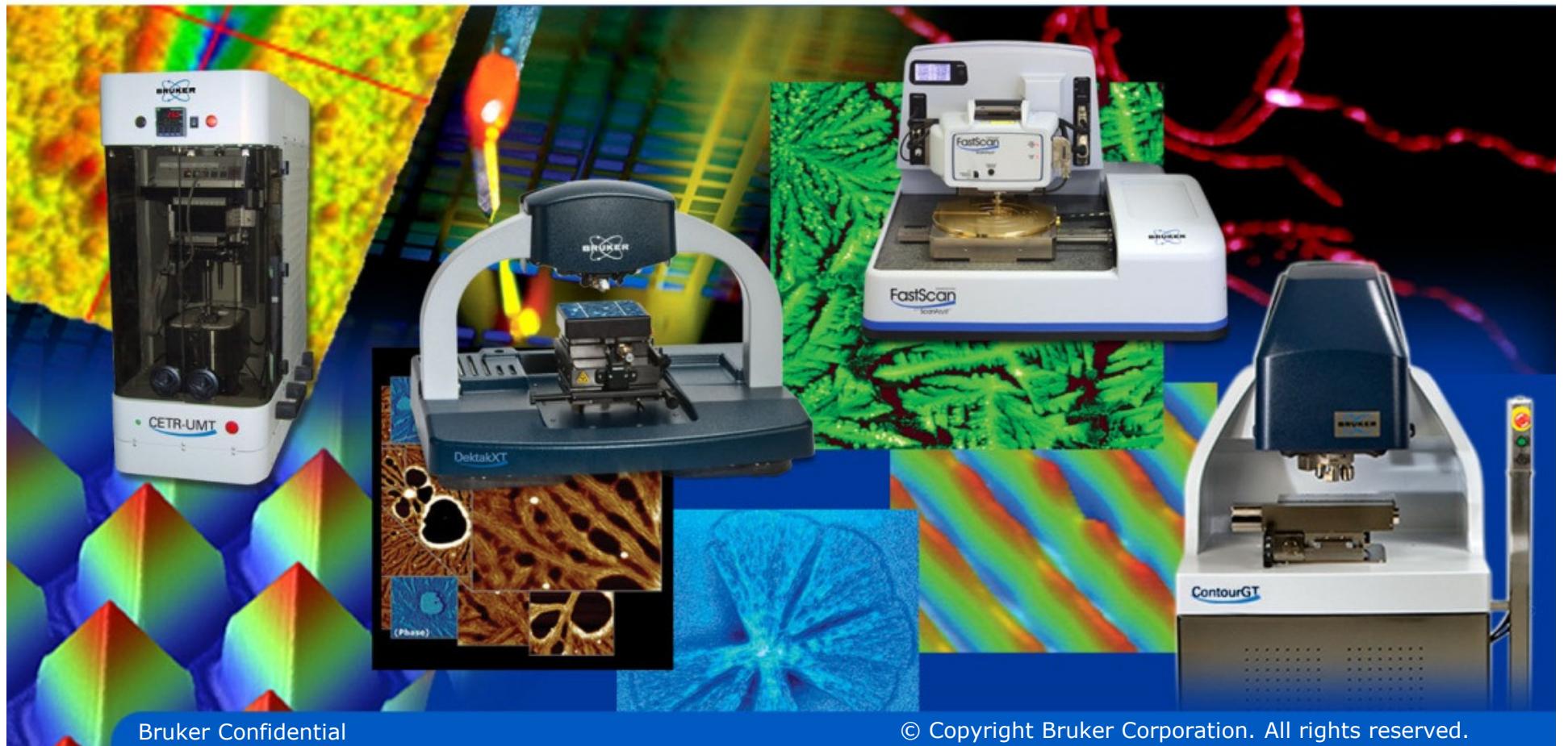


Advanced AFM Applications Training

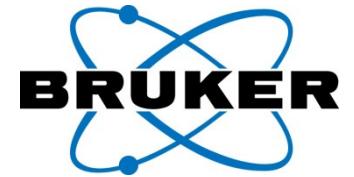


Fluid Imaging

Yueming Hua, Ph.D.

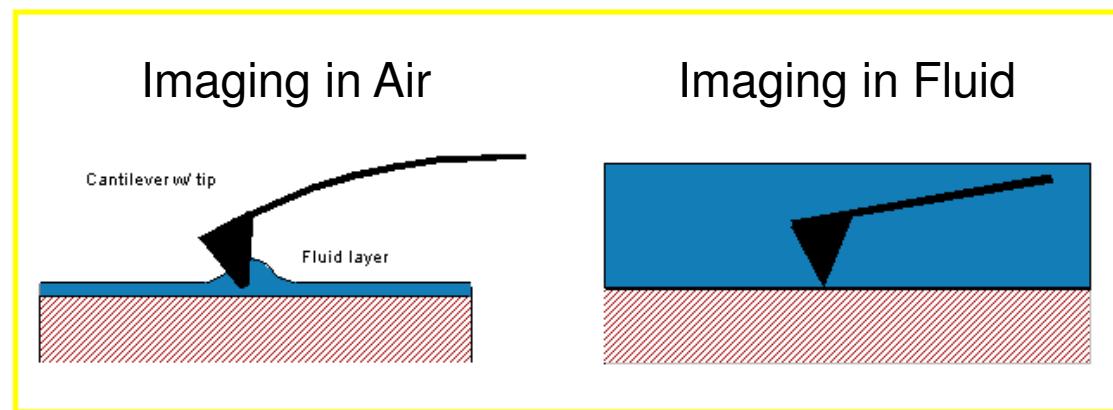


Why Fluid Imaging

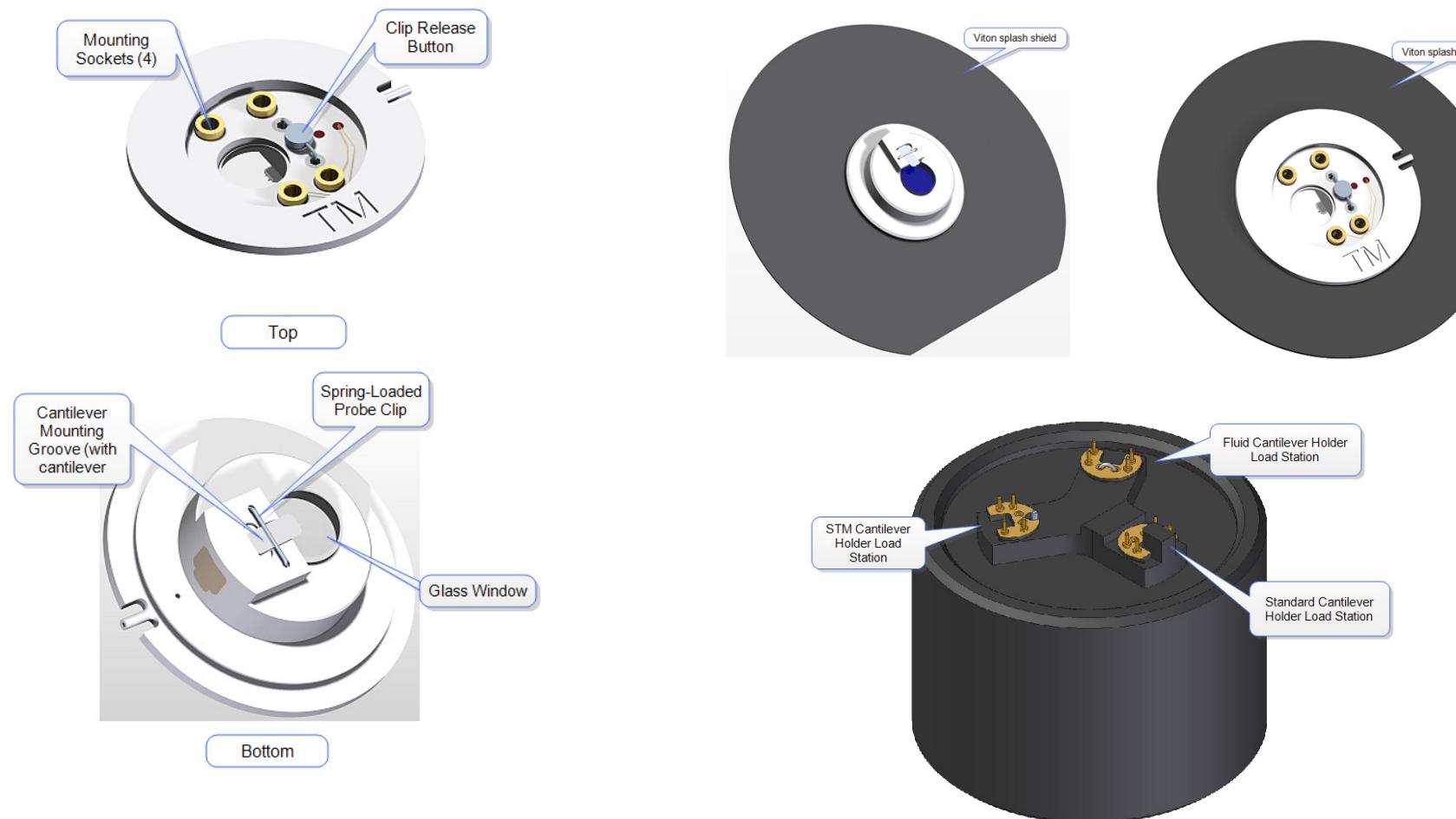
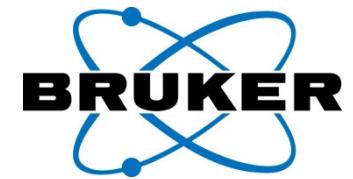


IN FLUID:

- Reduces adhesion (meniscus) forces
- Allow measurements in native environment (some polymer and majority of biological samples)
- In-situ study of sample surface during physical/chemical process:
 - Chemical reaction
 - Crystallization
 - Dissolution, etc.

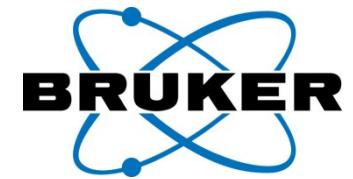


Required hardware for fluid imaging Icon AFM



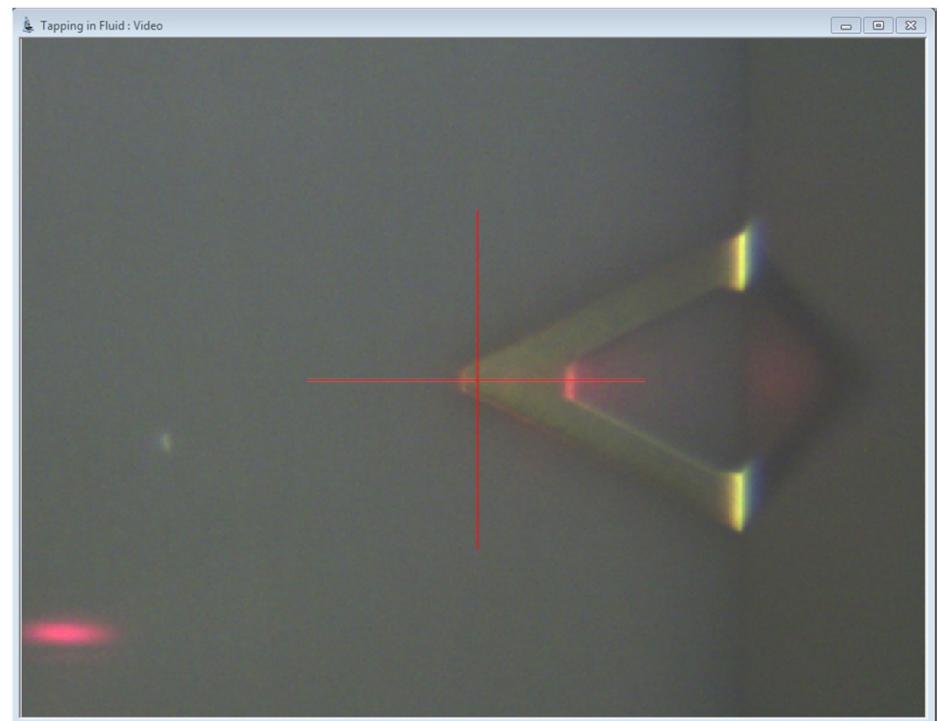
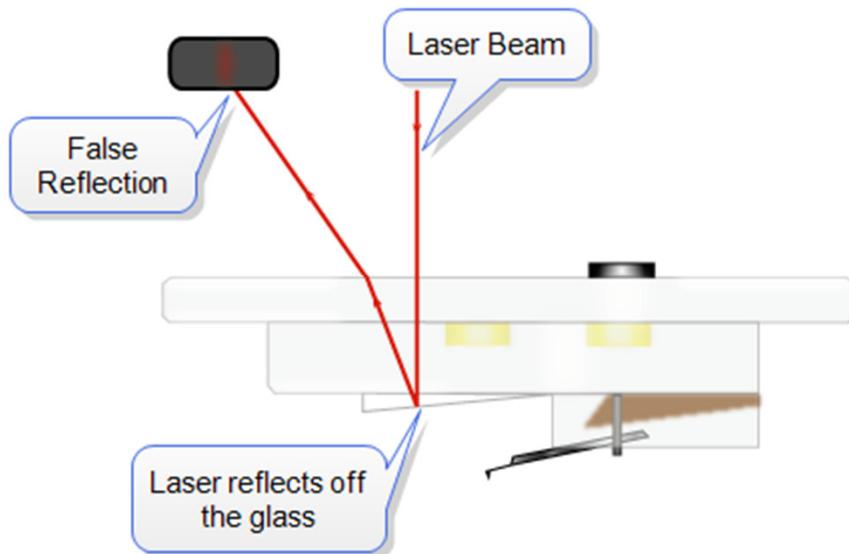
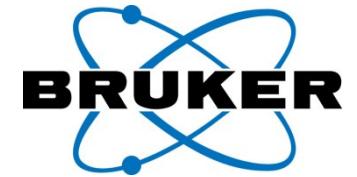
Precautions

Icon AFM



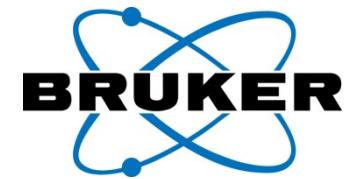
- **DO NOT use alignment station for laser alignment.**
- **DO NOT spill water into scanner.**
- **Set XY Closed loop sensor I gain to 8.**

False Reflection with Icon Fluid Cell



- Make sure to align the laser from cantilever onto the PSPD, not the reflection laser from the glass window
- If PSPD alignment is correct, small change of laser position will dramatic change sum signal

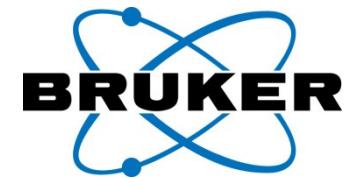
Required hardware for fluid imaging MultiMode AFM



