Tapping in Fluid SOP

Dimension FastScan Fluid Imaging

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Introduction to Fluid Imaging

This Standard Operating Procedure has been developed to help users image in fluid on the Bruker Dimension FastScan AFM. This SOP outlines step by step procedures and safety precautions designed to both ensure the AFM is not damaged in the process of fluid imaging and that the user acquires quality images. Fluid imaging is beneficial for imaging biological samples (e.g., DNA, proteins, lipid membranes, cells, etc.) in their natural environment as well as greatly reducing the tip-sample adhesive forces typically encountered when imaging in air.

WARNING: Use extreme caution while attempting to image in fluid! The equipment (Z scanner, etc.) is merely water resistant, not water proof, and very delicate/easily damaged. The approximate cost to repair or replace the AFM scanner head is anywhere from $5,000 to $25,000.

NOTE: *Do not allow fluid to sit on the scanner for extended periods of time, as seepage or solute (i.e., salt, biological materials, etc.) deposition may occur. If fluid is spilled on the scanner, wipe it off and dry it immediately. If fluid seeps into the electronics and damages the piezos, the Z-scanner will need to be replaced.*

Probe Mounting Procedure

1. Obtain a new fluid probe (e.g., ScanAsyst-Fluid+ or FastScan B, C, or D) from the probe cabinet and remove the FastScan probe holder (also known as the FastScan Z scanner, from its protective Pelican case, placing it gently on the probe holder mounting block (**Figure 1**).

Figure 1. The FastScan probe holder (also known as the FastScan Z scanner) and probe holder mounting block for the Dimension FastScan AFM.

2. Make sure the probe holder is clean and free of debris before loading the probe. (The sides of the probe can crack and generate debris if it's not handled with absolute care.)

   NOTE: *Debris lodged under the probe substrate will negatively impact the tuning curve by introducing unwanted (and often unstable) mechanical resonances and may eventually become dislodged, contaminating the sample or adhering to the probe, thereby interfering with imaging.*

   a. If debris is visible when viewing the probe holder under the microscope, first blow it off with the rocket-shaped bulb blower, then gently clean the window with a fresh lens tissue wetted with spectroscopic grade ethanol. Be extremely careful with the optical window and probe holder clip while cleaning as they can easily be scratched or otherwise damaged.

   b. If further cleaning is needed, consult the FastScan help files for instructions on using the Z scanner cleaning block.

3. Using tweezers, gently place the probe in the slot on the probe holder. Take care not to squeeze the probe too tightly with the tweezers, as that may generate small shards of silicon or silicon nitride that can lodge between the probe and the probe holder, leading to problems when tuning. Gently swing the spring clip up and over the probe into the closed and locked position to secure the probe (**Figure 2**).

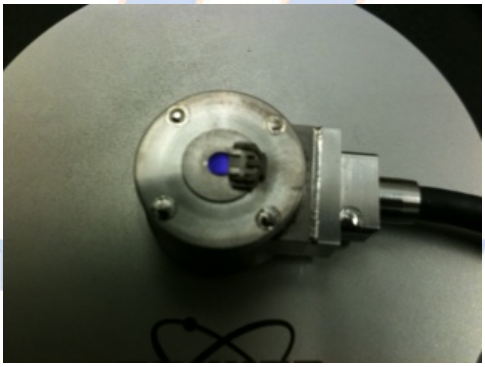

Figure 2. FastScan probe holder with spring clip secured in the locked position.

4. Use the optical microscope to view the probe's alignment within the slot on the probe holder. Carefully align the probe so the substrate does not directly contact any of the edges of the channel and is roughly equidistant from all three edges. Take care to only move the probe substrate a small distance off the back edge of the probe holder's groove. If the probe is either too far from or too close to the back edge of the groove, the tuning curve will be poor. Likewise, if the probe is lodged on the edge or back of the channel, it can affect the tuning curve and its stability.
5. After ensuring the probe is aligned properly in the desired location and the probe holder is clean/free of debris, close the spring clip to lock the probe firmly in place.
6. Using a micropipette, carefully deposit ~25-30 µL of filtered buffer solution onto the Z scanner, filling the small gap between the optical window and the backside of the probe so that it ideally surrounds and envelops the tip.

