# Chapter 11 Fluid Operation

This chapter addresses scanning probe microscope (SPM) imaging of samples in fluid using a MultiMode. Refer to the following for your specific area of interest:

## 11.1 Introduction

Imaging of samples in fluid is a growing application of AFM technology. This may be prompted by a desire to minimize surface forces on delicate samples, the need to observe biological specimens in their natural, fluid environments, and/or the necessity to make real time observations of samples undergoing electrochemical reactions (ECAFM). In order to conduct ECAFM observations with electrical potentials, it is necessary to connect an external potentiostat unit. Contact Bruker for more information.

Imaging samples under fluid eliminates attractive forces due to surface tension. This enables the sample surface to be imaged with a minimum of cantilever tip force—a decided advantage when imaging biological specimens and delicate materials.

Essentially, the procedure for observing samples under fluid is the same as that for Contact Mode or TappingMode AFM in air; however, special hardware is utilized to contain the fluid. In addition, minor adjustments must be made to correct for refractive effects as the laser beam transits air-fluid boundaries.

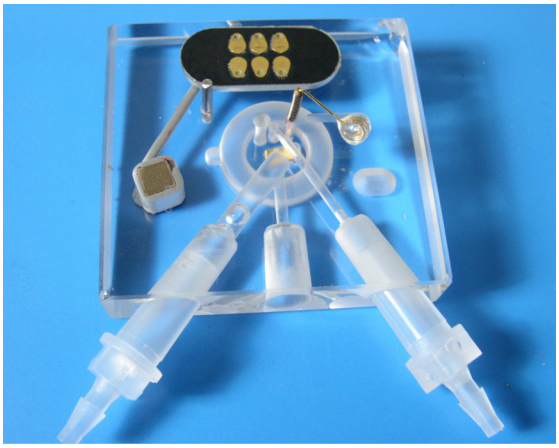
This chapter describes Contact Mode and TappingMode AFM operation of the MultiMode SPM in fluid, including loading the probe into the probe holder, mounting the probe holder into the head and aligning the laser on the cantilever portion of the probe and then engaging the probe tip with the sample.

This chapter assumes familiarity with Contact Mode and TappingMode AFM operation of the MultiMode in air. If you are not familiar with air operation of the MultiMode, refer to the procedures outlined in Chapter 9 and Chapter 10 before attempting to operate the MultiMode AFM with a fluid cell.

## 11.2 General Fluid Operation

The fluid cell consists of a small glass assembly with a wire clip for holding an AFM probe (see Figure 11.2a). The glass surfaces provide a flat interface so that the AFM laser beam may pass into the fluid without being distorted by an unstable fluid surface. The probe is mounted in a rectangular groove on the bottom of the fluid cell, and held in place by a gold-coated wire clip. A circular groove surrounds the probe and is used to seat the (optional) O-ring. Additionally, there are at least two fluid ports located on the front side of the fluid cell. These ports allow for the introduction and removal of fluid.

**Figure 11.2a** EC TappingMode Fluid Cell



There are two fluid cells that are commonly used for fluid imaging:

- Model MTFML is a fluid cell that can be used for imaging samples in fluid using either Tapping Mode or Contact Mode.
- Model MMTMEC is a fluid cell that can be used for imaging samples in fluid using either Tapping Mode or Contact Mode and has additional port for the insertion of a reference electrode and a counter electrode.

  **Note:** It is necessary to connect an external potentiostat to perform electrochemical measurements. Contact Bruker for more information.

The fluid cell comes with the following accessories:

- 10 glass cover slips (for use as sample substrates)
- silicone tubing
- 5cc syringe
- 4 male Luer fittings
- 2 female Luer fittings

- 2 tube clamps
- 6 silicone O-rings
- 6 fluorosilicon O-rings
- Silicone grease
- Lens paper

The male Luer fittings can be inserted into the fluid ports on the front of the fluid cell, and the opposite side of the fitting is connected to the silicone tubing. The female Luer fittings connect the silicone tubing to the syringe.

Use of the O-rings is optional. Instructions for operating the fluid cell with and without an O-ring are detailed in this chapter. The previous O-ring design was based on a circular cross-section. The new O-ring design has an “S” shaped cross-section with thin, flexible walls. This new design makes insertion of the O-ring easier, reducing the chances of leaking and minimizing imaging artifacts caused by lateral friction.