- Multimode fluid cell

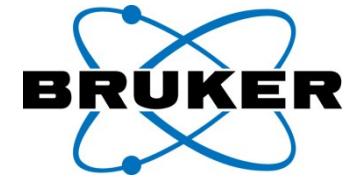
Precautions

MultiMode AFM



- **When imaging fluid samples, use extraordinary precautions against spillage. Fluids must not be spilled on or around components containing electronic parts**
- **Avoid spilling all corrosive fluids on exposed surfaces; otherwise, damage may result. In the case of a spill, immediately clean and dry all affected surfaces carefully**

Snell's law



$$\frac{\sin\theta_1}{\sin\theta_2} = \frac{v_1}{v_2} = \frac{n_2}{n_1}$$

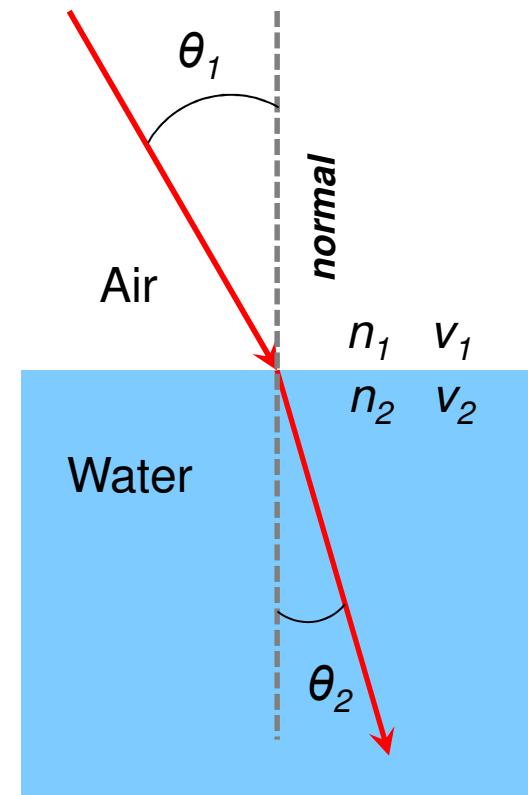
θ is the angle measured from the normal of the boundary,

v is the velocity of light in the respective medium (SI units are meters per second, or m/s)

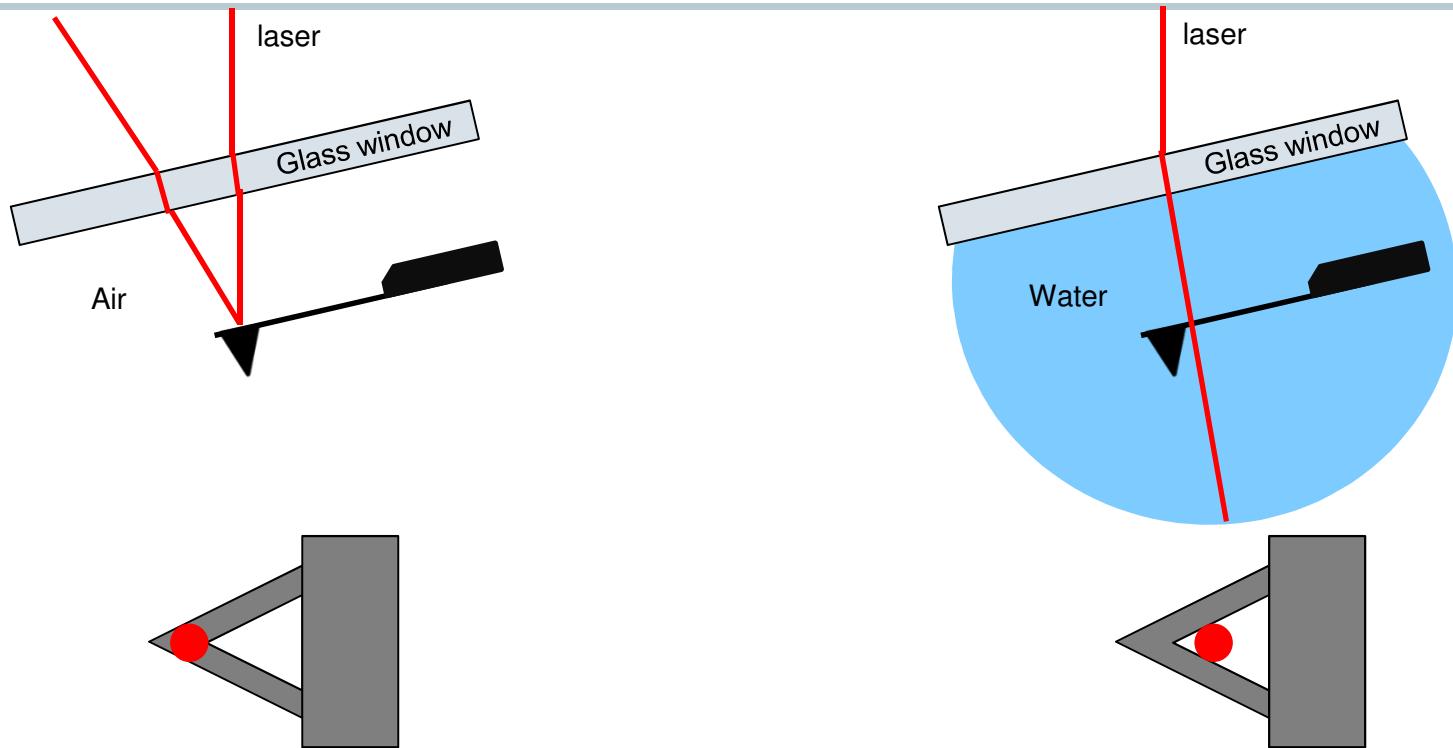
n is the refractive index (which is unitless) of the respective medium (n is about 1.00 for air and about 1.33 for water)

Two effects:

- The laser path changes when it enters from air to water with an angle
- Object in the water appears shallower than it really is

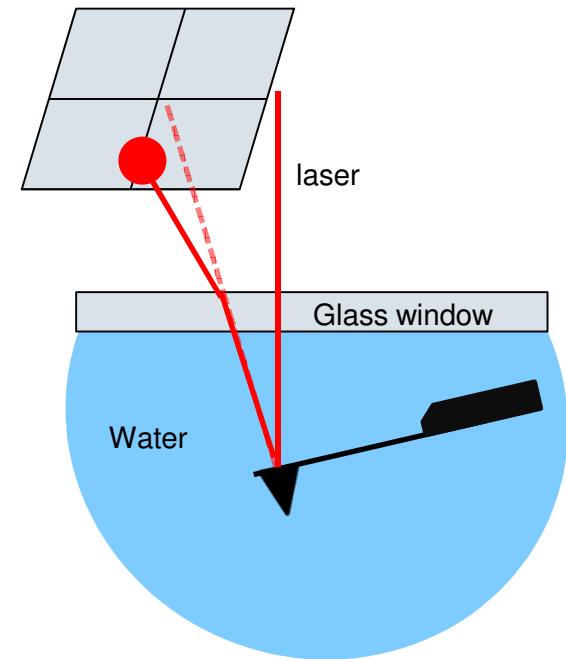
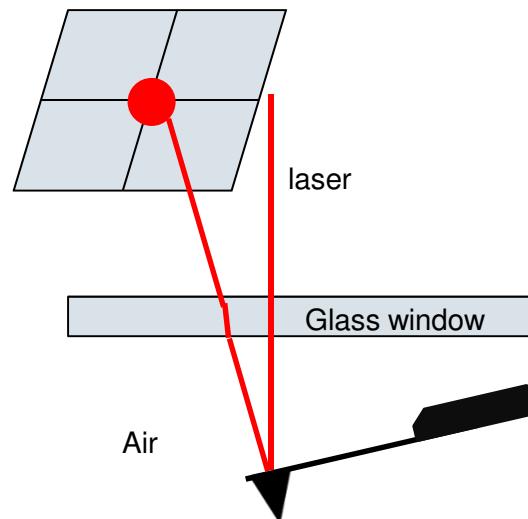


Laser Path Changes after Apply Water on Tip



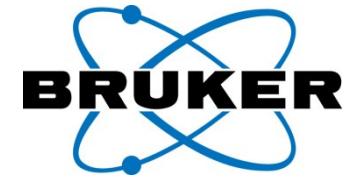
- A drop of water needs to be put on the cantilever to make engage in fluid easier (it is hard to wet a dry cantilever)
- Because of the refraction index change from air to water, the laser path will change after apply water onto the AFM probe
- For Icon fluid cell, after apply a water droplet on the cantilever, the laser spot will shift about 30um closer to the cantilever chip base

Laser spot position on PSPD changes



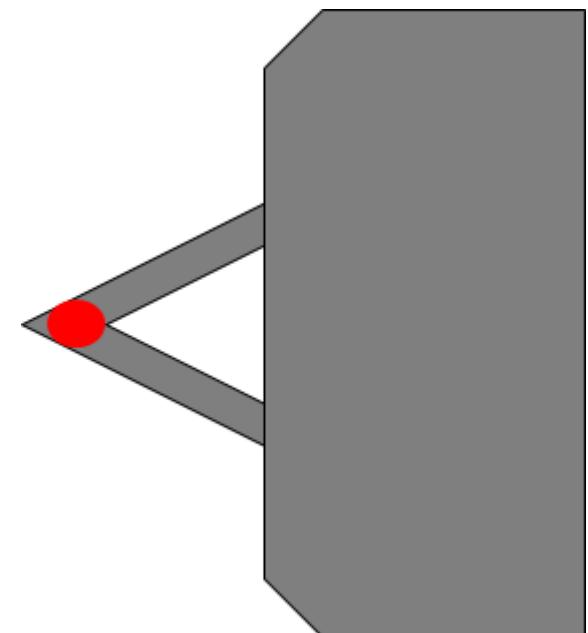
- Laser spot position on PSPD changes after adding fluid

How to Re-Align Laser After Adding Fluid

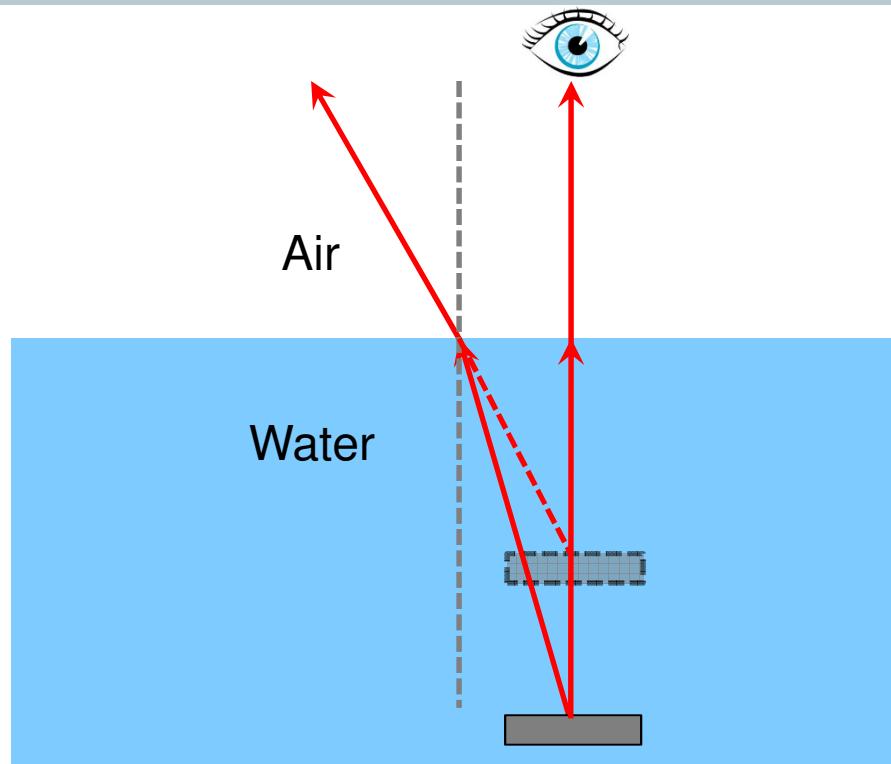
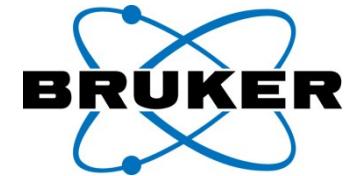


In fluid, we need to align the laser blindly:

1. Move the laser to left slightly so that it gets some sum signal, no need to maximize the sum at this step
2. Move laser up or down, maximize sum signal to confirm it's hitting one of the leg
3. Move laser to opposite direction and maximize sum signal to confirm it's hitting the other leg
4. Move the laser to between and minimize the sum signal, so we know the laser is at the exact middle of two legs
5. Move the laser to the left and maximize the sum, and now laser is at the best alignment position

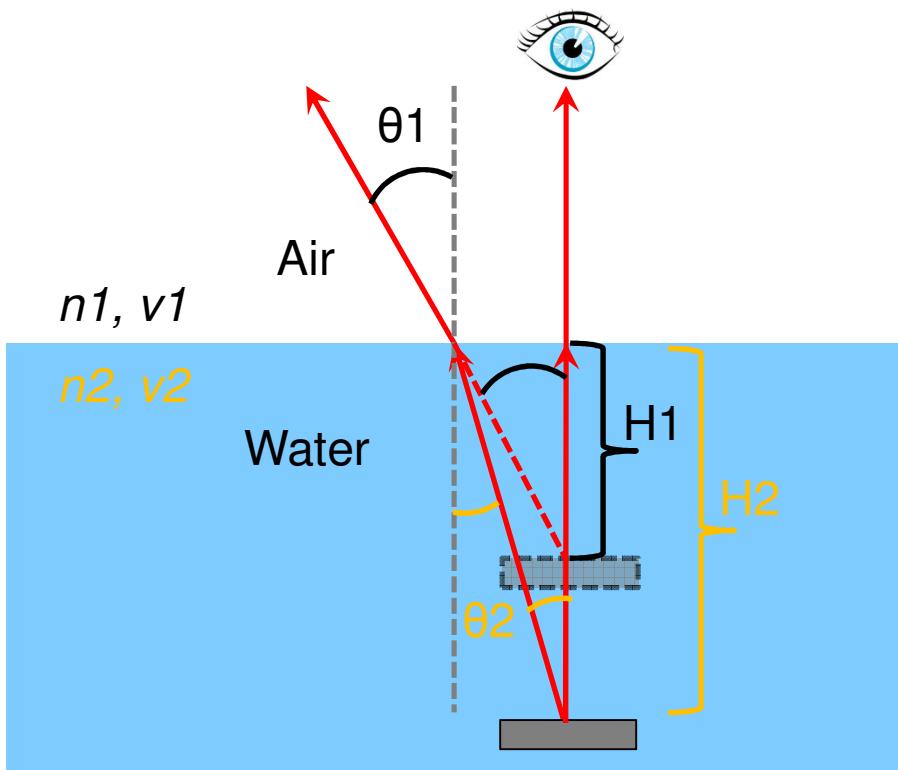
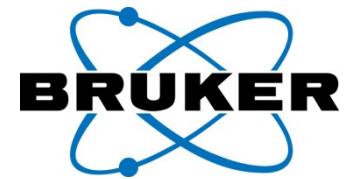


