point (voltage) values translate to higher applied force.

Spring constant, *k*, determination: 1) perform a Force curve (in CONTACT mode) on a clean, hard surface; 2) determine Virtual Deflection (cursors on free air approach (red) curve), select 'Virtual Deflection Line' from Set Sens in Calibration subtab (Force panel); 3) determine InvOLS (use Igor cursors (Crtl+I) on the contact region → Set Sens → Deflection). 4) Disengage tip; perform Thermal tune; Fit fundamental resonance peak to get *k*. *(Chapter 9)*

Auto Tune Drive frequency in AC mode: Align laser on tip; zero PD; Do thermal to get approximate resonant peak; enter frequency range that flanks this value; Click 'Auto Tune'; Engage tip/ image. *(Section 6.1)*

Picking Drive Frequency when imaging in fluid: Align laser; Do thermal; approximate resonant frequency of cantilever & enter in drive frequency value of Tune tab; select 'Append Thermal' ☑ checkbox; perform 'One Tune' with a ~15 kHz window; use cross cursor to select point on black frequency plot that is near the resonant peak of the thermal (red) scatter plot; select 'set drive frequency as '. Drive frequency updated in *Tune* & *Main* tabs. *(Section 6.2)*
Alternatively, perform thermal tune; manually pick frequency by placing Igor cursor on left side of peak; type this value into drive frequency; Engage tip; after engagement, use 'Hamster' wheel (front of controller) to fine tune drive frequency by monitoring the phase & amplitude images, and line traces. *(Section 6.2)*

Hard Engage Set point determination in AC mode: Pick a set point that is ~ 10 to 20% of the free air amplitude (value in Sum & Deflection meter). In AC mode, lower set point (voltage) values translate into higher forces. *(Section 6.1)*

Gentle Engagement (AC mode): Pick a set point that is ~ 5% of the free air amplitude; Click 'Simple Engage' to activate piezo; Thumbwheel down until tip engages (which is a false engagement). Adjust set point with Hamster to 'hard' engage tip; you may have to use thumbwheel to adjust piezo further, but it's already under feedback, so you're pretty safe. *(Section 6.1)*

Monitoring Phase during AC mode imaging: While imaging in air, you can monitor the Phase levels (Sum & Deflection meter) to determine whether the tip is in the attractive (non-contact) or repulsive (intermittent contact) regime with respect to the sample. ATTRACTIVE > free air phase > REPULSIVE *(Section 6.1.4)*

Saving images: click Save images checkbox in Master Panel. *Alternatively*, File → Save Graphics…; Filename, image size and file type of your choice, click 'path' → choose where you want to dump it; click 'Do it'

Saving images from ccd camera (top view optics model): Open AR Video Panel; name file; click Capture button; If images aren't already being saved.

Creating a 'Path' in Igor to save images: Misc → New Path; name your path; click 'Path'; click 'Do it'. *(see page 38)*

Exporting Line sections: In the line section graph panel that comes up, click on 'Edit', which brings up a new table with one column of data in it. 'Kill' it. Then go to Windows → New Table. Select the four waves in 'Columns to edit' that end with the following suffices:
(Filename-suffix)IHtT, QHtT, XHtT & _HtT; click 'Do It'

Real time Section analysis: During imaging, go to MFP IP→ Analyze Panel; Select Real Time ☑ checkbox; follow directions on. See Section 12.X.

Shutdown: Withdraw tip; turn off laser; if working in fluid, clean tip holder, dry.