### 11.2.1 Clean Fluid Cell and O-ring

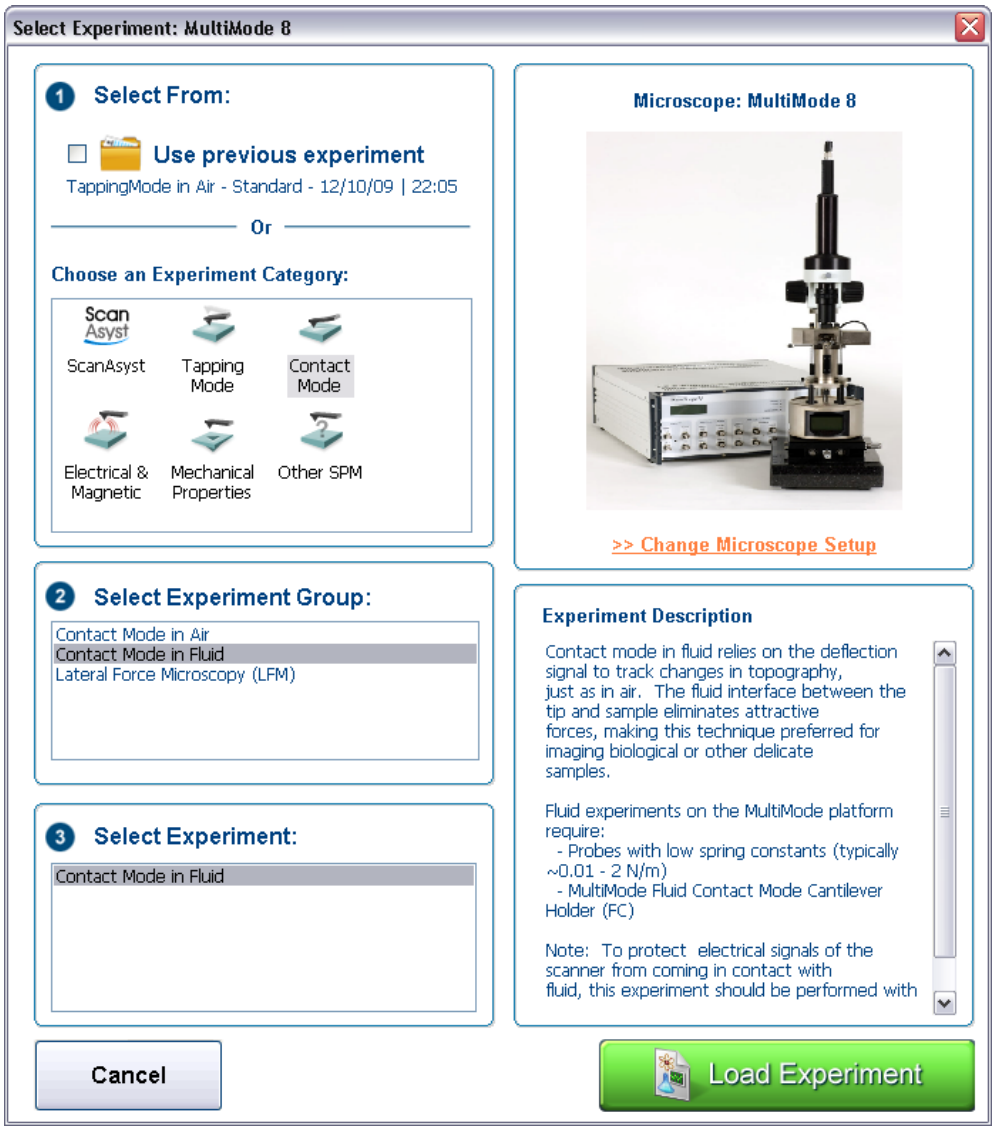
To reduce contamination problems and to obtain high-quality images, clean the fluid cell, and O-ring if applicable, as follows:

1. While soaking the fluid cell and O-ring in warm, soapy water, place a few drops of liquid dish soap on them. Use nonabrasive soap to prevent scratching the glass surfaces.
2. Gently rub the fluid cell and O-ring with a cotton swab or finger. Avoid scratching the glass surface with abrasive material.
3. Using distilled water, rinse the fluid cell and O-ring of all soap.
4. Using 0.2µm-filtered, compressed air or dry nitrogen, blow dry the fluid cell until all moisture evaporates.