Object in water appears shallower than it really is



- Because of the light path change at the air-water interface, the object in water appears closer to the interface
- On Icon fluid cell with sample immersed in water, if the real distance between tip and sample is 1000um, the appeared distance in the Icon optics is about 750um

How does the sample focus position changes in water



$$n_1 \sin \theta_1 = n_2 \sin \theta_2$$

$$\frac{H_1}{H_2} = \frac{\tan \theta_2}{\tan \theta_1} = \frac{\sin \theta_2}{\sin \theta_1} \times \frac{\cos \theta_1}{\cos \theta_2} = \frac{n_1}{n_2} \times \frac{\cos \theta_1}{\cos \theta_2}$$

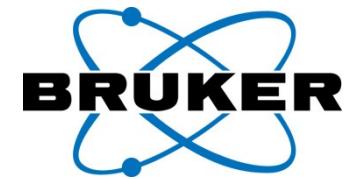
When look down from normal angle

$$\theta_1 = \theta_2 = 0; \cos \theta_1 = \cos \theta_2 = 1$$

$$\frac{H_1}{H_2} = \frac{n_1}{n_2}$$

- For air $n_1=1.0$, for water $n_2=1.333$
- If the sample is 1000um below the tip, in water it looks like only 750um below the tip

Focus Autocompensate in Fluid



2 Focus Sample

2.1 Select Focus Method



Sample (default) Tip Reflection

2.2 Autocompensate for fluid (only if focus is done in fluid)



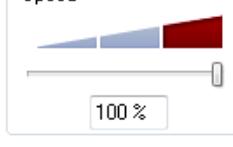
2.3 Adjust focus by moving scan head up or down



Scan Head



Speed

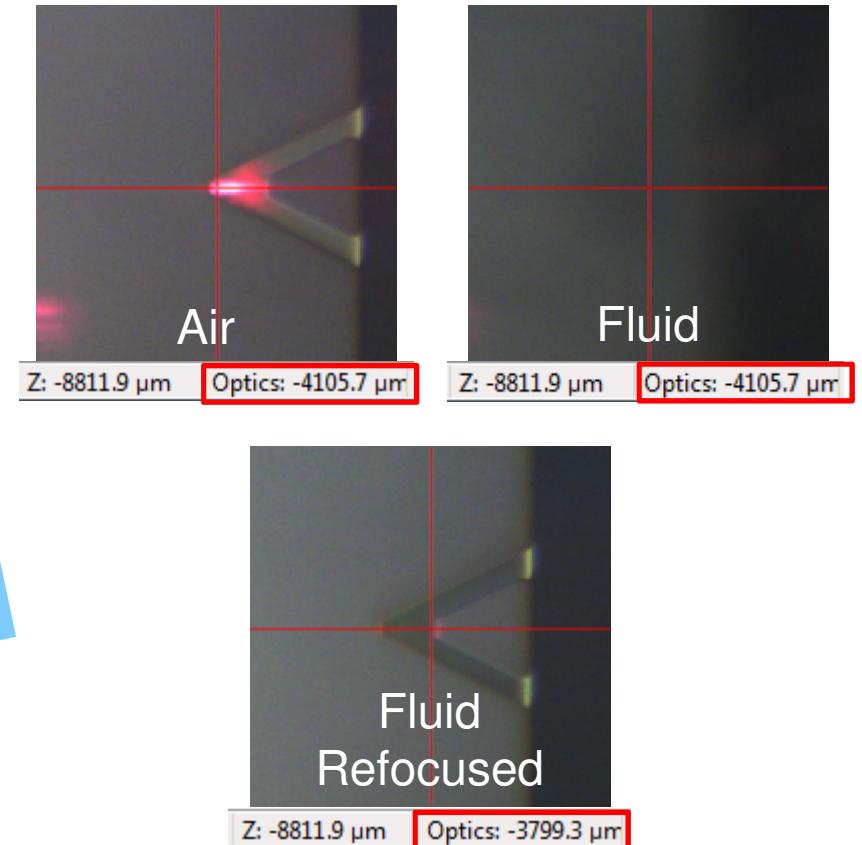
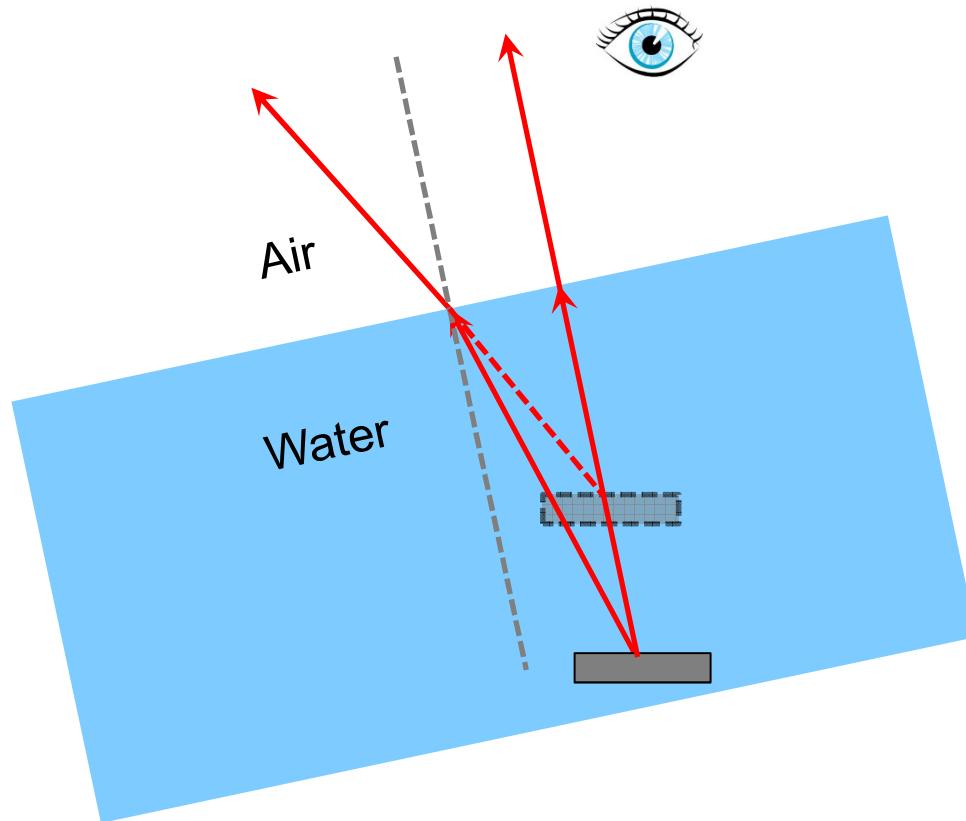
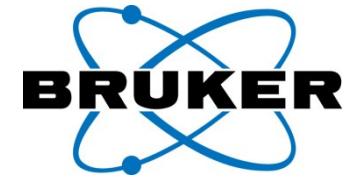


Be careful, adjusting focus position too fast may cause tip to contact surface



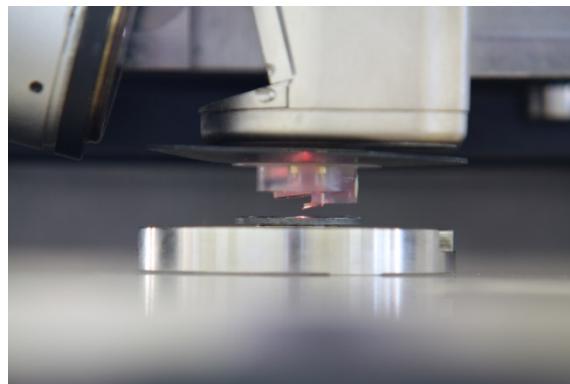
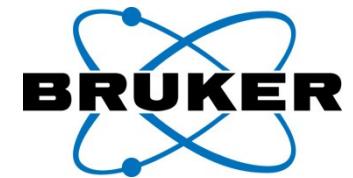
- **Question:** what will happen if we check this function when setup up tip-sample focus in air?

Object in water appears shallower than it really is



- If the glass window has an angle with respect to the sample surface, after adding fluid, the sample not only appear shallower, but also lateral position shifts

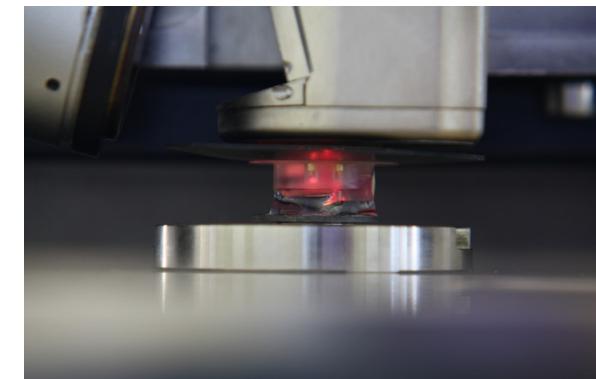
Icon Setup for Fluid Imaging Method_1



1. Setup tip-sample distance in air



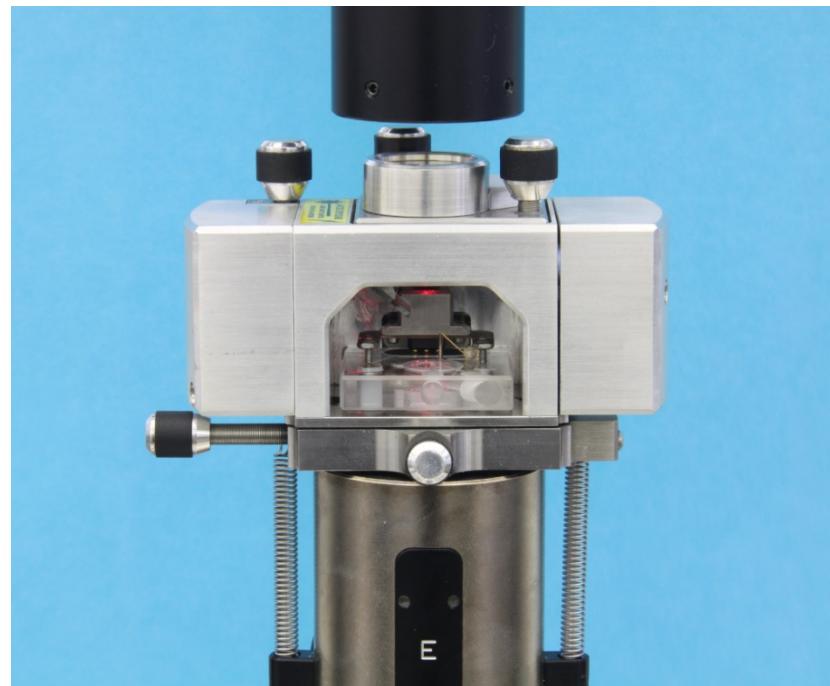
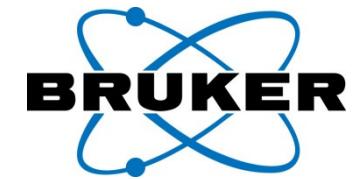
2. Apply fluid to sample and tip



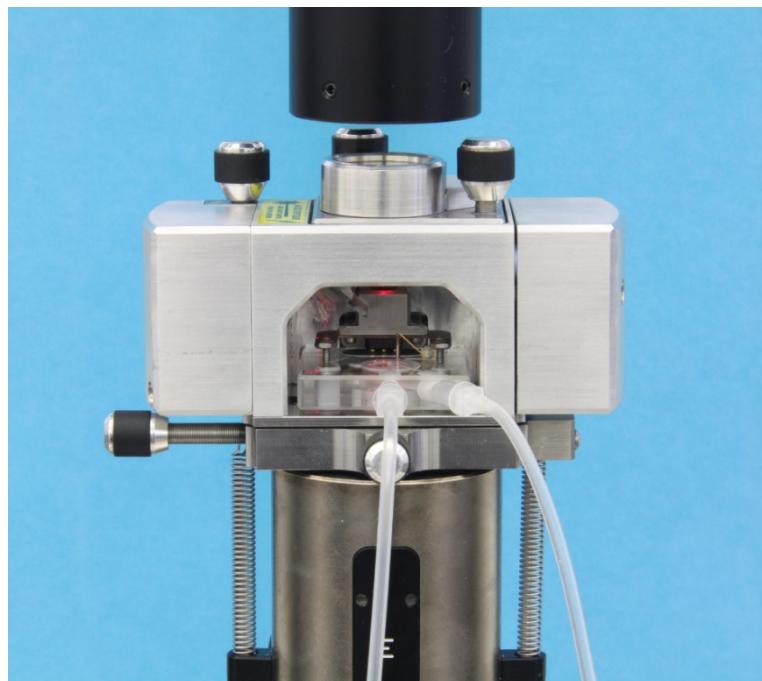
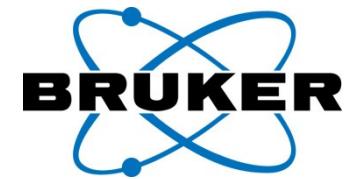
3. Re-install the scanner

- **Question:** what if the sample has to be in fluid all the time, and still want to used this method?

MultiMode Setup for Fluid Imaging Normal Setup

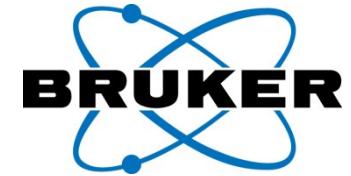


MultiMode Perfusion Cell Setup



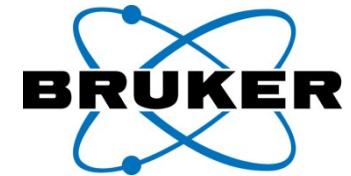
Icon Setup for Fluid Imaging

Method_1



1. Mount probe in fluid cell, mount on scanner and put scanner splash shield in place.
NOTE: For AFM studies in fluid, it is very important that the fluid cell is clean.
2. Align laser and set up the tip-sample separation while DRY, if possible.
 - Bring probe close to dry sample surface
 - Align (locate tip)
 - Navigate (focus surface)
3. Remove scanner, place a droplet of imaging fluid on the sample and on the cantilever. Why on cantilever?
 - Avoids air bubbles
 - Minimizes problems with cantilever interaction with the top of a drop

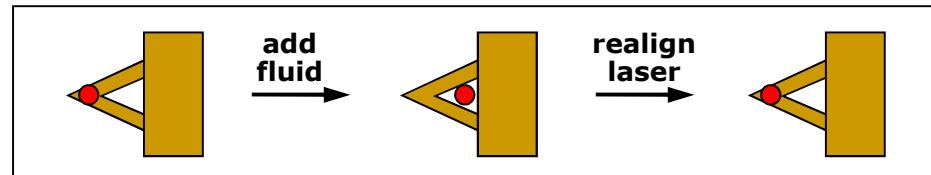
Icon Setup for Fluid Imaging Method_1



4. Put the scanner back in place. This will produce a meniscus of fluid between the sample and fluid cell. Add more fluid, if needed, to form an adequate-sized fluid droplet. Remember: the fluid will evaporate over time.
5. Realign the laser position on the cantilever.

