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Analysis of Chromatin by Scanning Force Microscopy

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1. Introduction

While much is known about the structure of the nucleosome, how a chain of contiguous nucleosomes rearranges to form a fiber is much less understood. Microscopical techniques with nucleosomal resolution (electron microscopy and the newly emerging probe microscopies) have been useful in understanding chromatin fiber structure. The most widely used probe microscopy for biological applications, the scanning force microscope (SFM), also known as the atomic force microscope, is capable of imaging samples under very mild conditions (for recent reviews, *see* **refs.** ***1–3***). Samples are not stained or shadowed or subjected to vacuum and are imaged under ambient room temperature conditions in air or in aqueous buffer.

In the SFM, a fine tip is scanned back and forth over an object placed on an atomically flat surface. The tip is deflected by the sample, and this deflection is recorded to provide a topographic map of the sample on the surface. Presently, SFMs operate reliably in ambient conditions in contact and tapping modes. In the contact mode, a constant force is applied from the tip onto the surface so that the tip constantly maintains contact with the surface. In the tapping mode, the cantilever is oscillated at high frequencies (>200 kHz) above the surface. The effect of the tip interacting with the sample is to reduce the amplitude of this oscillation, and this reduction is used to monitor the topography of the sample. In practice, tapping mode minimizes lateral dragging forces and is much more stable than contact mode in air and in liquid for imaging DNA and DNA-protein complexes. These protocols will only describe tapping-mode SFM.

We describe protocols for imaging chromatin fibers with the scanning force microscope for users with little or no prior experience with the microscope. The actual isolation of chromatin fibers from cells and their further manipulation, such as stripping of linker histone and subsequent reconstitution, have

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been described elsewhere *(4–10)*. In this methods paper, we describe protocols for imaging fixed chromatin fibers on mica in air, unfixed or fixed chromatin fibers on glass in air, and unfixed chromatin fibers on glass in buffer. As these protocols are based on the use of the NanoScope SFM of Digital Instruments (DI), we direct the reader seeking more information to the comprehensive spiral-bound DI Command Reference Manual (1996) and the DI Support Note No. 202 on Tapping Mode in Fluids with the MultiMode SPM *(11)*. Additionally, an excellent Internet directory of other probe microscopy companies, research groups, and journals is maintained by Stephan M. Altmann (www.rzuser.uni-heidelberg.de/~saltmann/spm.html).

2. Materials

1. Triethanolamine (Baker, Phillipsburg, NJ).
2. Electron microscope grade glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA; cat. no. 16120).
3. Clear ruby mica 0.5-cm diameter disks (New York Mica, NY) or mica (Asheville-Schoonmaker Mica Company, Newport News, VA).
4. Paper cutter board (local office supply; 12 in × 12 in).
5. Magnetic steel sample puck (Digital Instruments, Santa Barbara, CA [www.di.com]).
6. Scotch Magic Tape (3M, St. Paul, MN).
7. Coverglass 12-mm circles (Fisher Scientific, Pittsburgh, PA; cat. no. 12-545-80).
8. SPI Miracle Tip and other tweezers (SPI Supplies, West Chester, PA).
9. Nanopure water system (Barnstead, Dubuque, IA).
10. Coors porcelain combustion boat (cat. no. 22825-103) and Thermolyne type 1300 small benchtop furnace (VWR Scientific Products, S. Plainfield, NJ).
11. Fingernail polish (local drug store; color at the discretion of the researcher).
12. NanoProbe silicon tips (Digital Instruments; or Dr. Olaf Wolter GmbH, Wetzlar-Blankenfeld,Germany [ourworld.compuserve.com/homepages/nanosensors]).
13. Bungy cords (local hardware store). Concrete 10 kg block (local lumber yard store).
14. NanoScope IIIa SFM in the Tapping Mode (Digital Instruments).
15. Parafilm (VWR).
16. Gold coated, sharpened Microlevers (Park Scientific, Sunnyvale, CA [www.park.com]). Liquid cell and silicon O-ring (Digital Instruments).

3. Methods

3.1. Chromatin Dialysis (see Note 1 and Subheading 1.)

1. Dialyze chromatin three times, each time 3 h vs 2 L of 5 m*M* triethanolamine-HCl, pH 7.0, 0.1 m*M* EDTA.

3.2. Glutaraldehyde Fixation of Chromatin

1. Carefully break-open an ampoule of 10% glutaraldehyde.
2. To 99 µL of 0.1 mg/mL chromatin (A_{260} = 2.0) in microfuge tube, add 1 µL electron-microscope-grade glutaraldehyde. Upon addition of the heavy, viscous glutaraldehyde, one should see it sink into the chromatin solution.
3. Stir with plastic pipet tip. Place tube on ice and let it sit overnight. Do not shake.

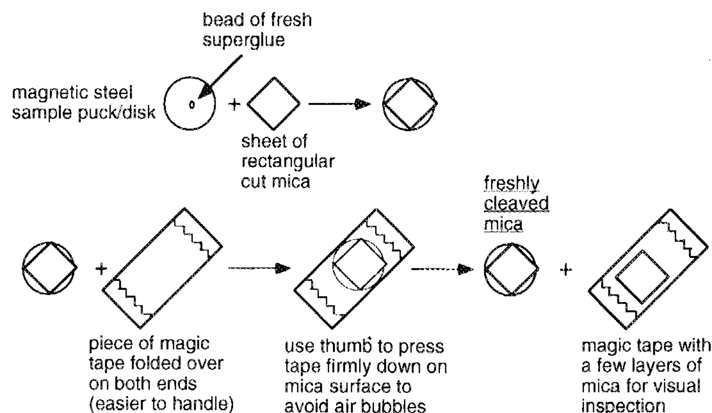


Fig. 1. Schematic of attaching mica with superglue to metal disk and then cleaving the top surface of the mica with Scotch Magic tape.

3.3. Preparation of Freshly Cleaved Mica (Fig. 1)

1. With small bead of superglue, attach mica (*see* **Note 2**) to magnetic steel sample disk/puck.
2. Place strip of $^{3}/_{4}$-in wide Scotch Magic Tape over mica.
3. Rub thumb over the tape to make an even seal over the mica.
4. Remove the tape to peel off a few layers of the mica.
5. Examine the peeled-off layers of mica on the tape, if the peeled-off mica makes a shiny smooth surface, then the remaining top layer of mica on the metal disk is also atomically flat and ready for deposition; if not, repeat the operation.