### 11.2.2 Select Mode of Operation

1. Click the **SELECT EXPERIMENT** icon. This opens the **Select Experiment** window, shown in Figure 11.2b.

**Figure 11.2b** The Contact Mode in Fluid **Select Experiment** window



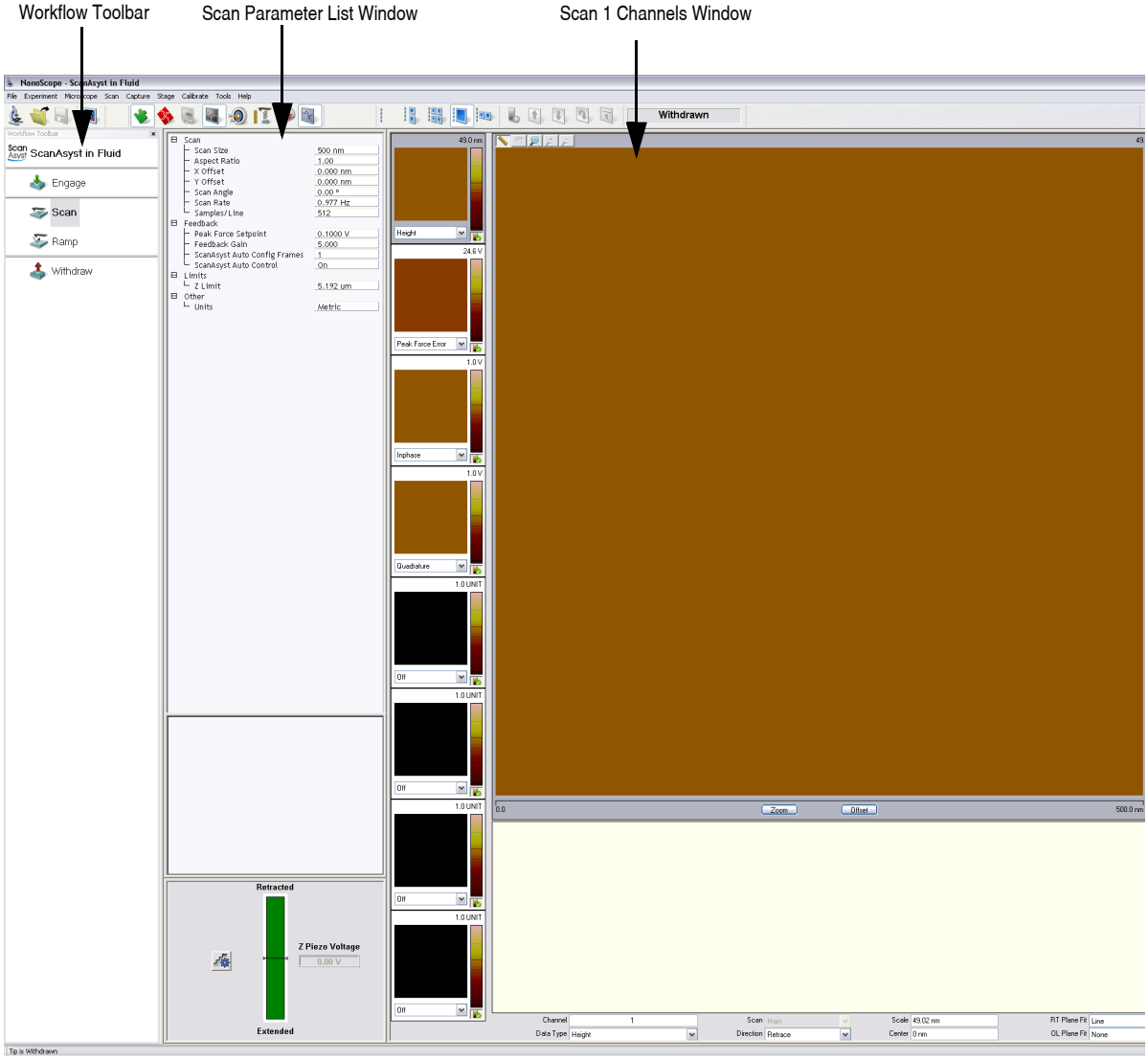

2. Chose a mode that supports imaging in fluid: **SCANASYST, TAPPING MODE OR CONTACT MODE** in the **Choose an Experiment Catagory** panel.

3. Select **SCANASYST IN FLUID, TAPPINGMODE IN FLUID** or **CONTACT MODE IN FLUID** in the **Select Experiment Group** panel.

4. Select **SCANASYST IN FLUID, TAPPINGMODE IN FLUID** or **CONTACT MODE IN FLUID** in the **Select Experiment** panel and click **LOAD EXPERIMENT**.

5. This opens the **Workflow Toolbar**, the **Scan 1** or **2 Channels** (depending on the mode) windows and the **Scan Parameters List** window, shown in Figure 11.2c.

**Figure 11.2c** Contact Mode in Fluid (Simple) configuration




## 11.2.3 Select the Probe

AFM probes featuring low stiffness cantilevers produce the best results for biological applications. We recommend sharpened tips to start: 100µm (“short”), V-shaped cantilevers with oxide-sharpened silicon nitride tips; for example, models NP-S (standard) or NP-STT (oriented twin tip). Stiffer etched silicon probes (model FESP, single beam, 225µm long) used at very small oscillation amplitudes also obtain good results. Experiment to find which probes work best for your sample.

**Note:** For additional information on selecting a probe, please refer to the applications note "*Choosing AFM Probes for Biological Applications*."

### 11.2.4 Remove Organic Contamination from the Tip

Contaminants on the tip may limit AFM resolution. You may use ultraviolet (UV) light to remove contaminants, as follows:

1. Place the fluid cell with installed tip face-up on a clean surface.
2. Position a UV lamp very close (3-5mm) to the fluid cell and irradiate the probe for 15-30 minutes at full intensity.

   **Note:** Washing probes in 1-5% SDS (Sodium Dodecyl Sulfate) is also effective. Glass areas may be cleaned using isopropyl alcohol or acetone.

### 11.2.5 Load the Fluid Cell with a Probe

The probe is held in a small pocket on the bottom side of the fluid cell by a gold-plated, stainless steel wire clip. A tiny coil spring mounted on the top of the fluid cell holds the wire clip against the probe.

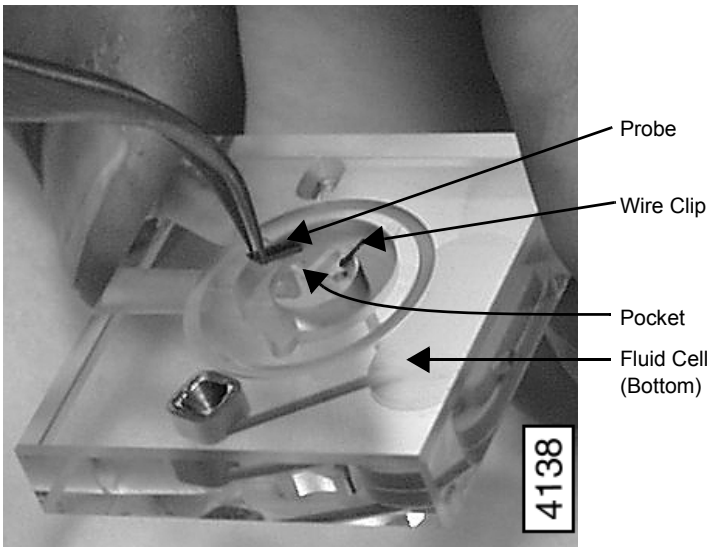
Load a probe into the fluid cell by performing the following procedure:

1. Hold the fluid cell with one hand and use the other hand to gently raise the wire clip by pressing the spring from beneath.

   **Note:** Do not press harder than enough to completely compress the spring!
2. Use the clear ball to the side of the spring to rotate the wire clip away from the pocket.
3. Use tweezers to slide a probe into the pocket. Lower the clip to hold the probe (see Figure 11.2d).
4. Verify that the probe is squarely set against one side of the pocket and flush against the back. Verify the probe is held firmly by the wire.

**CAUTION:** Avoid scratching the fluid cell glass surface with the tweezers or the probe, especially in the area under the probe.