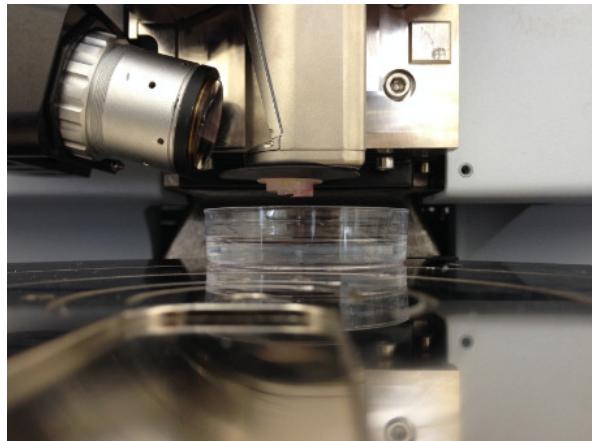
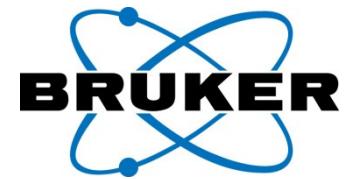
Due to the change in the refractive index of the imaging fluid vs. air, the laser spot will now be focused at a point behind the end of the cantilever. The change in refractive index will also alter the focus of the camera optics and you will no longer see your cantilever clearly.

Refocus the camera to see the cantilever if needed. Rotate the horizontal laser positioning knob COUNTERCLOCKWISE until your maximum sum signal is regained. Note: The sum signal will be higher in fluid than in air.

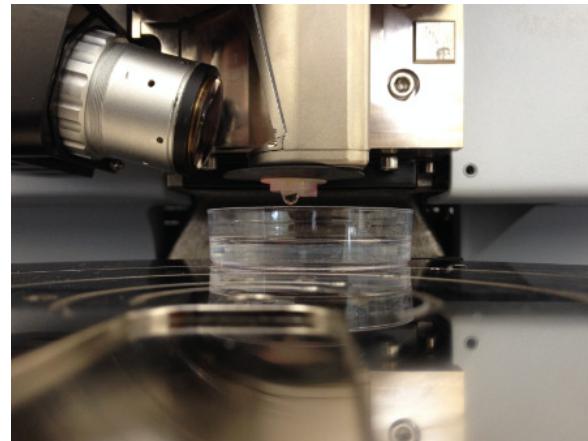


6. Adjust the photodetector, tune the cantilever (if operating in TappingMode™), and engage the tip on the surface.

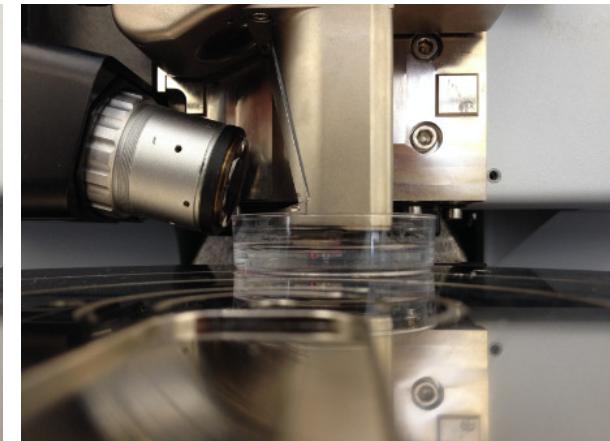
Icon Setup for Fluid Imaging Method_1



1. Mount the cantilever, focus on the tip and setup the laser alignment in air

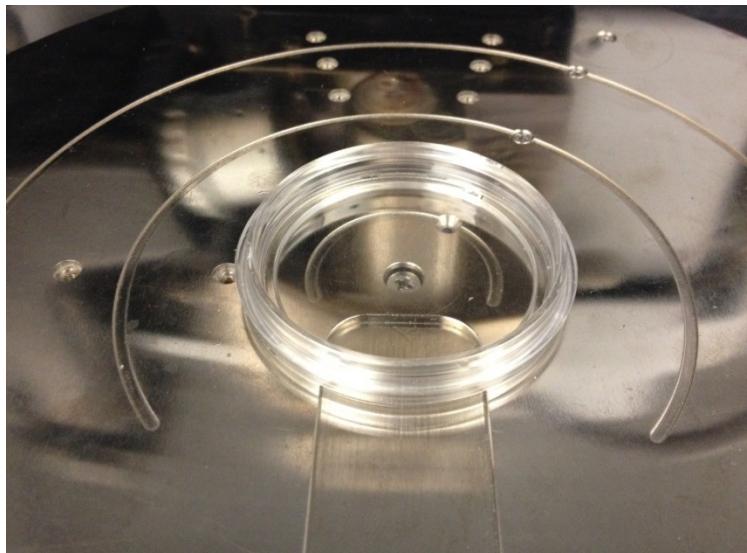
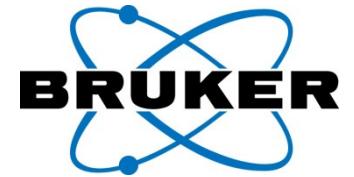


2. Apply a drop of fluid on the cantilever, and re-align the laser and PSPD, and re-focus on the tip

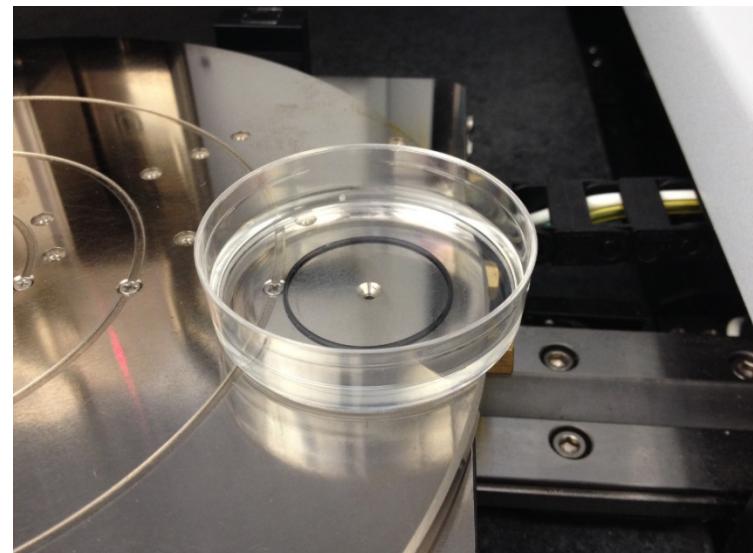


3. Move the Z stage down to setup the sample focus in fluid (use fluid focus auto compensation), and then engage the tip

Hold Petri Dish with Vacuum



50mm glass bottom petri dish



60mm PS petri dish

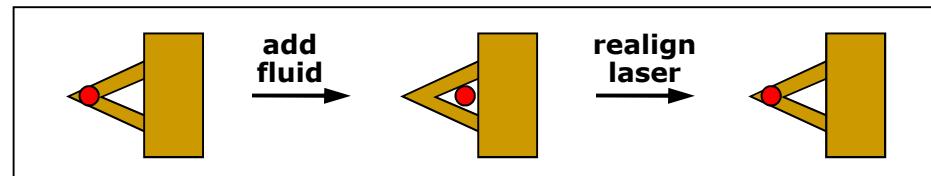
- On Icon AFM, petri dish can be hold on the sample chuck by vacuum
- When hold with vacuum, resonance frequency of the petri dish bottom will increase, and drum effect will be less

Icon Setup for Fluid Imaging

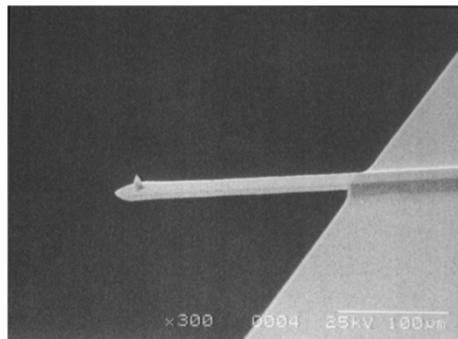
Method_1

Use the following procedure to setup fluid imaging on Icon AFM:

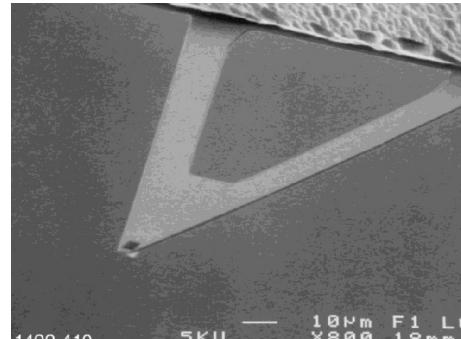
1. Set the XY sensor close loop I gain to 8 to accommodate the weight of the fluid cell
2. Setup the sample in water, and place it on the sample chuck
3. Mount a probe in fluid probe holder, mount it on scanner and put splashshield in place
4. Align the laser on the cantilever in air use shadow method (**don't use alignment station**)
5. Place a droplet of water on the cantilever. This will cause the laser alignment on the cantilever shift close to the cantilever chip base
6. Slightly move the laser alignment in X direction to re-align the laser on the cantilever with the water droplet on the cantilever
7. Mount the scanner and lower it until the droplet of fluid on the cantilever joins and forms a meniscus with the fluid on the sample.
8. Refocus the camera on the tip and the sample surface.
9. Adjust the photodetector, tune the cantilever (if operating in TappingMode), and engage the tip on the surface



Probe Selection for Fluid Imaging



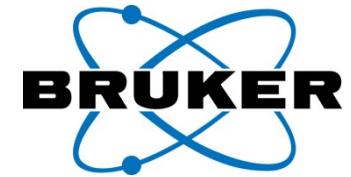
Etched Silicon Cantilever



Silicon Nitride Cantilever

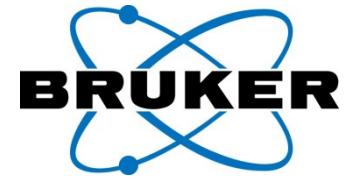
Name	Res. Freq.	Spring. Const.	Cantilever Geom.	Tip Material	Tip Radius, nm
SNL-10	18 to 65kHz	0.06 to 0.35N/m	Triangular	Silicon	2
DNP-10	18 to 65kHz	0.06 to 0.35N/m	Triangular	Nitride	20
DNP-S10	18 to 65kHz	0.06 to 0.35N/m	Triangular	Nitride	10
MSNL-10	7 to 125kHz	0.01 to 0.60N/m	Rectangular & Triangular	Silicon	2
MLCT	7 to 125kHz	0.01 to 0.60N/m	Rectangular & Triangular	Nitride	20
MSCT	7 to 125kHz	0.01 to 0.60N/m	Rectangular & Triangular	Nitride	10
SCANASYST-FLUID+	150kHz	0.70N/m	Triangular	Silicon	2
SCANASYST-FLUID	150kHz	0.70N/m	Triangular	Silicon	20
FASTSCAN-C	300kHz	0.80N/m	Triangular	Silicon	5

Imaging Modes in Fluid



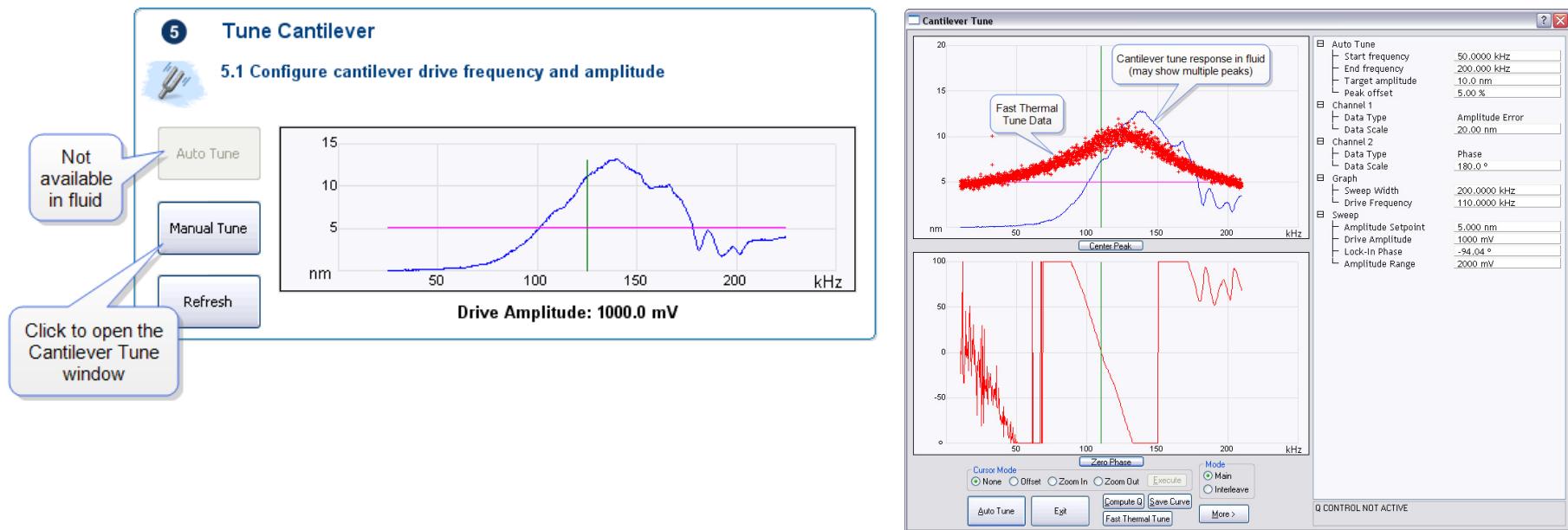
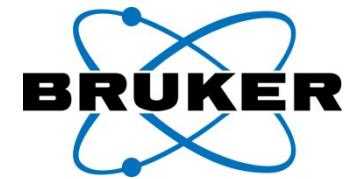
All three primary imaging modes can be operated in fluid:

- Contact mode
 - Easy to operate
 - Shear force may scratch delicate sample
- Tapping mode
 - Shear force is eliminated
 - Requires cantilever tuning to find the right frequency
 - Limited types of cantilever can work: SNL-C, DNP-C, ScanAsyst-Fluid
- ScanAsyst mode
 - Shear force is eliminated
 - No need to tune cantilever
 - Easy to operate