NOTE: *You may also deposit the fluid onto the probe after mounting the Z scanner, but you must be careful not to break the cantilever/tip.*

NOTE: *Cleanliness is paramount for successful fluid imaging! All samples and preparatory glassware must be rigorously clean. Solutions should be filtered immediately prior to use with a 0.2 µm or better syringe filter.*

7. Deposit enough imaging buffer onto your sample to form a droplet that rises ~1-2 mm above the sample surface (typically ~60 – 80 µL on mica).
8. Install the Z scanner on the FastScan head, taking care not to spill or allow the fluid to run off the scanner.

WARNING: Prior to installing the Z scanner, check to ensure the High Voltage indicator light on the FastScan head is not illuminated. Failure to do so could result in electrical shock to you and/or damage to the AFM head. If the NanoScope software is already open, you should always use "Change Probe" or "Change Head" option in software, as this automatically disables the high voltage and alerts the software that you are installing the Z scanner and/or a new probe.

   a. Select "Setup" in the Workflow Toolbar column. Click "Change Probe" (**Figure 3**).
   b. The "Change Probe" dialog box will appear. Click the "Move to probe loading position" icon (**Figure 4**). This will move the stage away from the head so the probe holder can be safely loaded.
   c. Select the type of probe being loaded (e.g., FastScan D) from the "New Probe of type" dropdown menu (**Figure 4**).
   d. Ensure the laser spot size selected via the switch on the right side of the FastScan head matches the size "recommended" in software for your chosen probe type (**Figure 3** and **Figure 4**).

9. After the Z scanner has been mounted and the probe type selected, align the laser in the center of the forward end of the cantilever. The closer the laser is to the apex of the cantilever, the longer the lever arm and the more sensitive the AFM will be to deflection of the probe. If you later experience issues with the AFM consistently false engaging, consider positioning the laser slightly farther back on the cantilever.

   NOTE: *You should periodically recheck the laser alignment during fluid imaging, as the laser spot can drift over time, drastically reducing the sum.*

10. From the navigate window, position the probe directly above the sample surface and lower the tip until the two droplets (tip and sample surface) merge. You should see a visible flash in the video camera feed when the droplets merge. Additionally, once the two droplets are merged there should be a substantial column of fluid (i.e., a "neck") between the tip and sample.

WARNING: Merging the droplets often results in the tip being closer to the sample surface than the standard sample clearance of 1 mm. When using the Z motor to bring the sample surface or tip reflection into focus, always begin by moving the Z motor up, not down.

11. Select the Experiment drop down menu from the toolbar. Select Engage Settings and in the window that opens, ensure sample clearance and SPM safety are set to 1,000 µm and 100 µm, respectively.
12. Close the Engage Settings window and use the Z motor controls to bring the tip to the 1000 µm (1 mm) clearance height where either the tip or sample surface is in focus (depending upon your software choice of Tip Reflection Focus or Sample Focus). Ensure the "Auto-Compensate for Fluid?" checkbox option is selected in the Focus workspace so that the index of refraction of the fluid (~1.30 for water) is taken into account.

Tuning the Probe

One advantage of tapping in fluid as compared to normal tapping mode AFM is that tip-sample adhesive forces due to absorbed moisture on the sample are removed. Reducing the adhesion permits lower drive and target amplitudes, resulting in lower forces and higher resolution images. Similar to normal tapping mode, tapping in fluid employs an offset from the natural resonance frequency but with a lower oscillation amplitude (~1-5 nm, similar to non-contact tapping in air) so that the probe engages and operates in the repulsive regime, but taps very softly. Following are instructions regarding how to tune the probe, as well as typical values for various parameters.