**Figure 11.2d** Load Probe into Fluid Cell




## 11.2.6 Sample Mounting

Secure a sample support (e.g., mica or a glass cover slip) to a magnetic stainless steel sample puck. Supports may be secured to the puck with epoxy. Select epoxy as follows:

- For non-critical applications, use Devcon 2-Ton Epoxy or 5-Minute Epoxy.
- For applications where contamination control is more critical, use a more inert, solvent-free epoxy such as Master Bond EP21LV or EP21AR or a hot melt adhesive.

**Note:** Follow the manufacturer's directions for mixing and curing to obtain the best resistance to leaching and chemical attack.

**CAUTION:** Do not use cyanoacrylate glue (e.g., SuperGlue™) for mounting samples in fluid.

### Method 1 (with an O-ring)

The O-ring protects the AFM scanner tube from spilled liquids. To mount the sample with an O-ring:

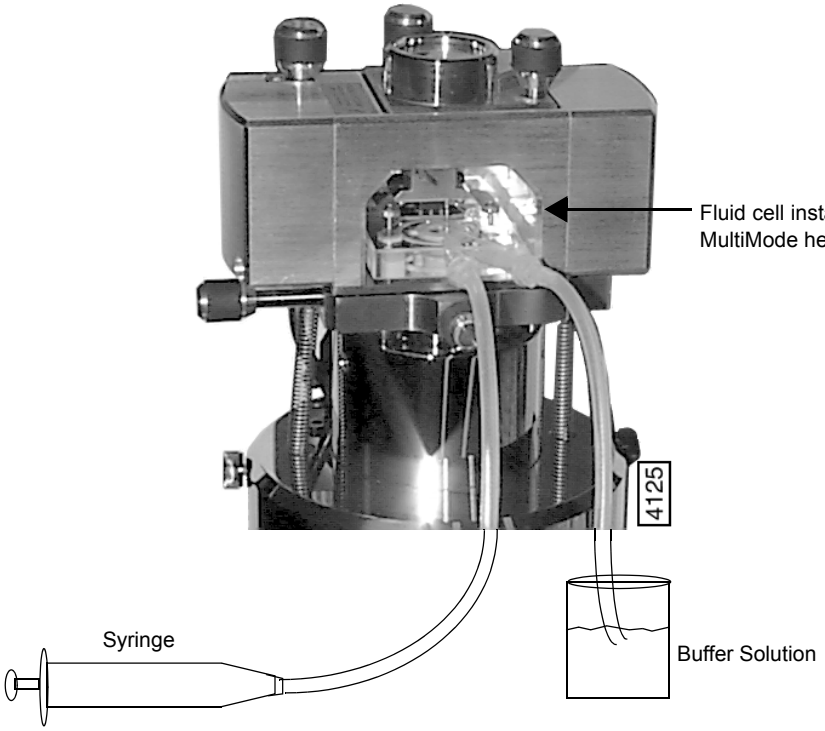
1. Install the protective O-ring into the fluid cell. Insert the O-ring into the recessed groove in the underside of the fluid cell. The O-ring slides up into the recessed groove.

2. Install the sample or sample support onto the scanner and insure the scanner is lowered enough to avoid damaging the probe when the head is mounted.

3. Mount the head carefully to avoid damaging the sample and secure the head in place with the two mounting springs.

4. Install the fluid cell in the AFM head and tighten the clamp to hold the fluid cell in place. Ensure that the O-ring is positioned properly between the sample and fluid cell. Position the fluid cell in the head to ensure that the O-ring creates a seal on the sample around the periphery of the fluid cell and does not overlap any edges of the sample. If necessary, use the XY translation knobs on the head to center the O-ring.

**Figure 11.2e** Fill the Fluid Cell with Liquid




5. Verify the head is leveled side-to-side and that the head is tilted slightly forward, so the tip is level when it contacts the surface.

   **Note:** If you are using a vertical-engage scanner, leveling problems are eliminated.

6. Using the **Tip Down** switch on the MultiMode base (and the coarse adjustment screws if not using a vertical engage scanner), lower the tip toward the sample until the tip is within ~30µm of the surface. This will bring the fluid cell close enough to the surface to ensure that the O-ring will create a seal against the sample.

7. Fill the fluid cell with liquid.

**Note:** To minimize the risk of fluid leakage, draw fluid to the fluid cell using the following technique:

- Attach a piece of silicone tubing to one of the fluid ports using a male Luer fitting. Place the free end of the tubing into a beaker containing the buffer.
- Attach a piece of silicone tubing to the other fluid port using a male Luer fitting.
- Fill the fluid cell with fluid by withdrawing the plunger on the syringe. This will draw fluid from the beaker containing the buffer through the fluid cell.
- Check for leaks and wick away any spilled liquid with filter paper until AFM components are dry.

8. Bubbles inside the fluid cell near or on the probe can interfere with the laser beam. Remove bubbles by performing the following procedure:

   a. Observe the fluid cell and probe through the viewing port using the OMV.

   b. Rapidly pull liquid through the cell with a syringe. If sufficient force is applied, the bubbles will be carried out of the fluid cell.

### Method 2: Without an O-ring

**CAUTION:** Without the O-ring, this method poses a potential spill hazard to microscope electronics and must always be undertaken with extreme caution. Limit the volume of liquid in the fluid cell to 30-50µl when possible.

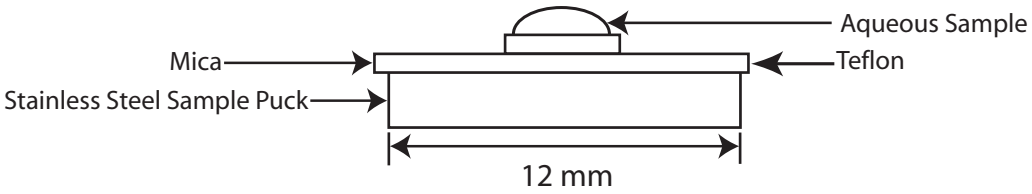
In many cases, it is possible to image a sample under a drop of fluid without use of a closed fluid cell. This technique is recommended for aqueous buffers when evaporation is not an issue (for example, in short experiments involving no use of volatile solvents).