3.4. Preparation of Cleaned Coverglass Circles (see Note 3)

1. Holding coverglass circle carefully with tweezers, rinse it first with a stream of Nanopure water from a squirt bottle, then with 95% ethanol. Allow ethanol on glass to evaporate.
2. With tweezers, place coverglass into center of Bunsen burner flame for about 0.5 s. It is only necessary for the coverglass to briefly turn orange in the blue Bunsen burner flame.
3. Remove coverglass from flame but hold it a few centimeters from the flame to allow it to slowly cool down (*see* **Note 4**).
4. Rinse again with stream of Nanopure water from squirt bottle. Allow to dry.

3.5. Alternative Preparation of Cleaned Coverglass Circles Using 600°C Benchtop Furnace (see Note 5)

1. Place porcelain combustion boat holding coverglass circles into furnace.
2. Carefully watch the temperature of the furnace rise to 600°C.
3. After 5 min at 600°C, turn furnace off, open furnace door, and let it cool off (approx 30 min) until you can safely remove porcelain combustion boat of coverglasses and rinse them with a stream of Nanopure water from squirt bottle.

3.6. Attachment of Cleaned Coverglass Circles to Magnetic Steel Sample Puck/Disk (see Note 6)

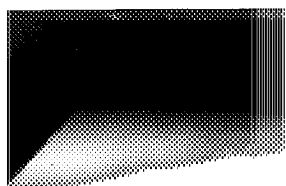
1. Place a small bead of fingernail polish on the metal disk and then place the cleaned coverglass circle onto the disk.
2. Press down carefully (to avoid cracking the glass) with tweezers.

3.7. Deposition of Chromatin onto Freshly Cleaved Mica or Cleaned Coverglass Circles

1. Pipet 20 µL of fixed or unfixed 0.1 mg/mL (A_{260} = 2.0) chromatin onto center of freshly cleaved mica or cleaned coverglass çircle surface. Wait 1 min.
2. Holding metal disk at 45° angle, place ten drops of Nanopure water onto the mica.
3. Without moving your hand holding the metal disk, use your other hand to blot one edge of the mica with No. 4 Whatman filter paper (**Fig. 2A**).
4. Turn on the nitrogen gas and check the flow by directing it towards your upper lip (**Fig. 2B**): the flow should be such that your lip can gently sense it.
5. Flux off (in one direction with respect to the plane of the mica) the remaining visible liquid with the nitrogen gas for up to 15 s (**Fig. 2C**).

3.8. Installation and Adjustment of Silicon-Chip-Holding Cantilever and Tip (see Note 7)

1. Insert the silicon chip by holding cantilever and tip into the chip holder: place the tapping mode chip holder on the corner of the keyboard (*see* **Note 7** and **Fig. 3A**).
2. With your right hand holding the tweezers with the silicon chip, use your left hand to push the chip holder against the keyboard, which releases the spring lever above the groove where the chip is actually mounted (*see* **Note 7** and **Fig. 3A**).
3. After placing the chip into the groove, release your left-hand pressure on the chip holder, and the chip will be secured by the spring lever (*see* **Note 7**).
4. Again, with your left hand, slightly press the back of the chip holder against the keyboard so that the spring holding the chip is released.
5. With the tweezers, carefully nudge the chip over to one side of the groove (*see* **Note 7** and **Fig. 3B**).
6. After the chip is properly positioned, mount the chip holder into the optical head of the microscope.
7. Manual alignment of visible red laser beam (*see* **Note 8**): cut a small piece of stiff paper (Whatman filter paper works nicely) roughly about 0.5 cm × 5 cm and insert it in front of the photodetector (**Fig. 4A**) to catch the red beam of the laser.
8. Use a monocular to determine where the beam is hitting the chip (*see* **Fig. 4B** and **Note 9**).
9. Once you find the beam, adjust it onto the chip, move it down a short incline on the end of the chip onto the cantilever (which looks like a small bee stinger sticking out of the chip), and then move it out to the end of the cantilever using the two knobs obscured by fingers in **Fig. 4B** (*see* **Note 8**).



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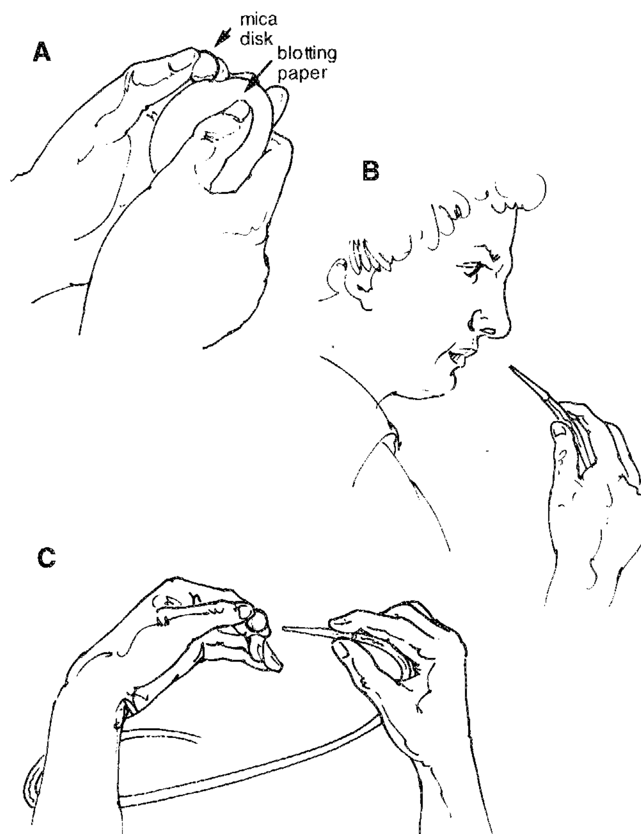


Fig. 2. **(A)** Drawing of the blotting process for imaging in air. The metal disk holding the mica (or coverglass circle) is held edgewise between the thumb and forefinger while the excess liquid is blotted on one edge (so that the flow is in one direction) with Whatman filter paper. **(B)** Blowing of N_2 gas towards ones upper lip to check the flow. One wants the flow to be fast enough that ones upper lip can detect it, but not so fast as to rip through the solution on the surface for samples that are to be imaged in air. **(C)** Removing by nitrogen flux the visible (by eye) liquid for samples to be imaged in air. This step takes at most 15 s.