Fluid Tuning Procedure

13. Ensure the probe type selected in software matches the one you are using (**Figure 3**).

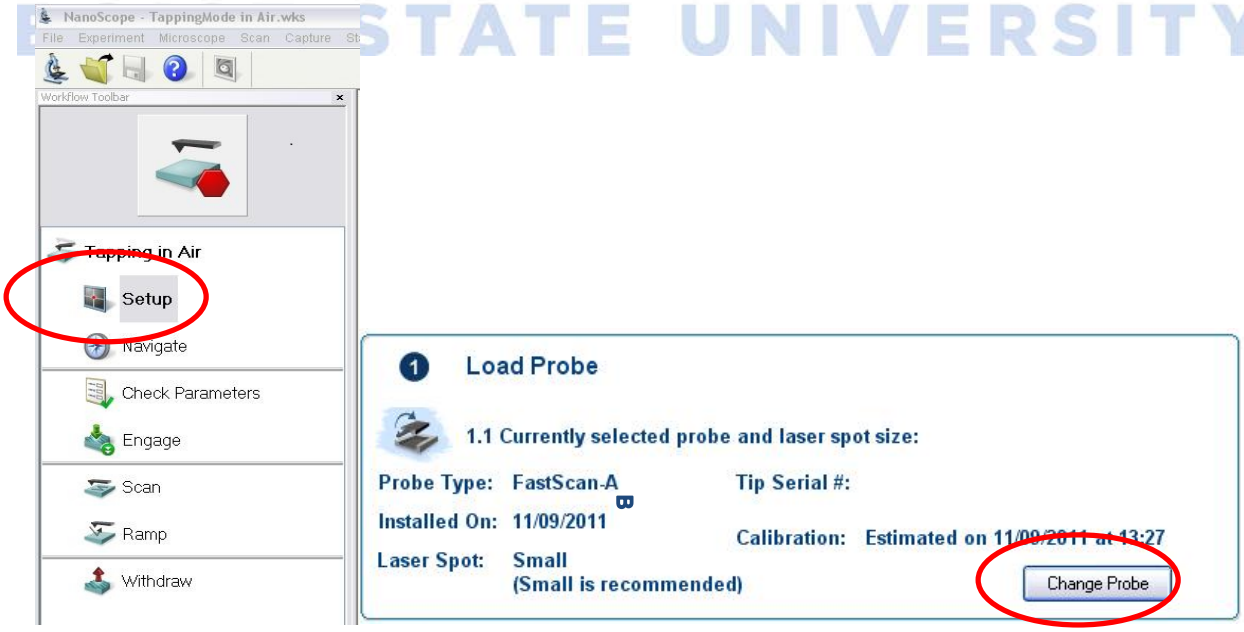

Figure 3. Workflow Toolbar column with Setup circled (left). Within Setup, the Load Probe option with the Change Probe button circled (right).

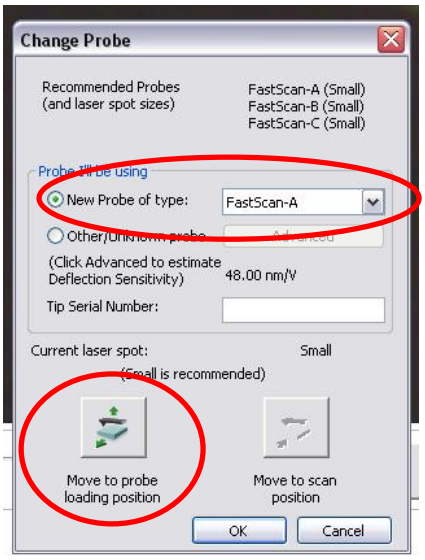

Figure 4. The Change Probe dialog box.

14. While in Setup, click on Manual Tune.
    a) Set the Auto Tune start and end frequencies to the range within which you would expect the resonant frequency to be found. In fluid, you should expect the resonant frequency to be one half to one third of the resonant frequency in air that is typically listed on the probe box.
    b) Set the Target Amplitude to 5 nm. This is a good default starting value to enable comparisons between different probes and evaluate the quality of the probe/tune.
15. Click Auto Tune to sweep the frequency range and obtain a tuning curve for your probe. Tuning in fluid will produce a multitude of peaks (**Figure 5**).
16. To figure out which peak within the tuning curve (blue trace) to choose, conduct a fast thermal tune to identify the probe's true natural resonant frequency. To be able to see the peak of the thermal tune (red crosses), adjust the 'Sweep Width' to ~100 kHz and the 'Channel 1 Data Scale' to 5 nm.
17. Select the "Offset" radio button under "Cursor Mode", then locate a gradual slope near the peak of the thermal tune to use as your Drive Frequency (green vertical line in **Figure 5**). Adjust the Drive Amplitude until the Drive Frequency you have chosen intersects the tuning curve at a height of ~1-3 nm.