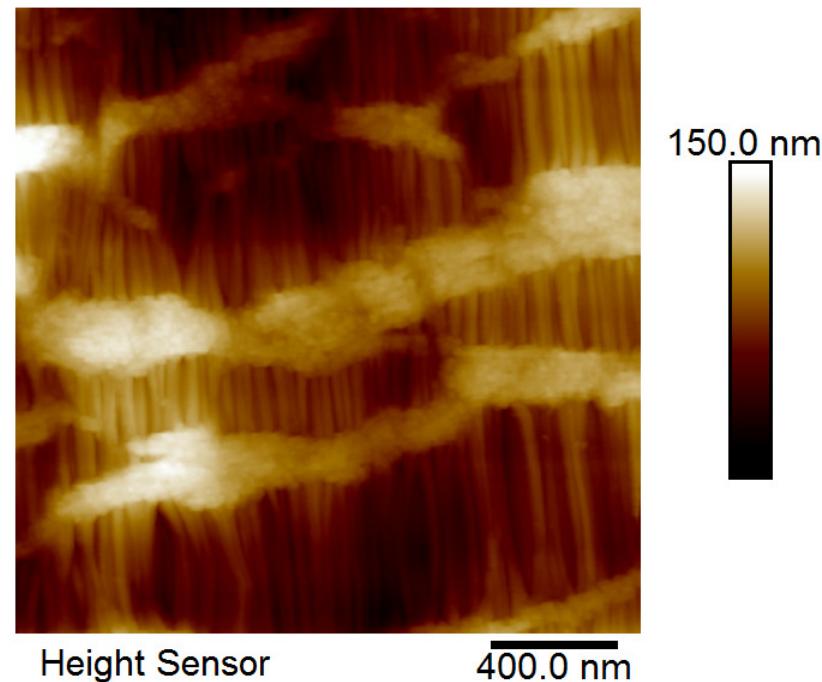
Tapping Mode

Cantilever Tuning in Fluid



- Typically there are multiple peaks in cantilever tuning curve in fluid, and peaks are much broader than in air (more damping in fluid and lower Q)
- Cantilever autotune does not work in fluid
- Use Fast-Thermal tune to determine the cantilever resonance frequency
- Manually offset the drive frequency close to cantilever resonance frequency in fluid

Tapping Mode in Fluid

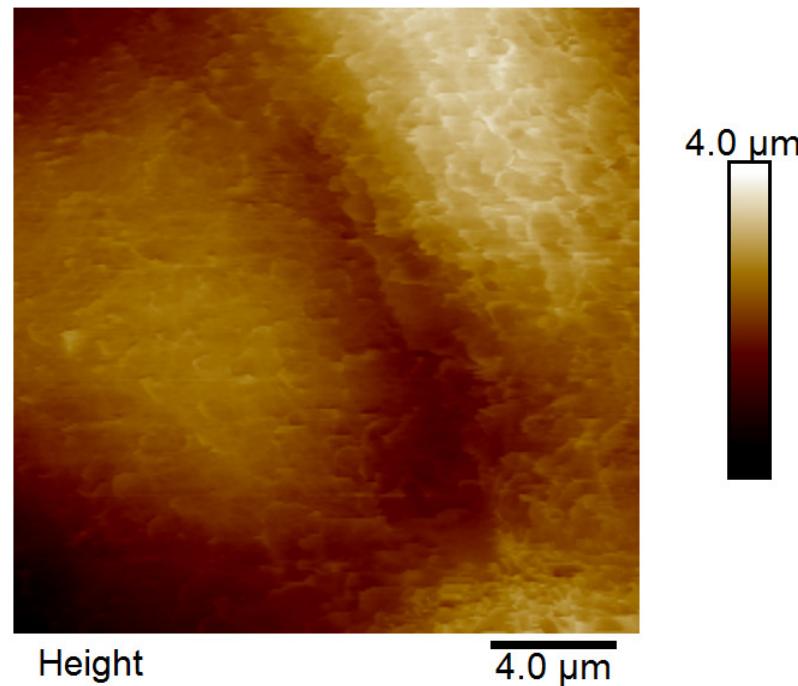
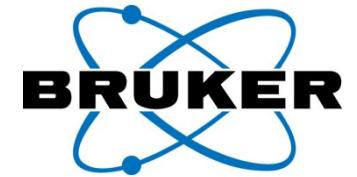


- Dampening effect in fluid is very significant
- Only certain cantilever is works well with Tapping mode in fluid:
SNL-C, DNP-C



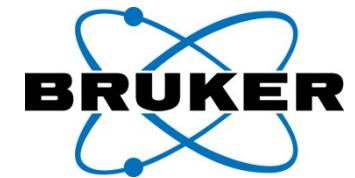
PeakForce Tapping Mode

1. PF-Tapping: Cell

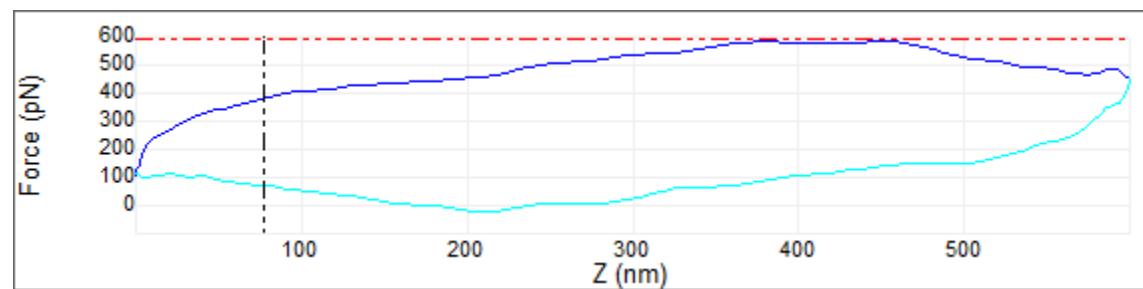
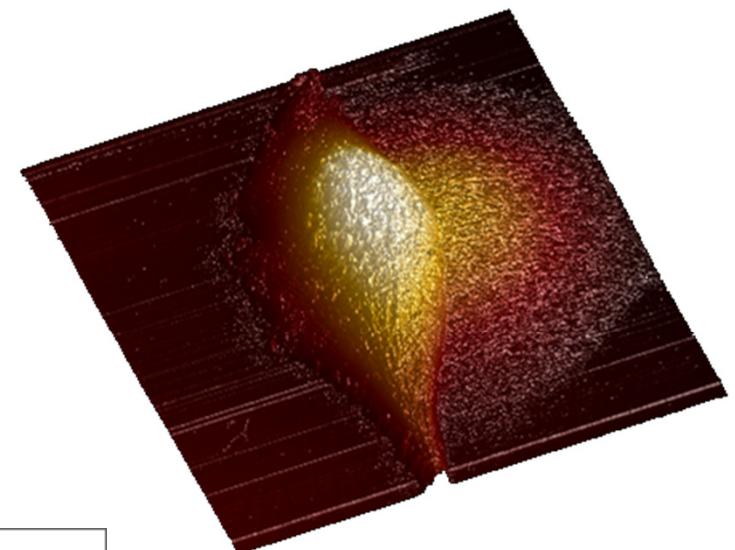
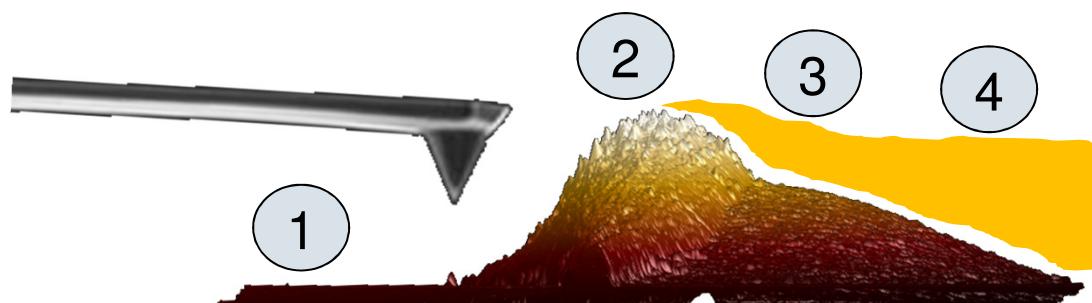


- PeakForce Tapping has direct force control during scanning
- PeakForce Tapping give high resolution topography and mechanical properties mapping same time

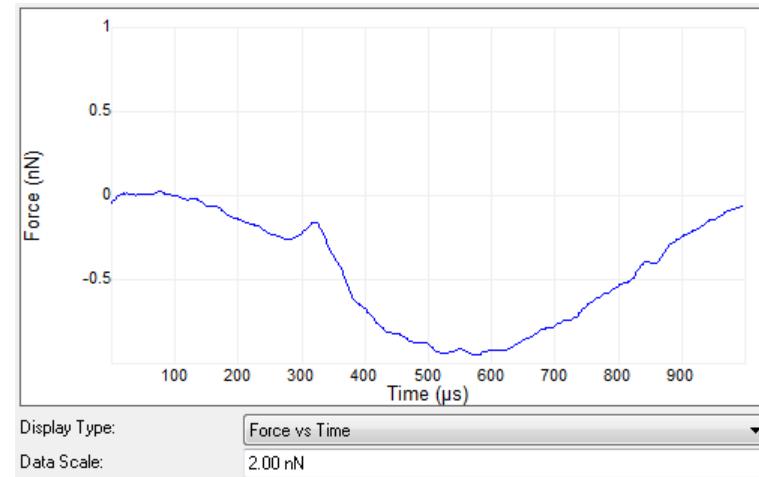
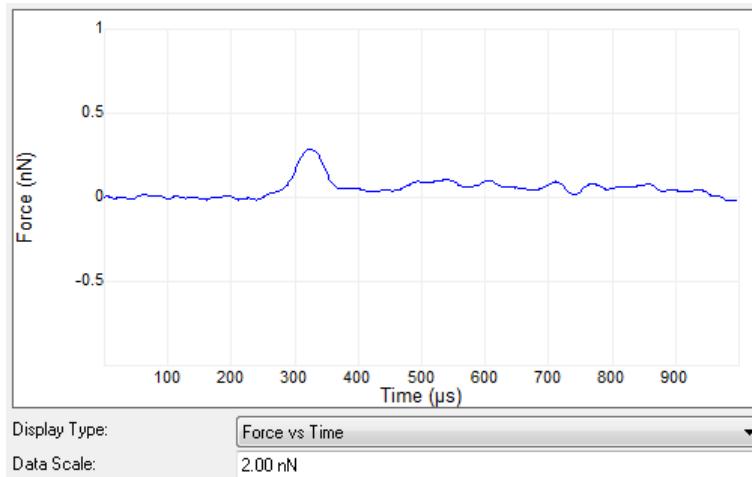
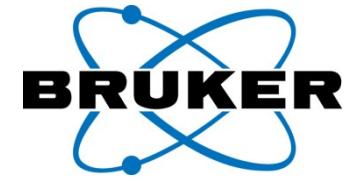
Force Background Changes Dynamically As The Tip Scan Over The Cell



Squeeze layer



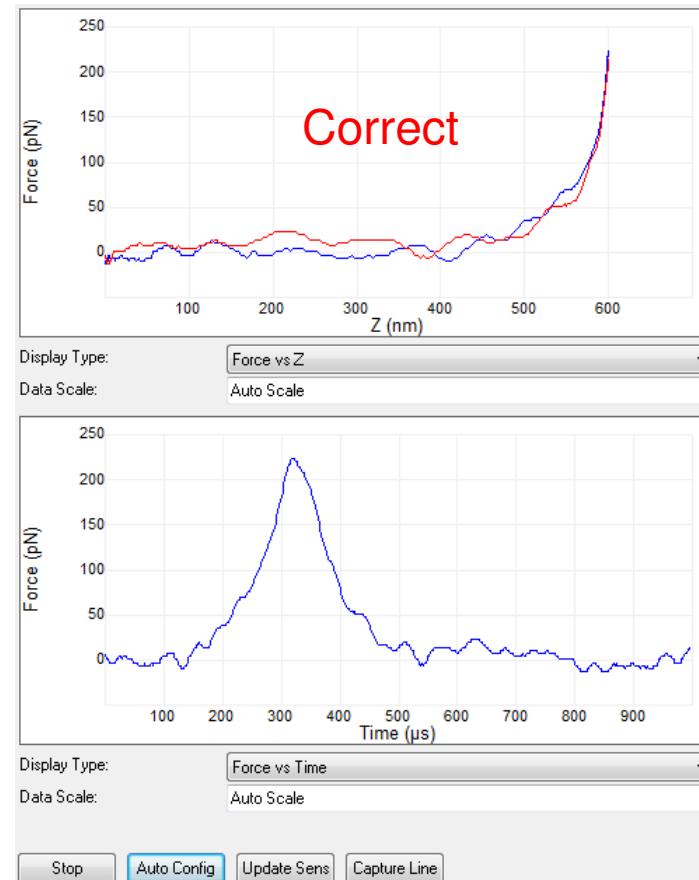
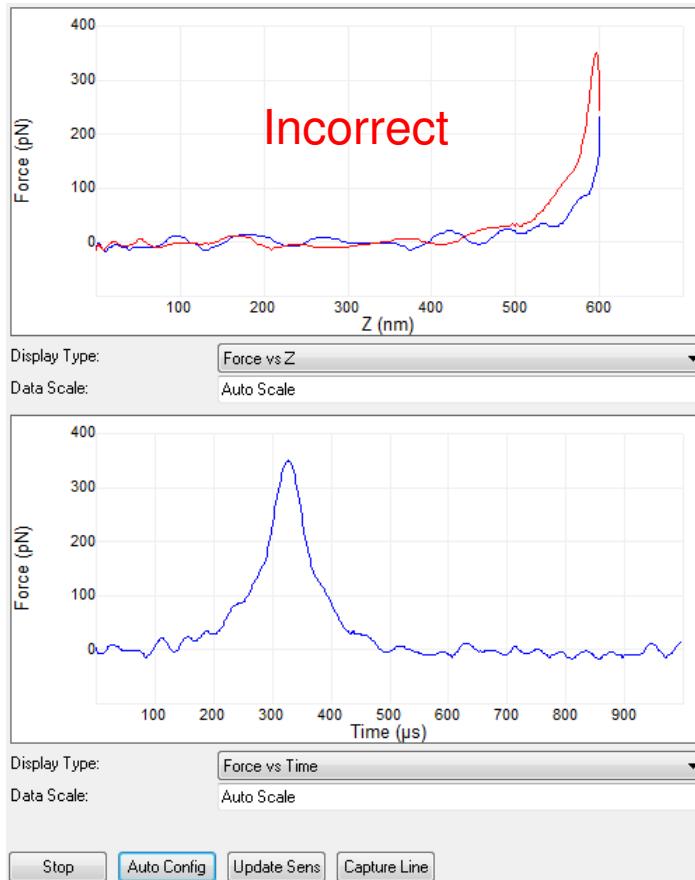
Force Background Changes