10. Return to using the short thin piece of paper to adjust the beam on the cantilever on the backside of the tip. Sharply focus the laser beam to a bright red spot on the paper strip. At this point if you move the focus controls in any of three directions, you should immediately lose the spot of the beam on the paper. Remove the paper slip.
11. Adjust the strength of the laser beam signal on the microscope photodetector (*see* **Note 10**): Maximize the inside-perimeter graphical signal of the microscope base oval display by manually adjusting the lever on the backside of the head of the microscope that tilts the mirror back and forth. Often a signal of approx 3.6 can

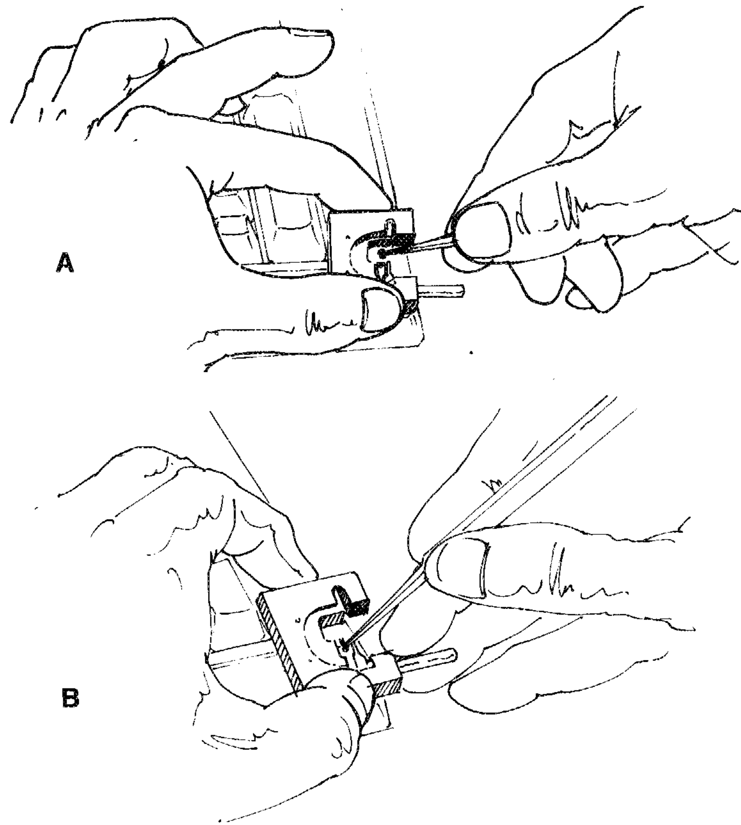
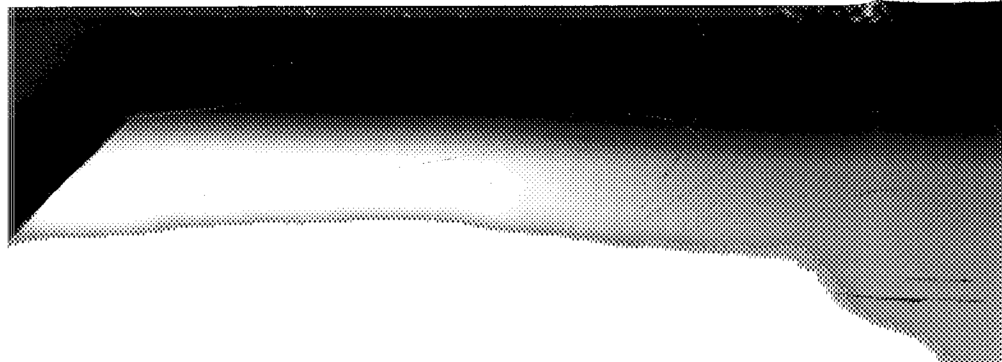
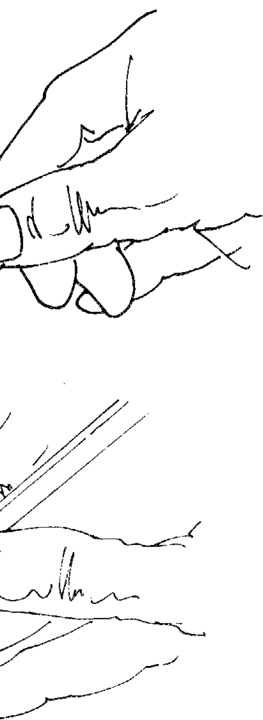


Fig. 3. **(A)** Insertion of a silicon chip holding the cantilever and tip into a tapping mode (in air) chip/cantilever holder. The backside of the holder is juxtapositioned against the slight incline of a computer keyboard to (i) release the spring which holds the actual chip and (ii) to provide an incline for the groove/slot into which the chip slides slightly-down into. **(B)** Tweezer adjustment of the silicon chip in the tapping mode (in air) chip/cantilever holder. Once the chip has been slid into groove **(A)**, then it has to be edged along one side of the groove to create more contact of the chip with the groove and avoid extraneous vibration of the chip. Please note that the angle at which the tweezers are used in **(B)** is different from that in **(A)**. Also notice that here the tweezers are being used not to hold but to nudge the chip over to one side.

be obtained. If you cannot obtain this graphical signal then you have to go back and check the tip, realign the laser beam, reboot the software, replace the tip, and so on.

12. Focus the signal on the center of the four-quadrant photodetector (*see* **Note 11**): rotate the knob (which finely adjusts the photodetector) on the upper left-hand corner on the top of the head of the microscope until the number in the center of the oval display is between –0.2 and 0.2.