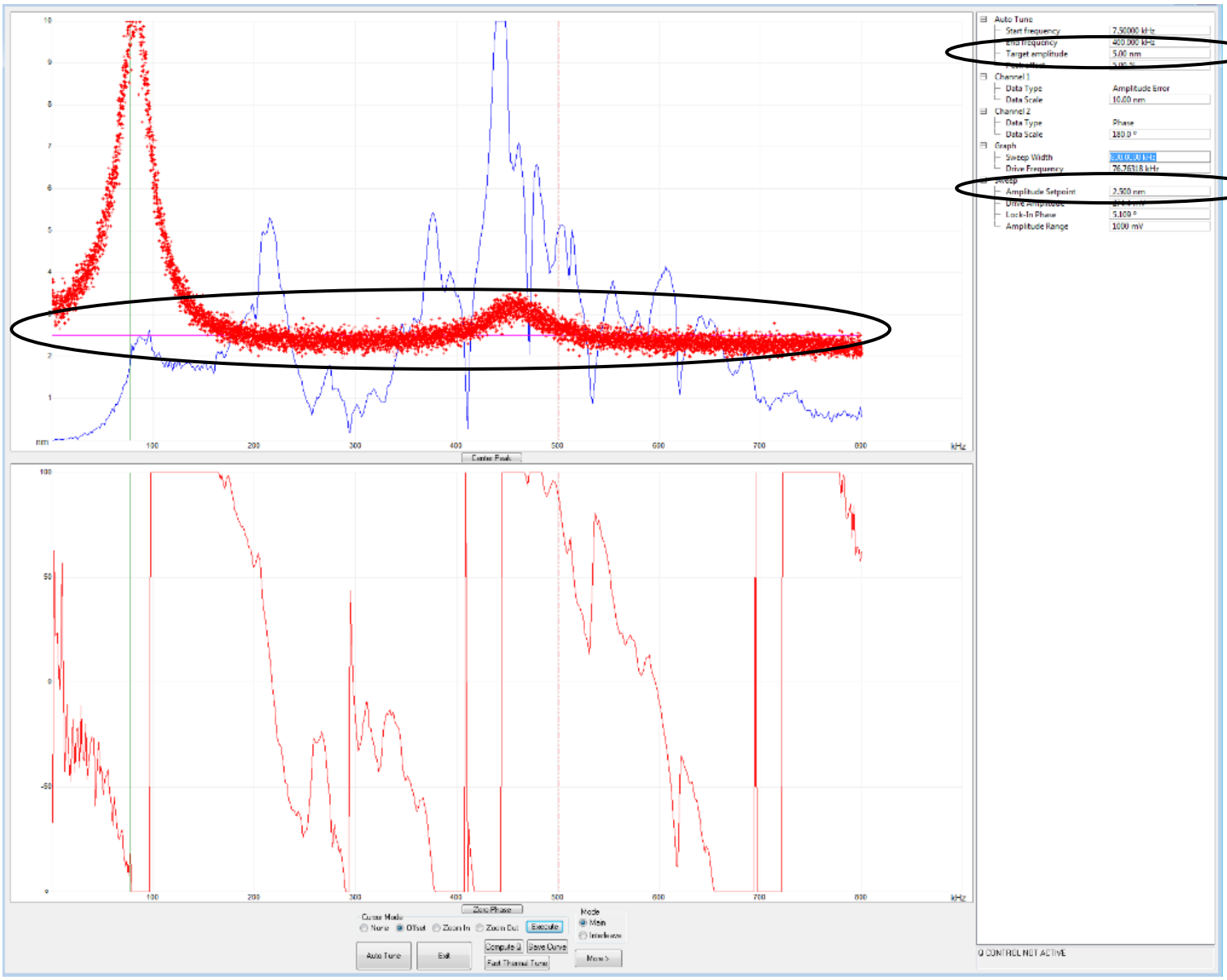

Figure 5. Representative fluid manual tune result showing the "forest of peaks" typically seen in fluid. The Target Amplitude and Amplitude Setpoint parameters are circled in black, as is the pink line indicating the current Amplitude Setpoint value. The Drive Frequency is indicated by the vertical green line.

18. Once the Drive Amplitude and Frequency are set, manually adjust the Amplitude Setpoint until the horizontal pink cursor line demarcating it intersects the tuning curve (blue trace) at the Drive Frequency (green vertical line) chosen in Step 17 above (**Figure 5**).
19. Use the navigational arrows to move to the desired scan location above your sample.