Force background changes when same tip scan at different locations

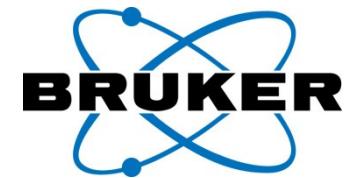
- Cell topography cause the force background to change when tip scan in different location
 - Zoom in scan on a smaller area to minimize background change
 - Use lower PFT frequency, e.g. 250Hz on Catalyst
 - Use taller tip

PeakForce Tapping Sync Distance



- Monitor the real-time force curve to make sure sync distance is correct

PeakForce Tapping Parameters

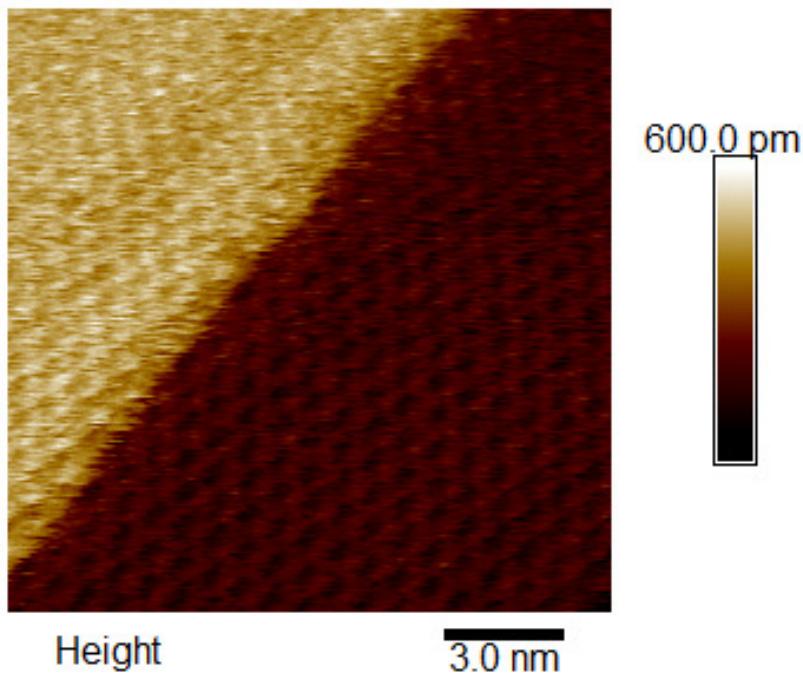
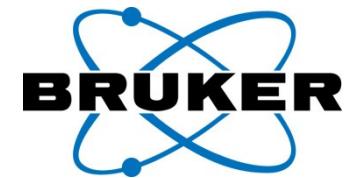


Feedback	
Feedback Gain	10.00
Peak Force Setpoint	300.0 pN
LP Deflection BW	20.00 kHz
ScanAsyst Noise Threshold	0.500 nm
ScanAsyst Auto Config Frames	1
ScanAsyst Auto Control	Off
Peak Force Tapping Control	
Peak Force Amplitude	300 nm
Peak Force Frequency	1 KHz
Lift Height	100 nm
Sync Distance New	162.0
Sync Distance QNM	162.0
Adhesion Algorithm	Threshold Crossing
Max Force Fit Boundary	90 %
Min Force Fit Boundary	30 %
Deformation Force Level	15 %
Cantilever Parameters	
Spring Constant	0.2400 N/m
Tip Radius	20.0 nm
Tip Half Angle	18.0 °
Sample Poisson's Ratio	0.500
PF Mapping Limits	
Limits	
Other	

Recommend Probes:

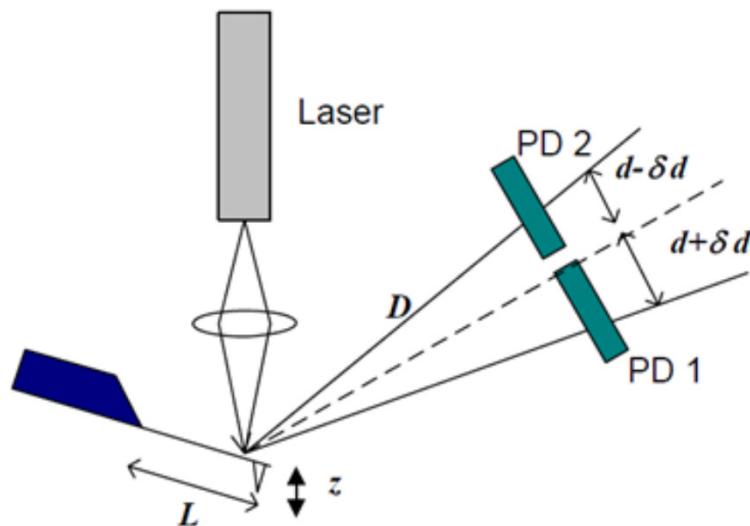
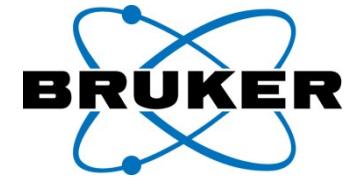
- DNP (short, thin cantilever, $K= 0.24 \text{ N/m}$)
- MLCT (second shortest V-shaped cantilever, $K=0.1\text{N/m}$)
- It is recommended that the longer cantilever on the same side of the substrate be removed before mounting the probe in the cantilever holder
- Fix the petri dish to reduce drum effect
- Use low PFT frequency to reduce hydrodynamic damping
- Adjust the lift height to remove background correct
- Scan smaller area if possible
- Increase the PFT amplitude if needed

2. PF-Tapping: Calcite



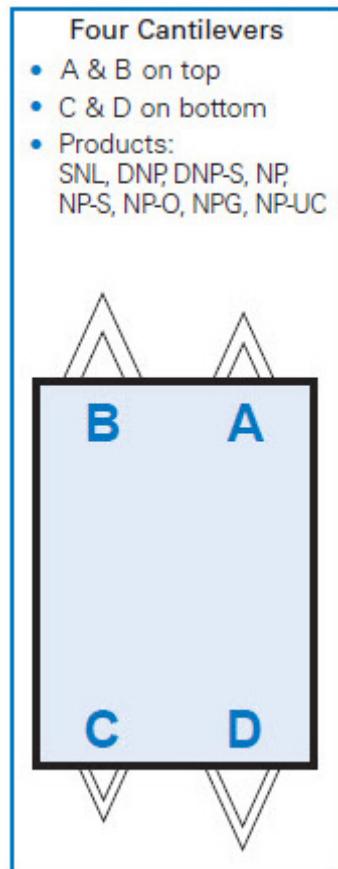
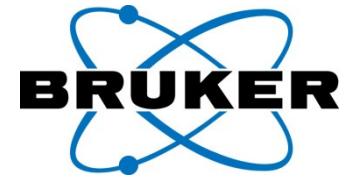
- Scanning Parameters:
 - System: MultiMode with **E** scanner
 - Probe: FastScan-C
 - Scan size: 15nm
 - Scan rate: 4Hz
 - PF Amp: 20nm
 - Engage setpoint: 0.04V
 - PF setpoint: 0.02V
 - Feedback gain: 2
 - Rounding: 0
 - Resolution: 256x256
 - Data channel: Height

AFM Cantilever and Optical Lever



- How sensitive is the cantilever is characterized by Deflection Sensitivity, unit: **nm/V**
- Short cantilever has better deflection sensitivity, **$\sim 1/L$**
- Short and soft cantilever is the best for Atomic Resolution
- Dirty fluid cell can deteriorate the deflection sensitivity
- Same cantilever on different systems has different Deflection Sensitivity

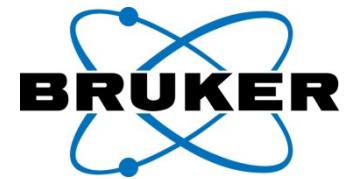
AFM Probe for Atomic Resolution



Shape	Resonant Freq. kHz			Spring Const. N/m			Length μm			Width μm		
	Nom.	Min.	Max.	Nom.	Min.	Max.	Nom.	Min.	Max.	Nom.	Min.	Max.
A Triangular	65	50	80	0.35	0.175	0.7	120	115	125	25	20	30
B Triangular	23	16	28	0.12	0.06	0.24	205	200	210	40	35	45
C Triangular	56	40	75	0.24	0.12	0.48	120	115	125	20	15	25
D Triangular	18	12	24	0.06	0.03	0.12	205	200	210	25	20	30

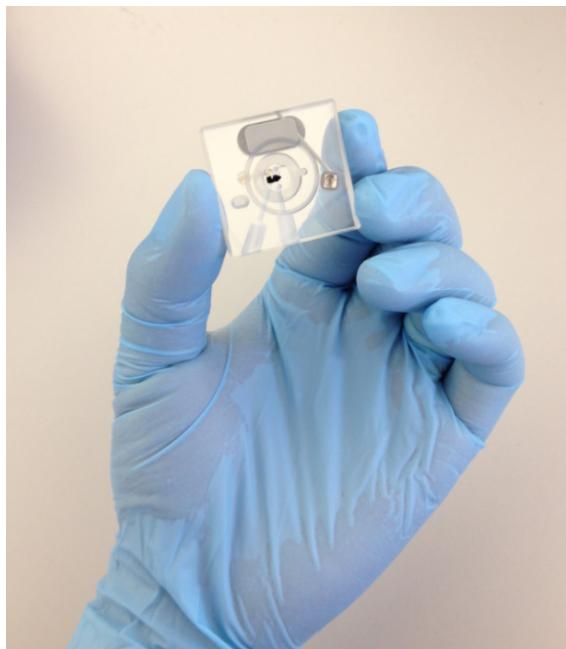
- Smaller and softer cantilever is the best choice
 - Better deflection sensitivity
 - Better force control
- Smaller cantilever has less hydrodynamic dampening when operated in fluid
- Recommended probes:
 - SNL-C, (120um length)
 - ScanAsyst-Fluid+, (70um length)
 - FastScan-C, (40um length)

Contamination Control



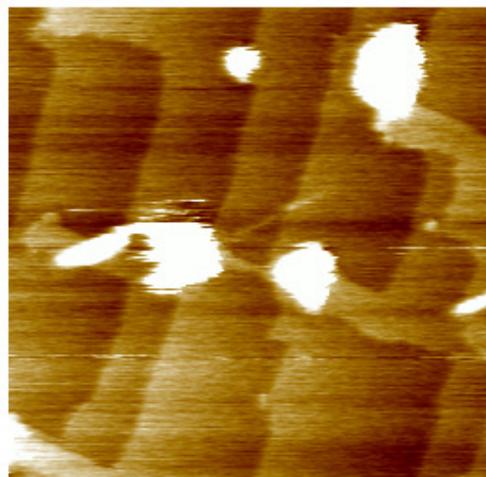
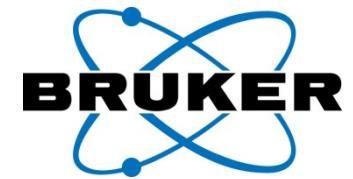
- Clean the tweezers with EtOH/IPA
- Wash both **fluid cell** and **sample** with a baby brush and soap water, and then rinse with DI water
- Dry with clean N₂ or compressed air, **NO Canned Air**
- Use a clean container to transport the fluid cell and sample

Contamination Control

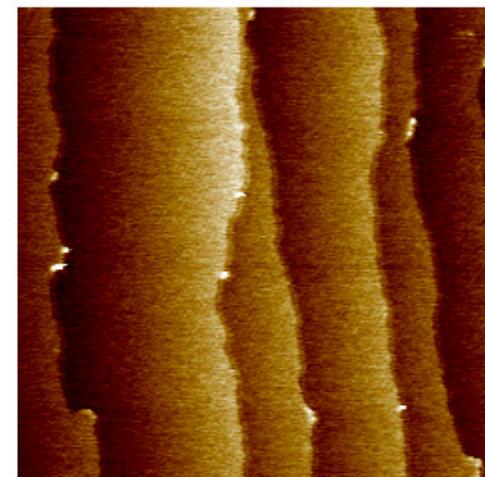


- Wear gloves when handle the parts
- Do Not breathe on the sample or probe holder
- Use **Analytical Grade Pure Water** as imaging media, regular DI water is not clean enough
- Use clean pipette to add water to tip and sample

DI Water vs. Pure Water



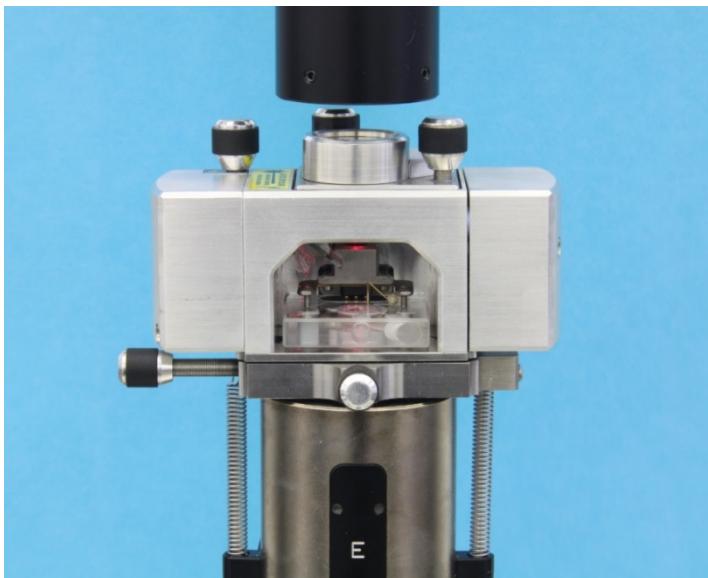
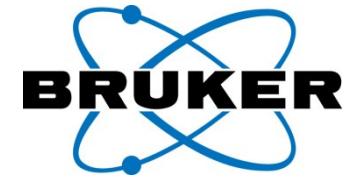
Calcite in regular DI water



Calcite in pure water

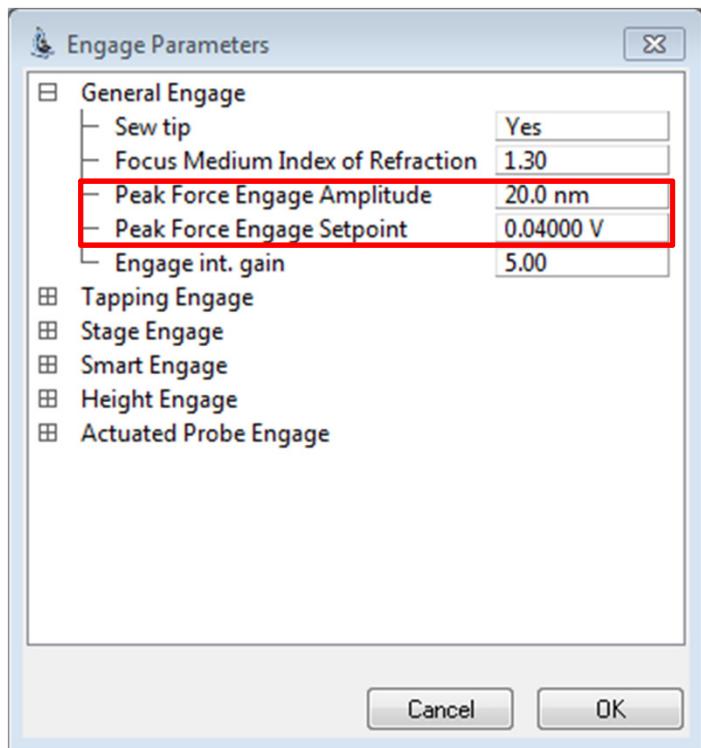
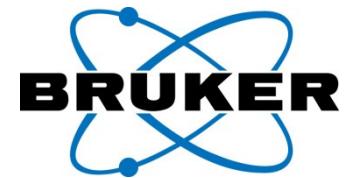
- Pure water is required for atomic resolution in fluid
- Regular DI, even after 0.2um filter, is not clean enough
- The soft particles from DI water deposits on calcite surface, and can easily contaminate the sharp tip

Setup AFM



- Setup the tip sample distance, and laser alignment in air, to minimize the operation once add fluid
- To further clean the sample surface, add a few drops of pure water on sample surface, wait for ~5mins, then remove the water. May repeat this a few times.