Limit risk of leakage as follows:

- Use a sample support larger than the puck.
- Use the recommended adhesive Loctite 770 to affix a Teflon cover over the steel sample puck. The Teflon should extend slightly over the edge of the sample puck. Use epoxy to attach the sample support to the Teflon.

The hydrophobic Teflon helps confine the solution without installing an O-ring (see Figure 11.2f).

**Figure 11.2f** Stainless Steel Sample Puck with Teflon Cover




You may load the sample on the support now, or you may inject it when the fluid cell is installed inside the AFM head.

Two variations of this method are possible: starting with a dry sample and starting with a sample in solution.

1. Complete the following if starting with a dry sample:

   a. Attach the sample support (e.g., mica) to a puck.

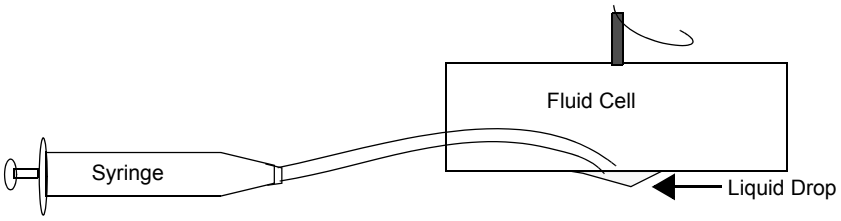
   b. Deposit your sample on the support.

      **Note:** It is easier to first align the AFM head and mounted fluid cell (probe installed) with the dry sample puck before installing the liquid-coated sample. Allow 0.5mm clearance between the tip and dry substrate surface.

   c. Occasionally air bubbles form in the fluid cell and block laser light. Reduce the chance of forming bubbles as follows:

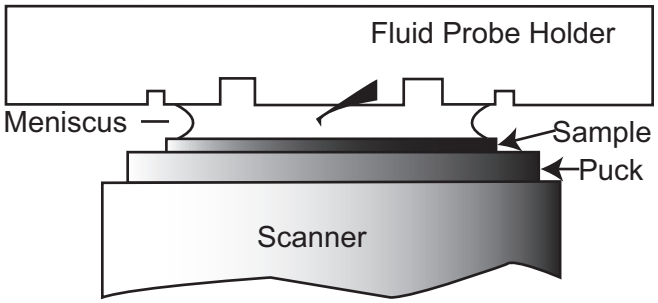
      - Before installing the fluid cell into the head, insert a syringe filled with liquid solution into a fluid port, or connect the syringe to the inlet tubing on the fluid cell.
      - Push enough fluid through the fluid chamber to flood the fluid cell port with buffer solution, allowing liquid to drip out the bottom of the cell.
      - Leave the buffer-filled syringe inserted. A small amount of buffer solution should be held to the bottom of the cell by surface tension (see Figure 11.2g).

**Figure 11.2g** Flush the Fluid Cell Before Installation to Reduce Bubble Formation




**Note:** The liquid is held by surface tension between the sample surface and the fluid cell (see Figure 11.2h). Because the aqueous sample changes the light deflection, the laser photodetector must be re-adjusted.

**Figure 11.2h** Imaging a Sample Covered by a Drop of Fluid




2. Complete the following if starting with a sample in solution:

    a. Place 30-40µl of your sample on the support mounted on the puck (the liquid should form a small dome over the support). During this placement, the sample should adhere to the support (e.g., mica).

    b. Install the sample support on the AFM scanner.

    c. Carefully install the fluid cell inside the head.

    - Inspect from the front to make sure the fluid is well confined to the mica area only.
    - Aim the laser on the cantilever and adjust the photodiode detector position (see Section 11.2.8).

    **Note:** Always verify that the microscope is dry and that all MultiMode surfaces are free of spilled fluid. Wick away moisture and droplets with filter paper.

    **Note:** Over time, evaporation of the fluid may necessitate replenishing the fluid cell using a standard micropipette or syringe.

### 11.2.7 Align the Laser

Use either of the techniques for aligning the laser on the probe (see Chapter 7, **Laser Alignment:** Section 7.2.) The following considerations apply:

- Refraction causes the laser beam path to bend slightly when entering and exiting the fluid surrounding an immersed probe as compared to the path in air. However, the basic process is essentially the same.
- It may be necessary to adjust the tilt of the mirror to position the laser on the photodetector. The laser position on the tip will not change with the introduction of fluid but the mirror will require adjustment.
- Although the top surface of the fluid cell has an anti-reflection coating, a faint reflection may still be visible. This reflection from the glass surface does not affect operation of the MultiMode, but it can be a source of confusion when aligning the laser. Ignore this faint reflection and focus on the much brighter reflection from the cantilever.
- The **Sum** signal on the display monitor typically displays less than **1V** when the laser is not aligned on the probe. The **Sum** signal should rise well above **1V** when the laser is reflecting off the probe.

### 11.2.8 Adjust the Detector Offsets and Setpoint (Contact Mode)

- Typically, samples are softer in liquid than in air. Before engaging, verify that there is not so large a difference between the setpoint and the vertical deflection signal as to damage the sample.
- Verify reasonable values for scan parameters (e.g., **Scan Rate**, **Scan Size**, **Integral Gain** and **Proportional Gain**). Gains are typically lower in fluid than in air.
- Once engaged, reduce the setpoint to minimize the tracking force.

### 11.2.9 Engage the Surface

1. Using the **Motor down** switch (and coarse adjustment screws if not using a vertical engage scanner), lower the tip until it is just above the level of the sample surface.