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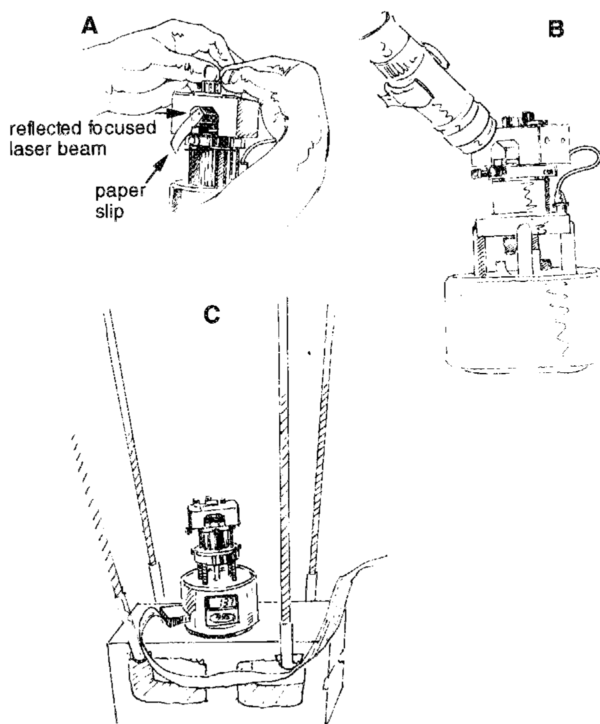

Fig. 4. (**A**) Manual alignment of visible red laser beam. Of the two knobs obscured by fingers, the knob on top right of the head of the microscope adjusts the visible laser beam left and right and the knob on top left back and forth with respect to the sample surface. The point is to adjust the laser beam to the end of the chip, down the beveled face of the chip, onto the bee-sting-look-like cantilever and out to the end of the cantilever on the backside of the tip. At this point the small piece of stiff paper inserted into the head of the microscope will catch the beam of the laser and thus can be used to determine the proper focus of the laser beam using the above mentioned two knobs. (**B**) 45° view of the tip with the monocular stand. This kind of manual setup with the 8×30 power monocular can be used to (i) visualize and adjust the laser beam onto the end of the cantilever and (ii) adjust the distance from the tip to the sample surface before placing the microscope on the vibration-free system and starting engagement. (**C**) Placement of the SFM on the vibration-free 10-kg concrete block attached to the ceiling by bungy cords.

13. Manually move the tip toward the surface: place the metal disk holding the substrate (mica or glass disk) with the sample onto the piezo scanner.
14. Place the head of the microscope on top of the piezo scanner and, if need be, secure the two attaching springs of the head to the base of the microscope.
15. Using the monocular to observe the location of the tip with respect to the surface (**Fig. 4B**), manually lower the tip toward the surface by rotating the knobs that manually raise or lower the head to the piezo scanner (*see* **Notes 12** and **13**).

Scan Controls	
Scan size:	0.00 nm
X offset:	0.00 nm
Y offset:	0.00 nm
Scan angle:	0.00deg
Scan rate:	1.97 Hz
Number of samples:	512
Slow scan axis:	Enabled
Z limit:	440 V

Channel 1	
Datatype:	Height
Z range:	15 nm
Line direction:	Trace
Scan line:	
Real time Planefit:	Line
Offline Planefit:	None
Highpass filter:	Off
Lowpass filter:	Off

Feedback Controls	
Integral gain:	0.50
Proportional gain:	1.00
Look ahead gain:	0.00
Setpoint:	0.00 V
Drive frequency:	300.000 kHz
Drive amplitude:	100 mV
Analog 2:	0.00

Interleave Controls	
Interleave mode:	Disabled
Integral gain:	
Proportional gain:	
Look ahead gain:	
Setpoing:	
Drive frequency:	
Drive amplitude:	
Analog 2:	
Interleave scan:	
Lift start height:	
Lift scan height:	

Other Controls	
Units:	Metric
Color table:	9
AFM Mode:	Tapping
Input attenuation:	1x
Engage Setpoint:	1.0
Min. Engage gain:	
Z modulation:	Disable

Fig. 5. Initial settings for major software controls.

16. Check that the head assembly is on a level plane with respect to the metal disk on the piezo scanner (*see* **Notes 12** and **14**).
17. Place the microscope on a vibration-free platform. In this example the microscope is placed on a 10 kg concrete block (**Fig. 4C**) hanging from the ceiling by bungy cords (*see* **Note 15**).

3.9. Software Controls and Imaging in Air

1. Run the microscope with the software controls (*see* **Fig. 5** for initial settings): place the Scan size, X offset, Y offset, and Scan angle at zero. Set Scan rate to about 2 Hz, Number of Samples to 512, Slow Scan Axis is Enabled, and the Z axis to 440 V.
2. In the next column of numbers, set the Integral gain to 0.5 and the Proportional gain to 1. (The Look ahead gain can be left at zero). Initially set the Setpoint to zero.
3. Set Datatype to Height, Z range to 15 nm, Line direction to either Trace or Retrace.
4. For the other controls, set the Color table at a setting that gives a reasonable (researcher's discretion) range of shades of color. Offline planefit is set to None, Z modulation is Disable, Highpass and Lowpass filters are Off, and Analog 1 is zero.

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or. Offline planefit is set to None, Z
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A

Cantilever Tune

Auto Tune Controls		Target amplitude:	1.00 V
Start frequency:	0.00000 kHz	Auto Tune	
End frequency:	500.000 kHz		
Graph Controls		**Main Controls**	
Sweep width:	500.000 kHz	Drive frequency:	300.000 kHz
Sweep sample count:	512	Setpoint:	0.00 V
Sweep graph range:	10.0 nm	Drive amplitude:	100 mV
Units:	Metric	Input attenuation:	1x
		Integral gain:	0.50
		Proportional gain:	1.00

Quit | Motor | Spring | Interleave Controls

B

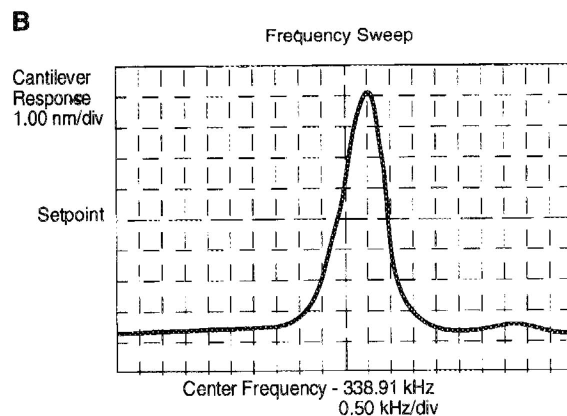


Fig. 6. **(A)** Initial settings for software controls before cantilever tune process. **(B)** Example peak in graph of amplitude of vibration of the cantilever versus frequency of vibration. The graph is centered on an inflection point of this peak before starting engagement.