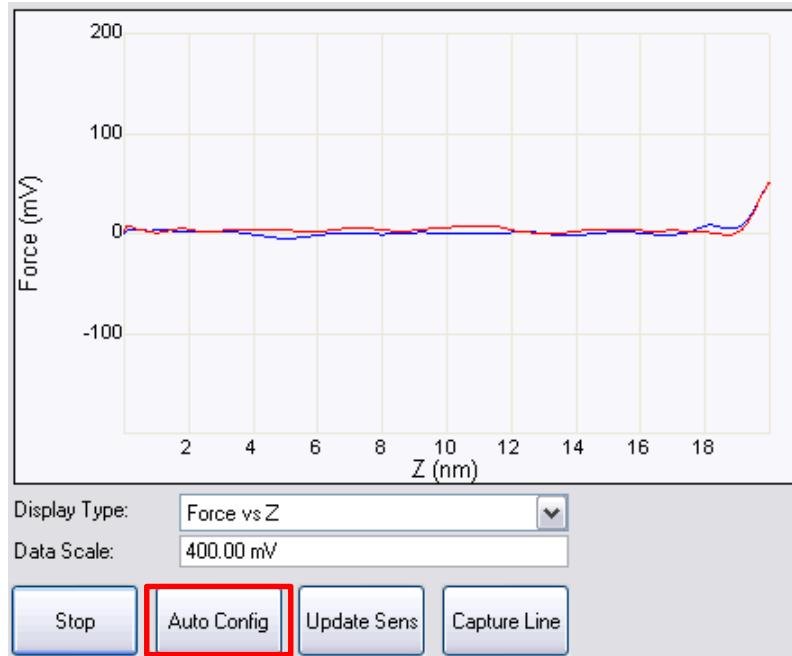
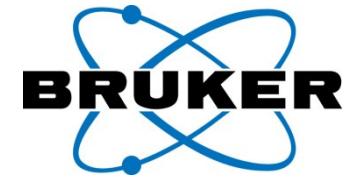
Prepare Tip Engage



Scan	Scan Size	10.0 nm
	Aspect Ratio	1.00
	X Offset	0.000 nm
	Y Offset	0.000 nm
	Scan Angle	0.00 °
	Scan Rate	3.91 Hz
	Samples/Line	256
Feedback	Feedback Gain	2.000
	Peak Force Setpoint	0.02000 V
Peak Force Tapping Control	ScanAsyst Auto Control	Off
	Peak Force Amplitude	20.0 nm
	Peak Force Frequency	2 KHz
	Lift Height	40.0 nm
	Sync Distance New	80.00
	Sync Distance QNM	80.00
	Adhesion Algorithm	Threshold Crossing
Cantilever Parameters	Spring Constant	0.7000 N/m
	Tip Radius	7.00 nm
	Tip Half Angle	18.0 °
PF Mapping Limits		
Limits		

- Set engage amplitude same as scanning amplitude
- Use minimum engage setpoint without causing false engage
- Turn off **ScanAsyst Auto Control**

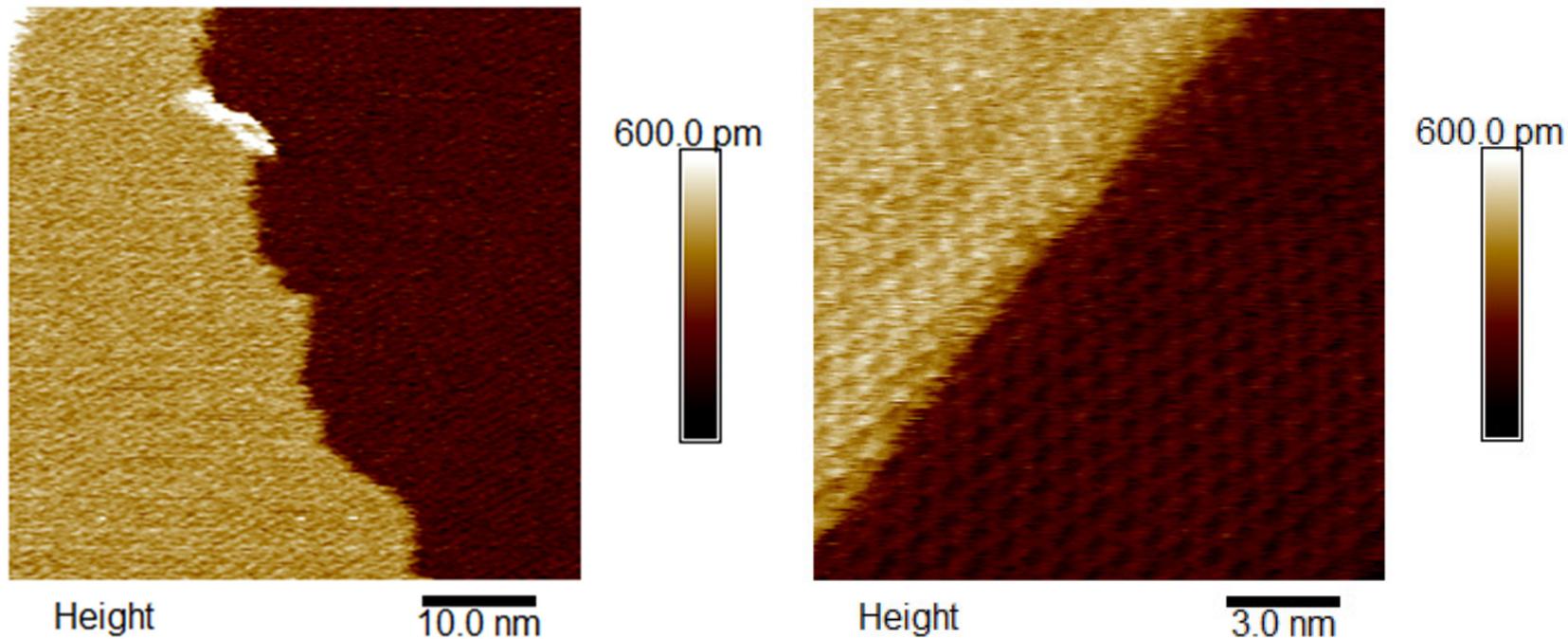
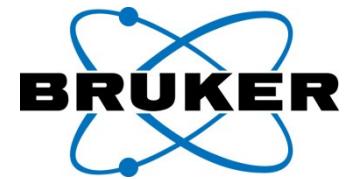
Optimize Scanning Parameters



Scan	Scan Size	10.0 nm
	Aspect Ratio	1.00
	X Offset	0.000 nm
	Y Offset	0.000 nm
	Scan Angle	0.00 °
	Scan Rate	3.91 Hz
	Samples/Line	256
Feedback	Feedback Gain	2.000
	Peak Force Setpoint	0.02000 V
	ScanAsyst Auto Control	Off
Peak Force Tapping Control	Peak Force Amplitude	20.0 nm
	Peak Force Frequency	2 KHz
	Lift Height	40.0 nm
	Sync Distance New	80.00
	Sync Distance QNM	80.00
	Adhesion Algorithm	Threshold Crossing
Cantilever Parameters	Spring Constant	0.7000 N/m
	Tip Radius	7.00 nm
	Tip Half Angle	18.0 °
PF Mapping Limits		
Limits		

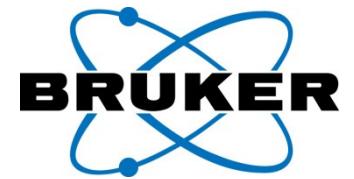
- Use Auto Config or manual type in Sync Distance New to make sure feedback on PeakForce
- Further adjust **PeakForce Setpoint** and **PeakForce Amplitude** if needed

Reveal True Atomic Structure

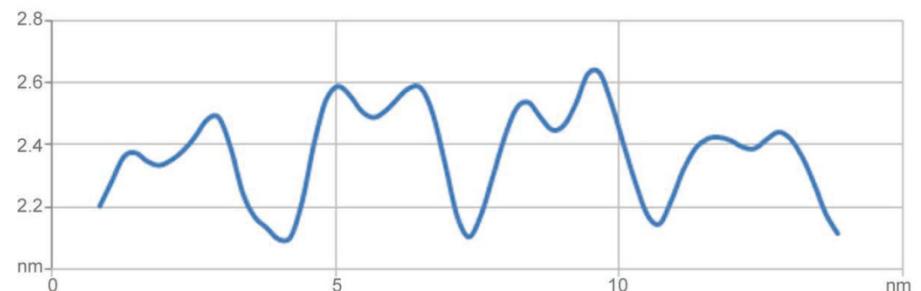
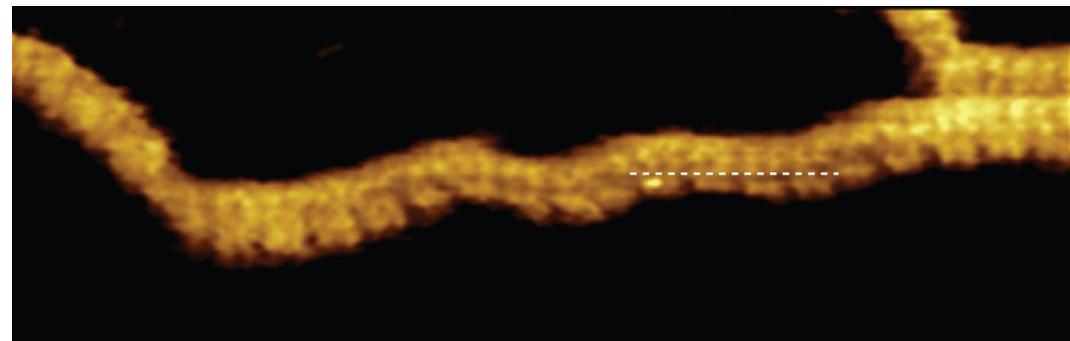
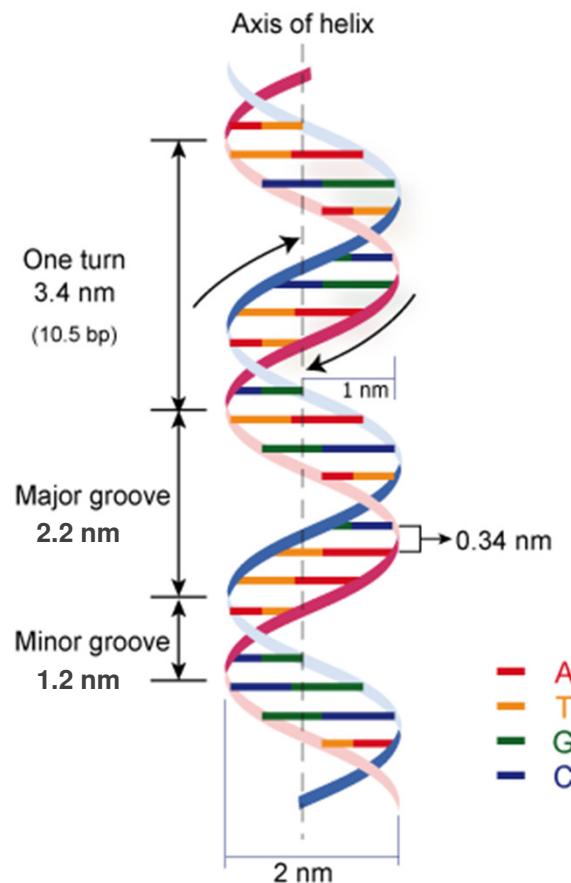


- Constantly monitor the scanning, use offset to avoid scanning over contaminations
- Use XY offsets and different scan size to find a good location for scanning
- Scan at **4Hz for 256x256** or **2Hz for 512x512** for zoom in scans on MultiMode
- Be patient, may still wait some time to reduce the drift

3. PF-Tapping: DNA

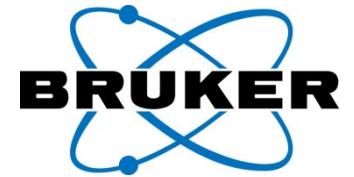


Schematic diagram DNA



- DNA diameter 2nm
- Major groove 2.2nm
- Minor groove 1.2nm

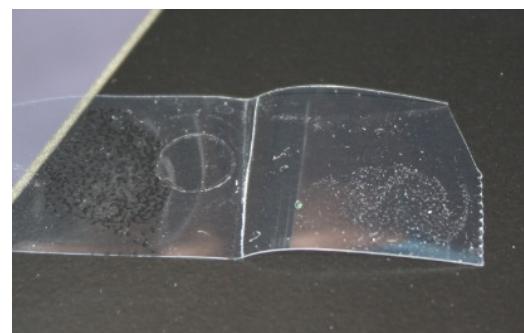
DNA Hi-Res Imaging



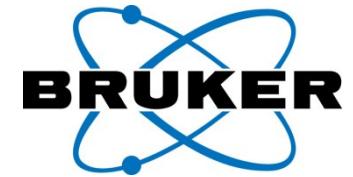
1. If possible, **first setup the tip sample distance in air at the beginning**, this will save the probe engage time during sample imaging
2. Wash Mica and fluid probe holder with soap solution and a baby tooth brush, and rinse them with DI water.