Engaging the Surface

20. Go to the Engage Settings (under the Microscope dropdown menu heading) and set the ***Engage Setpoint*** to ***0.90-1.00*** and the ***Engage Type*** to ***Standard***. This value is a multiplier of the amplitude setpoint. A multiplier of ***1.00*** will engage at 1.00 times the amplitude set point (a softer engage) or 0.9 (a more aggresive engage).
21. Prepare to engage as in normal tapping mode, using either Focus on the Sample or Focus on the Tip Reflection (as appropriate to your sample) to align your probe at the correct height above the surface.
22. Switch to the Check Parameters window in the Workflow Toolbar. Select the "Expanded" view (red puzzle pieces on the toolbar menu). Set ***Scan Size*** and ***X*** and ***Y Offsets*** to ***0*** in the parameter list.
23. Click Engage in the Workflow Toolbar. As soon as the computer beeps to indicate the tip has engaged, click on the tuning fork icon in the toolbar to retune near the surface. Use an ***Offset*** of ***50 nm*** and check to make sure the tuning curve hasn't changed significantly. Make adjustments as necessary.

    NOTE: *After retuning, the Z piezo meter bar graph may be red. This means the AFM has false engaged and the probe is not tracking the surface. If this is the case, slowly lower the Amplitude Setpoint, ~1 mV at a time, until the Z piezo meter just turns green.*

24. Gradually lower the Amplitude Setpoint in ~0.5-2 mV or ~0.1 nm steps until the probe just begins tracking the surface. The voltage indicated on the Z piezo meter should stop changing significantly at this point.
25. Once the probe is just barely tracking the surface, lower the Amplitude Setpoint another ~1-2 mV to ensure the probe will track well. Lower it further to improve tracking, but be warned that this may cause the tip to dull more quickly.

Imaging

Typical Parameter Values

Scan Size

- Scan size is dictated by the needs of the image recipient and the number density of nanostructures on the surface.
- For quick surveys, a **5 µm** scan size is recommended, assuming a relatively high sample density.
- For statistical surveys, **10-15 µm** scan sizes are recommended. However, the total number of images needed will depend on the nanostructure density and the number of structures necessary for statistical considerations.
- The largest recommended scan size is **~20-22 µm**. Above this size, the image resolution (due to max of **5120 Samples/Line**) will be worse than the tip radius of curvature (nominally 5 nm for FastScan series probes, but in practice typically closer to 2 nm) and nanostructures will begin to appear pixilated. Furthermore, more empty sample will be imaged, which can result in faster tip wear.

Samples/Line and Lines/Scan

- These are determined by the combination of the Scan Size and the lateral resolution needed. If fine details of structure are needed, then more Samples/Line and Lines/Scan will obviously be necessary. The max value for Samples/Line and Lines/Scan is 5120 (assuming a square image with an Aspect Ratio of 1.0).
- Recall that ultimately the resolution is limited by the tip geometry or the pixel size, whichever is greater. For FastScan series probes, the nominal tip radius of curvature is approximately 5 nm, making 5 nm the lower limit of resolution. However, this value is based upon the idea that the end of a tip will be dulled to ~5 nm upon engaging the surface in normal tapping mode. When engaging and operating as described in this SOP, the actual tip radius is typically closer to 2 nm.
- For large scans (10-20 µm square), standard settings for ***Samples/Line are between 3000-5120.*** Note that the Lines/Scan will adjust automatically based on the set Aspect Ratio (typically 1.0).
- The higher your resolution, the more noise is introduced into your image at a given scan speed (less signal averaging due to fewer surface interactions per point/pixel). Choose your resolution based on the purpose of image. If you just need to count nanostructures, use a lower resolution. If the image is for publication or presentation, go with a higher resolution.

Scan Rate

- Scan Rate is paramount in determining how well your probe tracks the sample surface: slower scan rates will result in better tracking. However, scan rate also determines how long your scan will take to complete. In addition, *scan rates in fluid are ~2-3x slower than comparable rates in air because of the decreased natural resonance frequency due to hydrodynamic drag.*
- Changing the Scan Rate changes the Tip Velocity. Tip Velocity is really the key parameter in obtaining a high quality image. Tip Velocity is determined by the product of the Scan Rate and the Scan Size. The lower the Tip Velocity, the better the tracking. However, lower tip velocities mean it will take longer for the scan to finish.
- The FastScan head has the ability to scan at very high rates, allowing for relatively quick imaging/short scan times. However, at very high scan rates (above ~4 Hz) small details (such as DNA nanotubes and Au nanoparticles) in large scans become distorted (smeared or streaked) because the tip velocity is too high, negatively impacting tracking.
- For smaller scans (< 2 µm on a side), "good" images can be obtained using ***Scan Rates*** as high as ***1-2 Hz (Tip Velocity = 4-8 µm/s)***.
- For larger scans, Scan Rate and acquisition time must be balanced, based on what is needed from the image.
    - For quick surveys where slight image distortion is not critical, a ***Scan Rate*** of ***1.5-3 Hz***, corresponding to a **Tip Velocity** of **~60 µm/s**, can be used.
    - For high quality, low distortion large area scans, a ***Scan Rate of 0.2-1 Hz (Tip Velocity = 8-20 µm/s)*** must be used.
- Overall, there are two general rules of thumb for Scan Rates:
    - *The smaller the scan, the faster the scan rate can be, and vice versa. (In the end, what matters is the Tip Velocity, which is the product of the Scan Rate and twice the Scan Size.)*