5. In the Interleave controls, the Interleave scan is Off.
6. Check the cantilever vibration frequency (*see* **Note 16**): select Cantilever Tune from the View menu (*see* **Fig. 6A** for Cantilever Tune startup settings).
7. With the mouse set the Z scan size, Z scan rate, and Graph range to the highest number possible.
8. In the graph of amplitude of the cantilever vibration vs frequency (**Fig. 6B**), find the (usually lone) peak of amplitude. Expand the X-axis from 0 to 500 kHz, locate the peak, and then use the Zoom in and Offset controls to decrease the Sweep width of the X-axis to 10 kHz, and center the peak such that the center vertical line of the plot intersects an inflection point on the left-hand side of the peak (**Fig. 6B**). Some NanoScope users use the inflection point on the right-hand side of the peak.
9. Check that the Setpoint on the microscope base is between 0.5 and 2.0 V (*see* **Note 17**).
10. Engagement of tip onto the sample surface: select the Engage command (under Motor Command window) and the microscope automatically approaches the tip to the sample surface. Once the microscope detects that the tip has engaged the surface, a beginning image will be displayed on the second monitor.

11. Adjust the Scan size to 2000 nm to create a 2000 nm × 2000 nm image.
12. If the image is fuzzy, carefully adjust the Setpoint until the force of the tip on the surface is sufficient enough to maintain stable imaging conditions. In tapping mode, this operation involves *decreasing* the Setpoint setting. We have routinely used a setting between 1 and 2 V for the Setpoint during imaging (*see* **Note 18**).
13. Inspect the gains. It may also be necessary to carefully increase the Integral and Proportional gains to improve the sensitivity of the piezo response to changes in heights of the chromatin fibers on the surface. If the gains are increased too high, it is easy to observe repeated noise in the image (for trouble-shooting *see* **Notes 18–22**).

3.10. Imaging in Liquid (see Note 23)

1. Cover the scanner with a thin film of parafilm to protect the piezo crystal from being destroyed by a short-circuit caused by the liquids used for imaging.
2. Place a Park Scientific Microlever tip in the liquid cell (**Fig. 7A**).
3. As the liquid cell is thicker than the dry cantilever holder, raise the microscope head sufficiently before placing the liquid cell inside it.
4. Focus the laser beam on a cantilever that has a spring constant of 0.5 N/m. You can use the monocular to look through the top of the microscope head to visualize the laser beam and to place it on the end of the chip (**Fig. 7B**).
5. After you have focused the laser beam onto the cantilever (using the piece of paper as described in **Subheading 3.8.**), then you can adjust the distance between the tip and surface: using the monocular, look directly between the liquid cell and the surface (**Fig. 7C**), and you should see a group of spots of reflections (**Fig. 8**) that indicate how close the tip is to the surface. The top and bottom spots are reflections of light from the laser on the tip and the surface, respectively (**Fig. 8**). Use the screws below the scanner to manually move the tip to the surface.
6. Deposit 20 µL of sample onto the cleaned coverglass.
7. Place the silicone O-ring on the surface and place the head of the microscope on top of the O-ring.
8. Now that the laser beam is being reflected through solution, the change resulting from the index of refraction requires that the beam be refocused slightly by tilting the mirror to regain the signal to the photodiode.
9. For tapping mode in liquid, set the Integral gain at 4.0 and the Proportional gain at 1.0. Set the Drive amplitude at 1 or 2 V (which is about 10X this setting for tapping in air).
10. In the Cantilever tune, look for a frequency peak usually approx 7 kHz before starting the engagement of the tip with the surface. Example images of chromatin fibers in buffer are shown in **Fig. 9** (*see* **Note 24**).

3.11. Image Analysis of Chromatin Fibers (see Note 25)

1. Determine (X, Y, Z) coordinates of each nucleosome in a fiber (*see* **Note 25**).
2. Calculate center-to-center distances and angles (*see* **Note 25**).
3. Plot histograms of Frequency vs Height, Center-to-center distance, or Angle.
4. Keep a record of analyzed images by printing a copy (*see* **Note 26**).

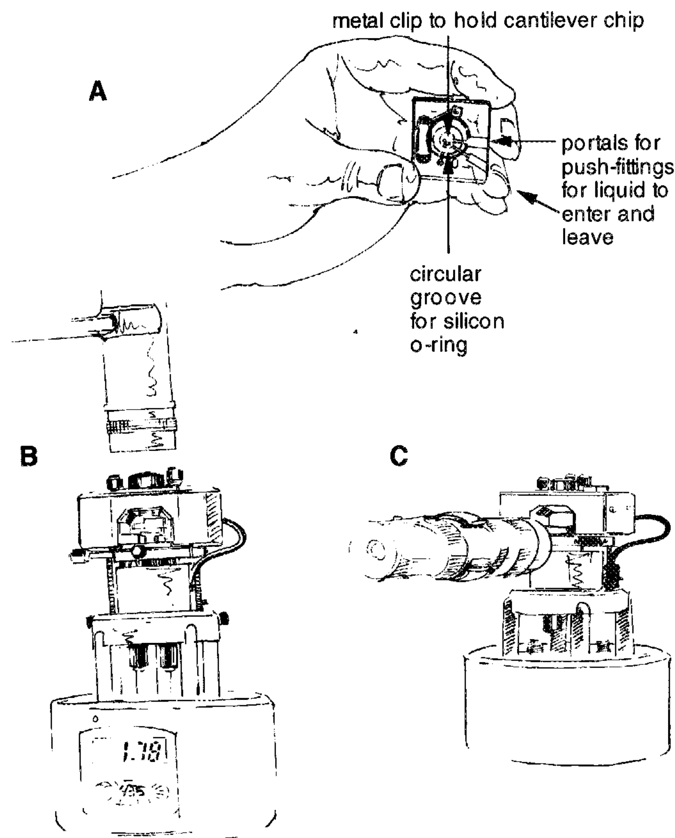


Fig. 7. **(A)** Carefully handling the glass tapping mode liquid cell from Digital Instruments. The word 'cell' is a slight misnomer as the imaging is done on the surface of the substrate. The liquid cell holds the cantilever chip, provides portals to allow liquid to be flushed through a chamber between the liquid cell and the substrate surface. This chamber is sealed with a silicone O-ring (not shown). **(B)** Downward view of the tip with the monocular stand. This kind of manual setup with the monocular can be used to visualize and adjust the laser beam onto the end of the cantilever for use with the liquid cell. **(C)** Horizontal view of the tip and the surface with the monocular stand. This kind of manual setup with the monocular can be used to manually bring the tip close to the surface without crashing it prior to engagement.