    - The **Motor down** switch is located on the MultiMode base.

2. Select **MICROSCOPE > ENGAGE** or click the **ENGAGE** icon on the toolbar. A pre-engage check begins, followed by Z-stage motor motion.

    - The motor begins to move the AFM head and probe down to the sample.
    - When the tip reaches the surface, the system should automatically stop, beep, and begin to image the sample.
    - In Contact Mode, if the system engages immediately or before the tip reaches the surface, try increasing the **Setpoint** approximately **2.0V**, then repeat this step.

### 11.2.10 Adjust Scan Parameters (Contact Mode)

1. Once engaged, adjust the scan parameters to obtain the best image.

    **Note:** This procedure is similar to operation in air, except that samples are often softer in fluid. Adjusting the applied force can be critical.

2. To avoid sample damage, reduce the **Deflection Setpoint** as low as possible:

    a. Stop when the tip pulls off the surface and the **Z Center Position** in the **RealTime Status** window jumps to **Limit** (**-220V**).

3. Increase the setpoint until the tip begins to touch the surface again and an image appears.

    - As an alternative, adjust the setpoint using Force Calibration. Refer to Chapter 14 for details.
    - Because the tip typically adheres to the sample surface much less in fluid than in air, it is possible to image at much smaller contact forces in liquid.
    - The optimal integral and proportional gains and scan rate may be different from air operation, because the dynamics of the cantilever change in fluid.

4. Set the two gains as high as possible (starting with the integral gain) without causing oscillation distortion to appear in your image.

5. Observe the agreement between the trace and retrace lines and adjust the **Setpoint**, **Gains** and **Scan Rate** to bring the trace and retrace lines into coincidence. This procedure will be similar to operation in air, with the following exceptions:

- Samples are generally softer in fluids. Adjusting the applied forces becomes more critical.
- Scan rates tend to be slower in fluid.

6. Choose a scan rate that is sufficiently slow to image without degrading your data.

### 11.2.11 Clean and Dry Parts When Done

1. When sample imaging is complete, drain the fluid cell and carefully remove it from the head. Avoid spilling fluid.
2. Rinse and dry the fluid cell, and O-ring if applicable, to prevent the buildup of salts or other contaminants on these parts.

**CAUTION:** When cleaning the fluid cell, use care to avoid scratching the glass surfaces in the center of the fluid cell where the laser beam passes.

## 11.3 Tapping Mode in Fluids

Operation of tapping mode in fluid provides the same advantages of tapping mode in air, with the additional ability to image samples under native liquid conditions. In fluid tapping mode, the probe is oscillated so that it only intermittently contacts the sample surface. This can reduce or eliminate lateral forces that can damage soft or fragile samples in Contact Mode. The following sections provide general instructions for tapping mode imaging in fluid.

**Note:** Before attempting tapping mode in fluids, it is recommended that the user becomes familiar with standard tapping mode operation in air (see Chapter 10) and Contact Mode in fluid.

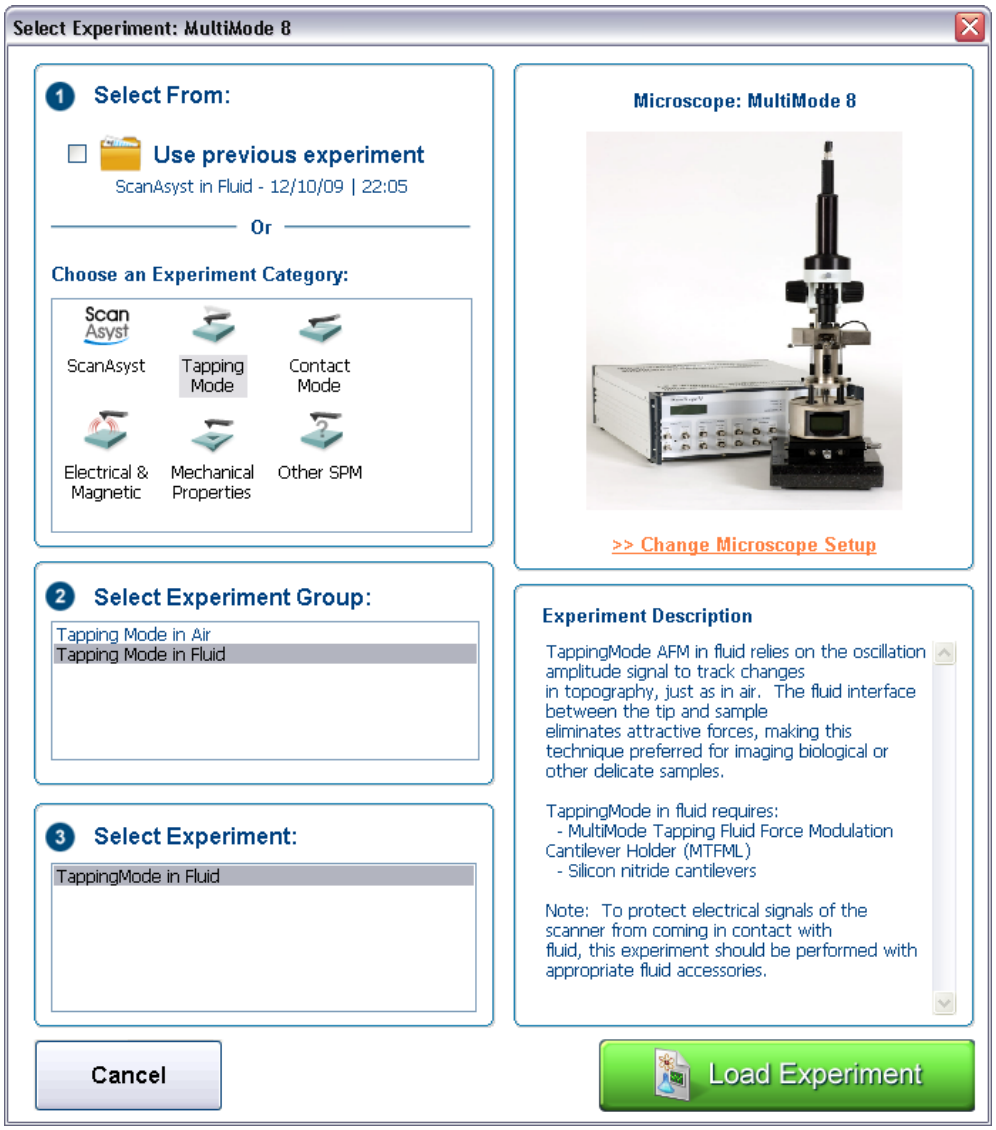
### 11.3.1 Procedure for Tapping Mode Imaging in Fluid