- *The slower the Scan Rate, the better the tracking (provided the I and P Gains are set correctly, as described below).*

Integral and Proportional Gains

- The I and P (Integral and Proportional) Gains are used to control and adjust how much/how quickly the probe responds to changes in sample height/topography. The higher the gains, the greater the feedback response to a given change in topography. Thus the gains can be used to improve sample tracking and reduce distortion of features in your image.
- Integral Gain has a larger effect than Proportional Gain.
- As the gains are increased, measurement noise is amplified; eventually the added noise outweighs the improved tracking. The noise can be viewed on the Amplitude Error channel.
- As a general rule, the lower the gains that can be used while still obtaining good tracking, the better the measurement/image obtained (due to lower noise).
- You should adjust the gains based on the quality of sample tracking you need.
- To fully resolve fine details such as Au NPs (nanoparticles), QDs (quantum dots), and DNA nanostructures, you should increase both gains until any streaking or distortion of features are gone (or at least minimized).
- How much gain is needed depends on both the Scan Rate and Scan Size. Faster and/or larger scans will usually require higher gains, while slower/smaller scans will require less gain to properly track the sample surface.
- Optimal gains will vary between samples (and potentially even between scans of different areas of the same sample).
- For large area, high resolution scans:
  - ***Integral gain is usually in the 1.5-3.0 range (but is Scan Rate dependent).***
  - ***Proportional gain is usually in the 7.00-10.00 range and ~2-10x the Integral Gain.***

Z limit

- This setting is used to control height resolution and inherent noise in the measurement.
- The lower the Z limit, the lower the noise and the greater the height resolution.
- However, reducing the Z limit imposes a restriction on how much the Z piezo can move.

Caution: If a feature in your scan exceeds the Z limit, it can stress the Z piezo to the point of damaging it (or the probe). Make sure the Z limit is set so that all features present in the scan can be imaged without damage, taking into account sample tip/tilt and thermal drift of the Z piezo with time as the electronics and sample chamber heat up.

- As noted above, the Z piezo is subject to upward thermal drift. This is particularly pronounced if the acoustic hood is shut, preventing convective cooling. Thus if the Z limit is very low and the scan time is long, the tip will eventually lose track of the surface.
- Also as noted above, the necessary Z piezo range can also be affected by how flat the substrate is glued on the puck (i.e., how perpendicular the sample surface is to the probe). As a general rule, the larger the Scan Size, the larger your Z limit will need to be due to sample tip and tilt.

- The relative flatness of the sample can be seen on the height channel. Change *RT Plane Fit* to *None* and observe the change in height across the sample at its top and bottom using the Scan Down and Scan Up features.
- For large area, high resolution non-contact scans on relatively flat samples with limited (i.e., low height) topographical variation (e.g., DNA nanostructure surveys) the ***Z limit should be ~1.5-2.75 µm.***
- Only use low Z limits (≤1 µm) when the scan is relatively small/quick and it is certain that the sample is relatively flat (limited tip/tilt) and has no large topographical features.

Conclusion/Disclaimer

By following the above general guidelines, experienced/qualified AFM users should be able to obtain excellent quality high resolution tapping mode AFM images in fluid. Keep in mind that the precise optimal values of the various scan parameters will depend on the individual sample/scan and desired image quality. It therefore requires practice to become proficient and obtain the best images possible.

This document is intended as a guide to help experienced AFM users new to tapping mode AFM in fluid. Users should always take caution to preserve and protect the AFM from unnecessary damage or stress. Most importantly, if a user is unsure of how to perform a specific task they should **ALWAYS ASK FOR HELP OR SEEK ASSISTANCE FROM THE SSL STAFF AND/OR A MORE EXPERIENCED USER BEFORE ATTEMPTING ANYTHING.**