4. Notes

1. Dialysis removes small organic contaminants and extraneous salts that may interfere with either the deposition or the imaging steps.
2. If the mica is cut as a rectangle, it is much easier to peel than as a circle. The easiest way to cut the mica is with a paper cutter board.

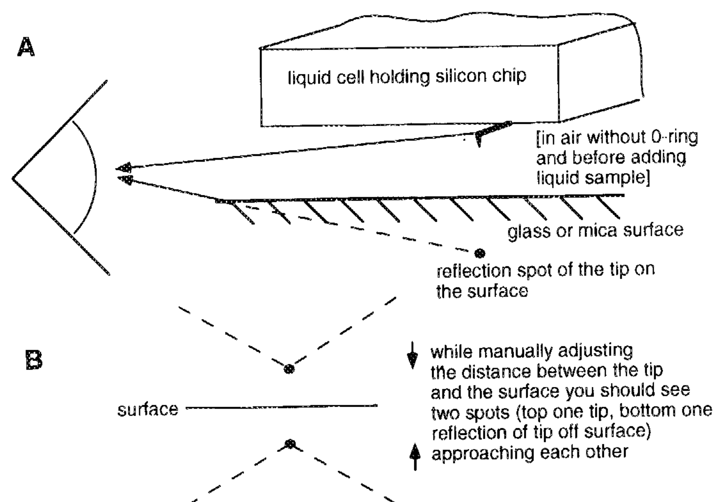
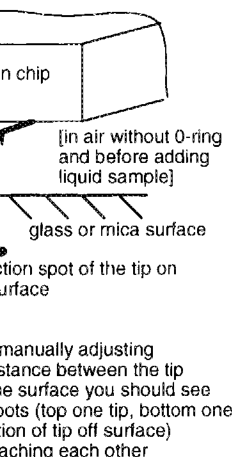


Fig. 8. **(A)** Schematic of the view when looking through the monocular between the liquid cell and the surface before inserting the liquid sample and the silicone O-ring. One should see the spot on the tip and the reflection spot of the tip on the surface. Inner groves for the O-ring which is placed around the tip to seal the chamber are not shown. **(B)** Depiction of the red spots of reflection that one sees through the monocular as one adjusts the distance between the tip and the surface before adding the liquid sample and the silicone O-ring.

3. Coverglasses from the manufacturer have a thin film of oil that must be flamed or baked off and then rinsed before use.
4. This precaution will prevent the glass from cracking as a result of a fast cooling. Typically, however, 50% of the coverglasses are lost to cracking during this early manipulation step.
5. This protocol requires a 600°C bench top furnace. Use proper equipment (i.e., asbestos-like gloves) and care to avoid personal injury.
6. Coverglass circles can be attached to the metal disks either by the use of doublestick tape or by the use of fingernail polish.
7. As there is a push-button on the backside of the chip holder which releases the spring -lever holding the chip, insertion of the chip is easier to perform at a slight incline, such as on a corner of a computer keyboard as indicated in **Fig. 3**. The chip then slides in at an incline and does not fall out. To avoid unnecessary vibration, it works best for one of the long thin edges of the chip to be adjacent to one edge of the groove; thus, once the chip is in place, it is necessary to manually adjust it (**Fig. 3B**) with the tweezers over to one side in its slot or groove, because this groove is slightly wider than the chip.
8. The point of this step is to adjust the laser beam to bounce off the cantilever on the backside of the tip, reflect onto the mirror and then reflect onto the four-



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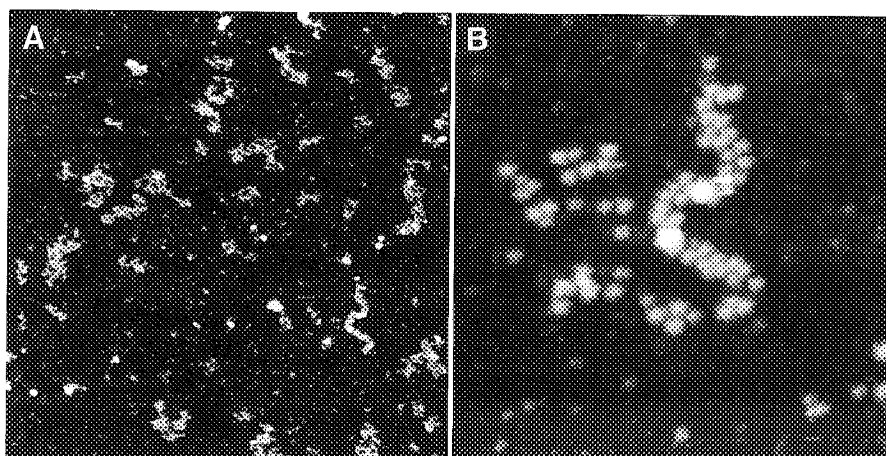


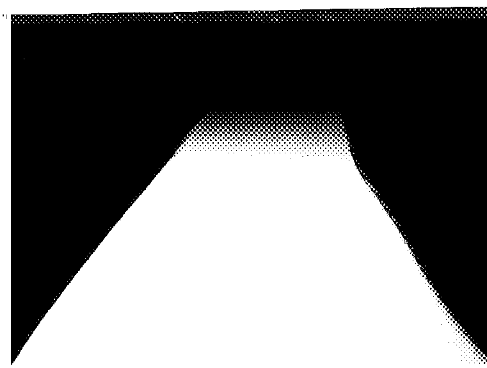
Fig. 9. SFM image from **ref. *10*** of unfixed chicken erythrocyte chromatin fibers imaged in 5 m*M* triethanolamine, pH 7.0, 1 mM sodium butyrate, 0.1 m*M* EDTA on coverglass. Image sizes are **(A)** 1000 nm × 1000 nm and **(B)** 500 nm × 500 nm. Heights are encoded in shades of gray with low regions depicted in dark gray and higher regions in lighter shades in a scale of 0–15 nm.

quadrant photodetector of the SFM. There are two knobs on the top center of the head of the microscope (see where fingers of both hands are in **Fig. 4A**) that adjust the beam left and right and forward and backward.