1. Follow the directions described in the Contact Mode section (see **Section 11.2.1** on page 194) to clean the fluid cell and O-ring. Clean and mount a probe and then the sample.
2. Align the laser on the free end of the cantilever portion of the probe.
3. Adjust the mirror to maximize **SUM.**
4. Center the photodiode to give a deflection signal near **0V**.
5. Set up the system for tapping mode operation:

6. Set the mode selection switch on the left side of the MultiMode base to **TM AFM**.

7. Click the **SELECT EXPERIMENT** icon. This opens the **Select Experiment** window, shown in Figure 11.3a.

**Figure 11.3a** The **TAPPINGMODE IN FLUID Select Experiment** window




8. Select **TAPPINGMODE AFM** in the **Choose an Experiment Catagory** panel.

9. Select **TAPPINGMODE IN FLUID** in the **Select Experiment Group** panel.

10. Select **TAPPINGMODE IN FLUID** in the **Select Experiment** panel and click **LOAD EXPERIMENT**.

11. Manual Cantilever Tune:

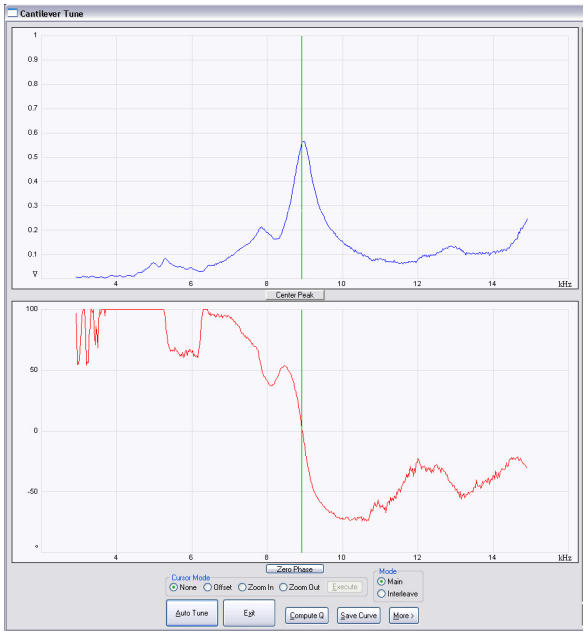
- This is similar to the Cantilever Tune process used for standard tapping mode in air. Unlike operation in air, the cantilever resonance will be largely damped by liquid and the Auto Tune function cannot be used.

- When viewing a wide bandwidth cantilever tune sweep, observe that there is no single, well-defined resonance peak, but, rather a number of broader maxima. Manually select a peak.

- Select **MICROSCOPE > CANTILEVER TUNE** or click the **TUNE** icon on the NanoScope toolbar to select a drive frequency. The optimal drive frequency can depend upon sample, fluid and fluid volume inside the fluid cell. Experiment to find the best drive frequency for specific imaging conditions. Two frequency ranges that are commonly used are 16-19kHz and 8-12kHz; higher frequencies have also been used. Start with a **Sweep width** of 20kHz. Users of TappingMode in air will notice that there is not a single well-defined resonance, but instead a large number of broad peaks. The peaks are resonances of the fluid cell and fluid, and do not usually depend so much on the cantilever dimensions.

  **Note:** For the short, narrow Silicon Nitride probe recommended for soft samples (e.g., model DNP), the resonant frequency in fluid is a broad peak centered around 10kHz (see Figure 11.3b). Best results are achieved by tuning the cantilever to a peak between 7-12kHz. Higher and lower frequencies have also been used depending on the type of probe employed. Start with a **Sweep Width** of **20kHz** and a **Drive Frequency** of **10kHz** in the **Sweep** panel.

**Figure 11.3b** A 100µm, Narrow-legged, $Si_3N_4$ Cantilever Fluid Tune Curve




- Manually adjust the **Zoom In** and **Offset** functions in the **Cantilever Tune** window.

**Note:** If the expected peak does not appear in the spectrum, choose another peak, engage on the surface and disengage immediately. With the tip closer to the surface, the peak at 8 to 9 kHz appears.

Adjust the **Drive Amplitude** until a desired probe RMS amplitude is obtained.

- An RMS amplitude of **150 mV** is appropriate for soft samples and typically results from a **Drive Amplitude** of **250-500mV**. A **Drive Amplitude** >**0.3V** generally works poorly with soft samples.
- For rougher samples, target **300-600 mV** RMS amplitude. Experiment to find what level of probe oscillation gives best results for specific applications.

a. Set the **Scan Rate** to **1Hz**.

b. Set the **Integral Gain** to **0.5**.

c. Set the **Proportional Gain** to **0.7**.

d. Set the data **Scale** to **50nm**.

e. Select the Channel 1 **Data Type** as **HEIGHT**.

f. Select the Channel 2 **Data Type** as **AMPLITUDE ERROR**.

g. Optional: Select the Channel 3 **Data Type** as **PHASE**.