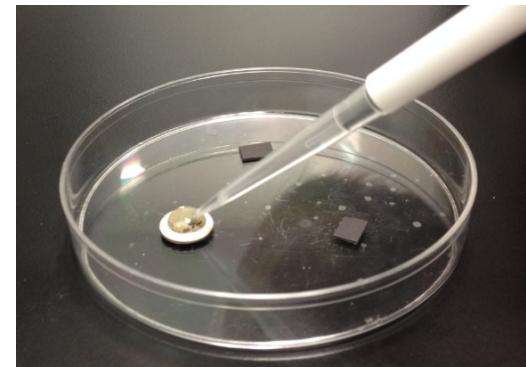
3. Rinse Mica and probe holder with Ethanol wash bottle, and then dry them with N2 gun. And store them in a clean Petri dish, wait for use.
4. Use Scotch tape to make a clean peel off the top layer of Mica surface. Make sure the whole layer is peeled off by checking the peeled off Mica on Scotch tape.



DNA Hi-Res Imaging



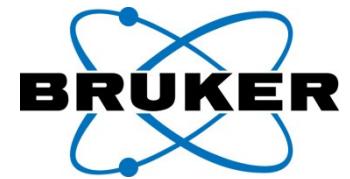
-
5. Filter the NiCl_2 and HEPES buffer solutions with 0.2um syringe filter before use. **DO NOT filter DNA solution**. Apply 20 μL of NiCl_2 solution on the Mica surface, and cover it with Petri dish. Incubate for 2 minutes. Then add 10 μL prepared 2ug/mL solution and 20 μL of HEPES buffer, and mix by pipette.



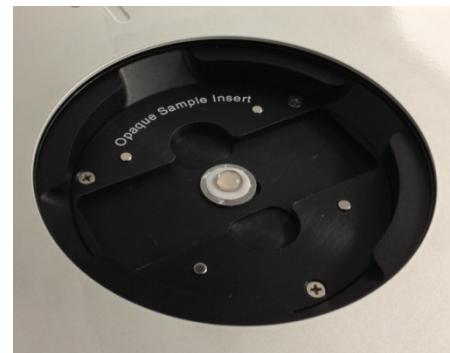
6. Cover the sample in a petri dish to incubate for 15 minutes before imaging. If needed, put a small piece wet paper tower inside petri dish to reduce the solution evaporation.



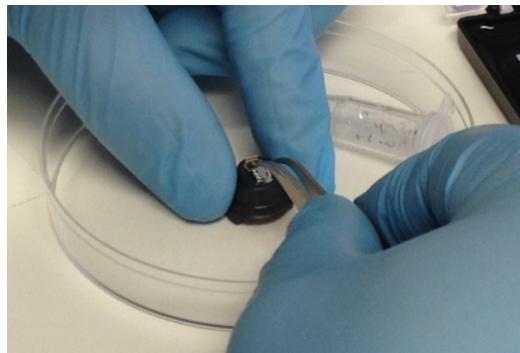
DNA Hi-Res Imaging



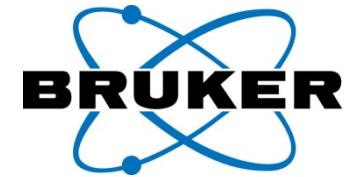
-
7. Mount the sample on the AFM, and wait for imaging. When handle the sample, use tweezers to holder the edge of the sample, and make sure the tweezers does not touch the solution. Otherwise the solution will be contaminated.



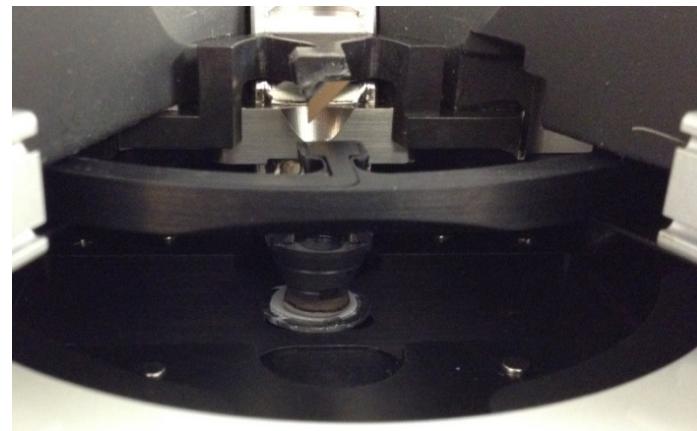
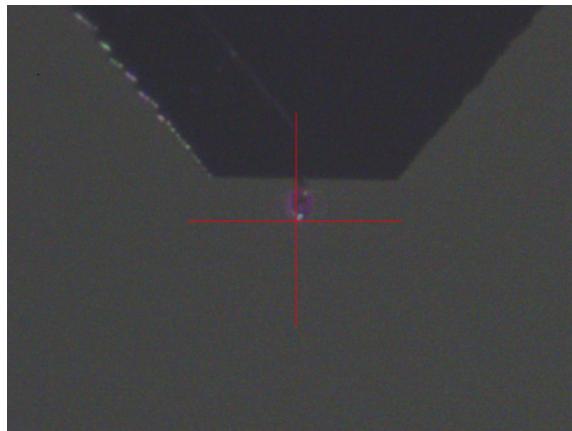
8. Mount a probe on the fluid probe holder and then mount it on to the AFM head.



DNA Hi-Res Imaging



-
9. Align the laser in air first. Add a drop of imaging buffer solution to the probe holder to wet the cantilever, then place the head on the AFM. Make sure the probe is submerged in the solution on sample. Move the stage if needed to position the probe to roughly the center of the sample..

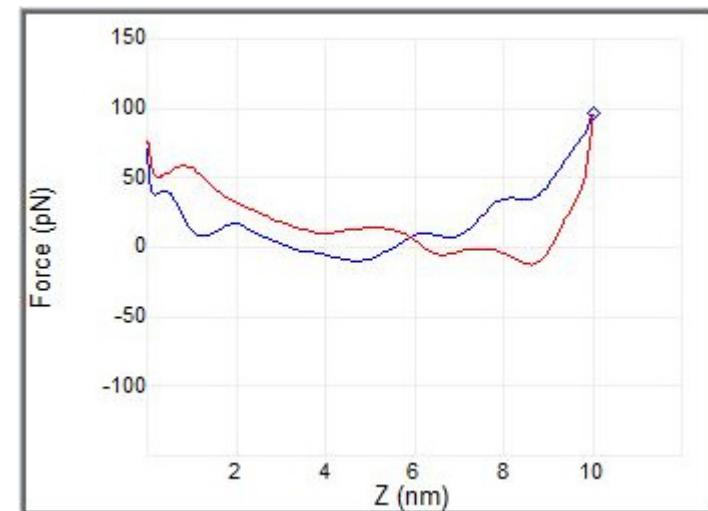
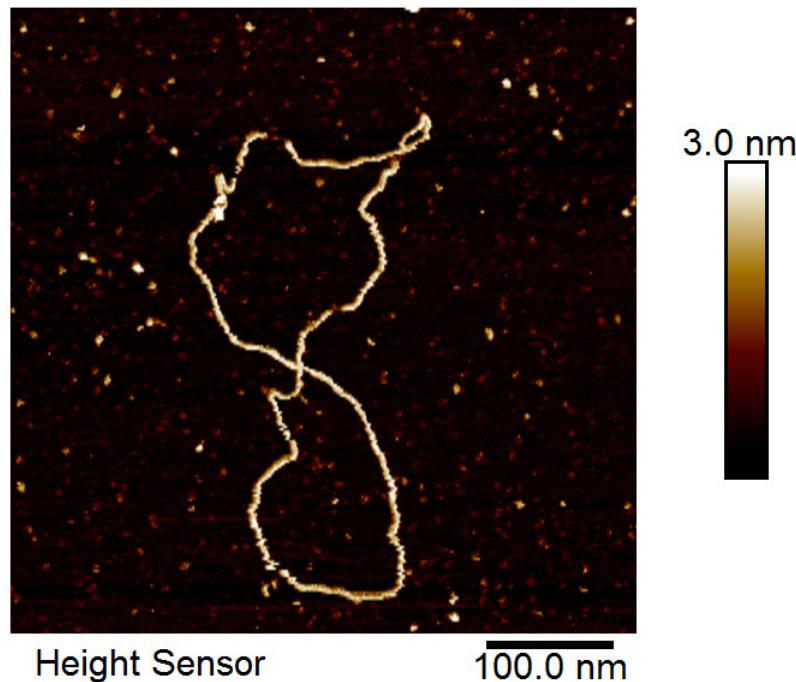


10. Set engage setting to 20nm amp, 0.075V engage setpoint, gain 3; scanning setting size 0nm, ScanAsyst control Off, 20nm Amp, and 2KHz. Then click engage.
11. Once tip is engaged, reduce the engage setpoint to 0.02V-0.05V as needed, because every time if the auto config is triggered, it will use the engage settings to optimize the parameters.
12. Reduce setpoint to 200pN, scan size to 1-2um, scan resolution 128x128, Z limit 1um to search for DNA. Use XY offset if needed when searching for DNA.

DNA Hi-Res Imaging

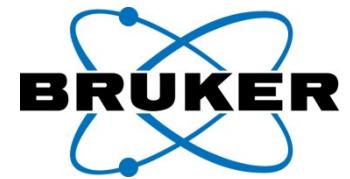


13. Once find DNA, zoom into 500nm on DNA, reduce setpoint to 100pN, PF Amp 10nm, lift height 5nm. The height of DNA should be about 2nm. If the tip is sharp, the width of DNA is about 4-6nm.

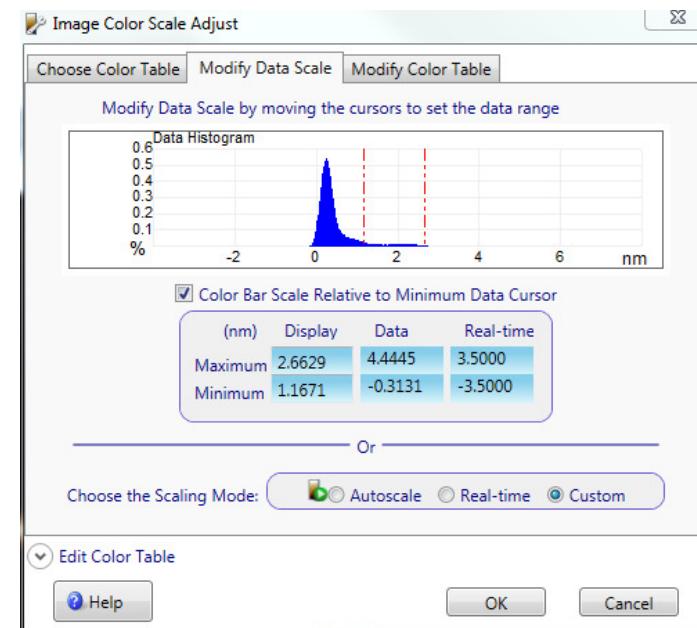
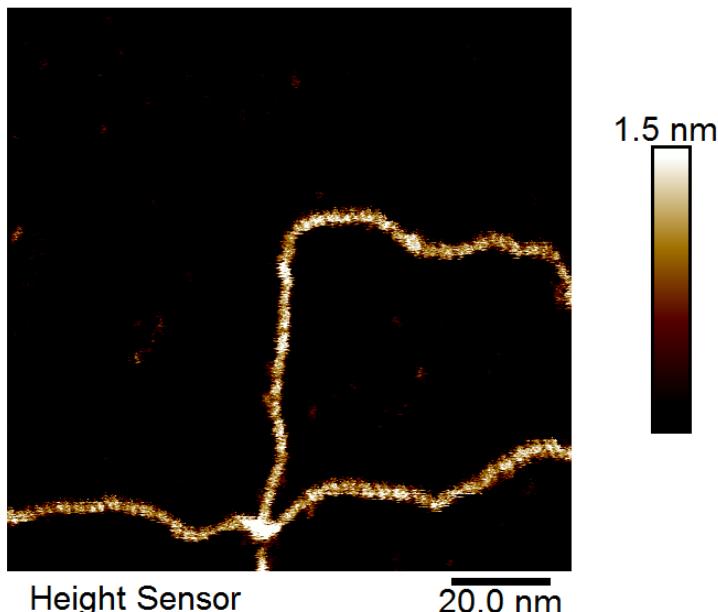


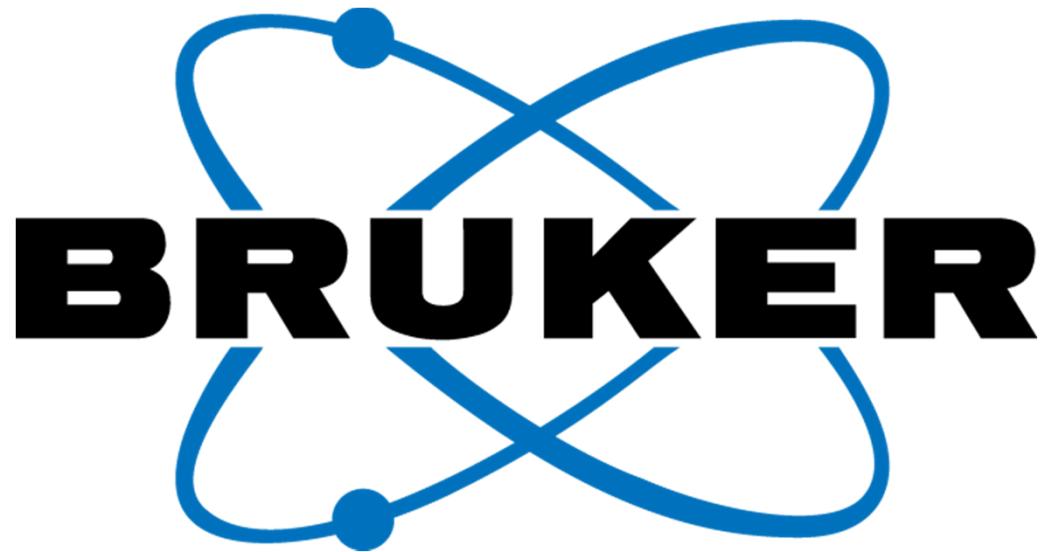
14. Further reduce PF setpoint to 80pN if possible, and scan at 500nm for a few frames to let the drift stabilize.

DNA Hi-Res Imaging



15. Depends on the sample preparation, some part of the DNA may firmly attached to the Mica surface, some part of the same DNA may still floating in the buffer solution. If image shows zigzag pattern when tip scan across DNA, this means this part of the DNA is still floating. Find a clean area where DNA is firmly attached to the Mica surface, and zoom into that area with 200nm scan size.
16. Set scan rate 1.2Hz, 512 resolution, open both trace and retrace topography data channel, and change the scan angle to scan along the DNA. Scan a few frames to let image stabilize, and collect data. If drift is still to high, set scan resolution to 512x256.
17. Image process: first flatten the image, then in color setup set range to ~2.0 nm, offset the range to get best contrast.





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