9. Digital Instruments supplies an 8 × 30 power monocular with a Bogen stand for viewing tips. However, a high-power clear-focus zoom monocular (1–6 zoom) with stand can be separately and additionally obtained from Nissho Optical Co. Ltd., (Japan, model no. ZMM1) through Labtek (Scotts Valley, CA).
10. On the base of the microscope are two liquid crystal displays. The lower oval display indicates
    a. Graphically the strength of the detected laser beam signal in a range from 0–12.
    b. Numerically how well the beam is centered on the four-quadrant photodetector.
11. Once a maximum graphical signal is obtained, one needs to adjust how well the signal is focused on the center of the four-quadrant photodetector. This digitally displayed number is the difference between the intensities of the signals on opposing sides of the photodetector.
12. The knobs that manually raise or lower the head to the piezo scanner are located above the base of the microscope and directly under the central scanner. In concert with a third motor-driven screw, these three knobs/screws raise or lower the tip relative to the mica surface. Digital Instruments now also makes scanners that are completely motor driven without manual screws.
13. Through the monocular the body of the cantilever, the spot of the laser beam reflecting on the end of the cantilever, and the spot of the laser beam that spills

over the end of the cantilever onto the surface can be seen. A rule of thumb about how close to bring the tip to the surface is to lower the tip until these two laser spots are as close vertically as the horizontal length of the cantilever.

14. This leveling is accomplished by running the motor-driven screw up or down with the switch on the upper right side of the microscope base as well as manually adjusting the two forward screws below the scanner.
15. Some bungy cords do not work well. One has to experiment with different bungy cords or double them up until a sufficient damping of high-frequency vibrations is obtained when using a 10-kg concrete block. Alternatively, one can purchase a tripod/bungy-cord/flat-concrete-block assembly from Digital Instruments. Another possibility are the soundproof imaging cabinets supplied with bungy cords and a heavy base from Molecular Imaging (Phoenix, AZ [www.molec.com]). Once the bungy cords have lost most of their elasticity, they need to be replaced, usually once every year or two.
16. For tapping mode force microscopy, it is necessary to find the correct frequency, generally in the range of 250–350 kHz, to vibrate the cantilever in air.
17. When leaving cantilever tune, the Setpoint will appear automatically on the microscope base in the liquid-crystal display above the oval display. Setpoint values outside the range of 0.5–2.0 V indicate that the cantilever tune needs adjustment such as lowering or increasing the Drive amplitude (usually within the range of 30–250 mV, though generally no greater than 150 mV). Once the cantilever has been tuned, leave these menus, and now the system is ready for engagement (under the Motor Command).
18. Both the Setpoint and the gains should be adjusted slowly with small increments, as too great a change can lead to tip damage, irreversible loss of imaging quality, and the need to replace the damaged tip.
19. An incomprehensible image can be caused from not having a fresh, atomically flat surface on the mica. It may be necessary to peel the mica with Magic tape several times, each time always with a fresh piece of tape. Image the mica surface alone without sample as a control.
20. Sample is imaged as below the surface (e.g., *see* **Fig. 10A**). This contrast inversion problem can likely be attributed to anomalies with tapping mode in air (for extensive discussion *see* **ref. *12***). At this point one has to 'play' with the software controls (i.e., minimize the Setpoint, adjust the Scan angle, reduce Scan speed to 1 Hz, change Scan size to 1000 nm × 1000 nm, carefully adjust Integral and Proportional gains, etc.) in order to obtain an image with the sample above the surface (**Fig. 10B**). It may be necessary to disengage from imaging, adjust tip with tweezers or replace it, and then return to imaging.
21. We have found that it is difficult to get good images of fibers of unfixed chromatin fibers on mica in air. It was only possible to get nucleosome resolution of chromatin fibers deposited from a low salt buffer such as 5 m*M* triethanolamine-HCl, pH 7.0. Addition of just 10 m*M* NaCl to this buffer allowed the histones to dissociate from the DNA during the deposition process (deposition, rinsing, blotting, N_2 fluxing). Under these conditions, the images show mostly naked DNA



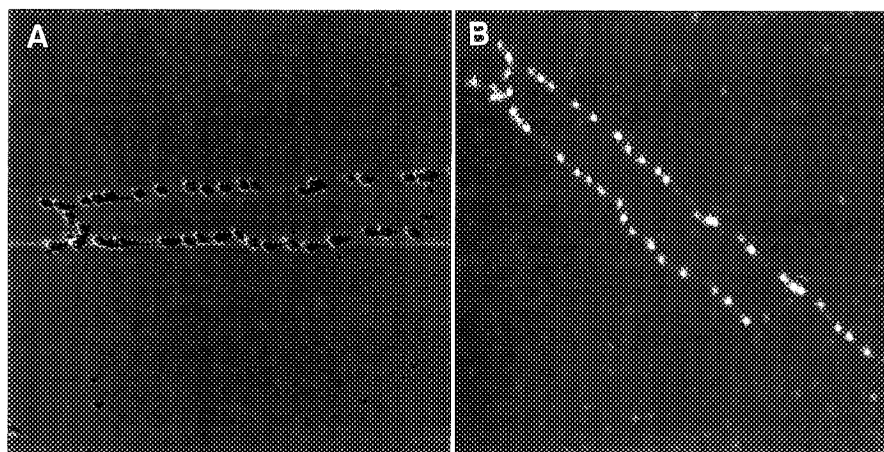


Fig. 10. **(A)** SFM image where the nucleosomes appears to be below the surface of the mica. **(B)** SFM image of exact same fiber upon adjustment of software controls (*see* **Note 20**). Glutaraldehyde-fixed, linker histone-stripped chromatin fibers were deposited from 5 m*M* triethanolamine-HCl, pH 7.0, and imaged in air. Image sizes are 1000 nm × 1000 nm. Heights are encoded in shades of gray with low regions depicted in dark gray and higher regions in lighter shades in a scale of 0–0 nm.

and a few histone octamers. It is much easier to get images of nucleosomal fibers of unfixed chromatin on glass in air.

22. If the images show too few or too many fibers in the field, the easiest way to change the fiber concentration is to adjust the deposition time accordingly. The deposition time can be increased up to typically approx 30 min before the 20 μL drop dries, or can be decreased to a minimal time by rinsing immediately after deposition. Alternatively, one can use slightly more or less concentrated chromatin to begin with. While the 0.1 mg/mL deposition concentration of chromatin is about 100 times the concentrations used for the deposition of DNA restriction fragments, we find that this concentration works reasonably well for chromatin fibers.
23. The set up for imaging in liquids is different from that used in air imaging applications. One needs to set up the microscope completely with the liquid cell (**Fig. 7A**) before the sample is deposited. The steps can be separated into
    a. Protecting the scanner with a piece of Parafilm or thin rubber before starting.
    b. Placing the Park Scientific Microlever tip into the liquid cell, placing the liquid cell into the microscope head, and adjusting the laser beam to bounce off the cantilever on the backside of the tip and focus onto the photodiode.
    c. Adjusting the distance between the tip and the surface.
    d. Depositing the liquid sample onto the surface of freshly cleaved mica or cleaned coverglass and letting it settle for a period of time.

e. Rinsing the sample with the same buffer to remove excess chromatin not on the surface.
f. Placing the wet sample in the microscope, placing the silicon O-ring onto the surface, and placing the head onto the silicon O-ring surrounding the sample to seal the liquid chamber.
g. Tuning and engagement of the cantilever.

24. For imaging fibers in buffer, we have observed that the chromatin is more sticky to the glass if the chromatin has been isolated in a procedure in which all buffers contain 1 m*M* sodium butyrate. The presence of butyrate, a deacetylase inhibitor, maintains the lysines in histone tails in chromatin in higher states of acetylation which appears to result in the chromatin fibers sticking better to the glass surface in buffer. One should keep in mind that the butyrate treatment keeps the histones in a more acetylated state than is found in 'native' chromatin.
25. We have used Alex, a program written in Matlab (Mathworks, Natick, MA) by Mark Young and Claudio Rivetti for the Silicon Graphics Workstation (SGI, Mountain View, CA) for the analysis of our images. The source code for Alex is available through the world wide web at alice.uoregon.edu. In Alex, we have used a homemade routine, "Measure_Chromatin", which records the X, Y, and Z coordinates of each mouseclick on the top of each visible nucleosome in a fiber. (Alternatively, it is possible to use the NanoView program written by Gerry Leatherman in Delphi [Borland, Scotts Valley, CA] for Windows 95/Windows NT. NanoView source code is available from [www.molec.com].) From these coordinates, then it is straightforward to calculate
    a. The center-to-center distance of two adjacent nucleosomes.
    b. The interior angle formed by the intersections of two lines formed by centers of three successive nucleosomes.
    c. The height of each nucleosome observed in the fiber.

    Given coordinates (X_1,Y_1,Z_1) and (X_2,Y_2,Z_2) of two adjacent nucleosomes, the center-to-center distance is calculated by:

$$D_{1,2} = \sqrt{[(X_1 - X_2)^2 + (Y_1 - Y_2)^2 + (Z_1 - Z_2)^2]}$$

    Given coordinates (X_1,Y_1,Z_1), (X_2,Y_2,Z_2), (X_3,Y_3,Z_3) of three sequential nucleosomes, the angle (∅) is calculated from the law of cosines:

$$\cos(\varnothing_{1,2,3}) = \frac{[(D_{1,2})^2 + (D_{2,3})^2 - (D_{1,3})^2]}{(2\, D_{1,2}\, D_{2,3})}$$

    It is simple to assign the coordinates to nearly every nucleosome within images of linker histone-depleted beads-on-a-string fibers. Deciding which nucleosomes are successive in images of three-dimensionally organized, native chromatin fibers is not so straightforward. However, in these three-dimensionally organized fibers, especially at monovalent ionic concentrations of 20 m*M* and less, it is usually possible to see the repeating structure of the nucleosomes. Typically, the nucleosomes that would take the shortest path to form the fiber are taken to be adjacent, although this choice is somewhat arbitrary. The analysis should be repeated at

least once with the same images to ensure against operator bias. The important point to remember with measurements, distributions, and statistics is that they should only be used to quantitatively support morphological differences easily seen by eye.

The present Alex and NanoView software only recognize v. 4 images of NanoScope files; in earlier DI versions, the Z-height is incorrectly read in the image header files. DI v. 3 images can be resaved as version 4 images using DI software. It is also possible to make similar kinds of measurements noted above in the analysis protocol using Digital Instrument software, although it is much more tedious and time-consuming.

26. If the user would like to print or make color slides of images, a simple way is to take photographs of the screen with 100 ASA color print or slide film and a 35 mm camera equipped with either a macro lens or a 70–200 mm zoom lens. The camera is mounted on a tripod approx 3 m in front of the screen with the zoom lens all the way out (Use of the 200-mm lens reduces the problems from the curvature of the computer screen.) After focusing, photographs are taken in a darkened room at 0.5 s with f-stop of 5.6, using a cable release. The user can experiment with exposure times longer than 0.25 s and f-stops of 5.6 or greater (i.e., f-stop of 8, 11, 16). White text will become over exposed and fuzzy in photographs or slides especially during longer exposure times. Alternatively, one can import tiff images into a program such as Adobe Photoshop (www.adobe.com) and then print the images on a color dye-sublimation printer at a cost of approx $5–$10 per page. An economical alternative is to print gray scale images of the screen directly to a laser printer using the DI software.

7. Conclusions

We have described protocols for imaging chromatin with the SFM. With the drawings of the various procedures, it should be possible for the experienced chromatin biochemist/beginning SFM user to obtain similar results. While the tapping mode in air experiments are routine, we should caution the reader that the imaging in buffer experiments are not, and they require extra time, care, and experimentation.

Acknowledgments

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