- The RMS amplitude must be adjusted when the AFM tip is near the sample surface (<50µm).
- Verify that the tip is very close to the sample surface when tuning the probe in fluid.
- Adjust and optimize these settings for each imaging condition and sample.

12. Center the laser spot on the photodiode detector.
    - Adjust the photodiode until deflection is roughly zero.
    - The deflection signal can drift when the probe is first in fluid, so it is best to adjust just prior to engaging.

13. Click the **ENGAGE** icon on the toolbar.to bring the tip into tapping range.
    - The NanoScope software automatically selects a setpoint, then stops the engagement when the surface is detected.
14. Adjust the setpoint when engaged.
    - The best topographic images are usually obtained at setpoints 10-20 percent less than the RMS amplitude before engaging.

- The setpoint may be optimized by observing and optimizing the image quality.

- It may be necessary to check the cantilever tune after engaging. Click the **TUNE** icon on the NanoScope toolbar. When prompted for a **Tip Offset**, enter **100-200nm**. Observe the cantilever tune spectrum and the offset the **Drive Frequency** if required.

### 11.3.2 Optimizing Image Quality

Adjust the setpoint by monitoring image quality, as follows:

1. Select an appropriate Scan size. Wait until the current scan line is over a tall feature, then **DISABLE** the **Slow Scan Axis** (Expanded Mode). Check the agreement between the Trace and Retrace displays.
2. Increase the setpoint in small increments until the probe pulls off the surface and the **Z Center** position voltage goes to retracted (i.e., **-220V**).
3. Reduce the setpoint in small increments until an image appears.
4. Continue reducing the setpoint until the image is optimized.
    - The best images are obtained at setpoints just below where an image appears.
    - The NanoScope Controller attempts to keep the cantilever oscillation amplitude constant during the scan.
5. Optimize the **Integral gain** and **Proportional gain** so the **Height** trace shows the sharpest contrast and there are minimal variations in the **Amplitude** trace (the error signal).
6. Optimize the **Scan rate** to obtain the sharpest image.

## 11.4 Troubleshooting Tips

### 11.4.1 Cantilever Tune Plot Looks Poor: Loose Probe

The **Cantilever Tune** plot can be used as a diagnostic tool. Become familiar with its characteristics when good images are obtained. If the plot looks substantially different from previous successful experiments, there may be a problem with the fluid cell. For example, the probe may be loose in its holder.

Check the clip which holds the probe in place, and verify the probe is not loose. Contact Bruker for assistance if needed.

## 11.4.2 Laser Sum Signal Absent or Weak: Air Bubbles

Verify that all bubbles are removed from the probe. Bubbles may attach themselves to the probe, causing the laser beam to be diffracted. While bubbles can sometimes be removed by forcing fluid through the fluid cell, it is often necessary to dry the fluid cell with absorbent paper (e.g., Kimwipe).

Degassing the fluid prior to use in the AFM reduces bubble problems.

## 11.4.3 Poor Image Quality

### Contaminated Tip

Some types of samples (e.g., certain proteins) may adhere to the cantilever or tip. This reduces resolution, resulting in blurred images.

If tip contamination is a problem, it is necessary to protect the tip against contamination. There are two ways this may be accomplished:

1. If the sample is adhered to a surface through adsorption (e.g., diffusion of protein onto mica):
    a. Diffuse the sample substance into the substrate, then rinse with buffer.
    b. Lower the tip into a fluid containing little or no stray substances which may adhere to the tip.
2. If the sample is short-lived and must be imaged quickly, it may be possible to mask the tip against contamination by bringing the tip into gentle contact with an uncontaminated substrate surface:
    a. Put the MultiMode into Contact Mode and engage the substrate surface using a **0.1nm Scan Size**.
    b. While the tip is kept in gentle contact with the substrate surface, add the sample substance to be imaged and allow it to diffuse/settle onto the substrate.
    c. After a diffusion/settling period has lapsed, quickly lift the tip from the substrate surface.
    d. Switch to **Tapping Mode** and image the sample before it becomes contaminated.

### Dull Tip

Change to a new probe. AFM probe tips can become dull during use and some unused tips may be defective. Check the probe type being used. Oxide sharpened silicon nitride probes are usually much sharper than standard silicon nitride probes.

#### Multiple Tip

Change or clean the AFM tip. Probes can have multiple protrusions at the apex of the tip, which result in image artifacts. Features on the surface appear two or more times in an image, usually separated by several nanometers.

### 11.4.4 Lost Particulate Samples: Attracted to Cantilever

Some particulate samples such as proteins may prove difficult to find directly beneath a cantilever if the cantilever has remained stationary during a diffusion or settling period. This may be due to the fact that some types of particulates are more attracted to the cantilever than to the substrate intended to support them. The result is a "shadow" on the substrate directly beneath the cantilever where fewer sample individuals can be located; they are stuck to the cantilever instead. If you suspect this problem, shift the imaging site to a new location away from where the probe had remained motionless.

### 11.4.5 Drift in AFM Image Because O-ring Slid Across Sample Surface

Set up the fluid cell so there is minimal lateral movement of the optical head with respect to the sample once the O-ring is installed. Keep the head level while positioning the tip close to the surface to minimize tip lateral motion during engagement. Other countermeasures:

- Unlike three-point supports, the vertical engage scanner allows the tip to approach samples without lateral offset, eliminating stress on the O-ring during engagement. For more detail, contact Bruker.
- Lightly coating the area of the O-ring which contacts the sample surface with silicone grease from the fluid cell kit allows the O-ring to slide across the surface, minimizing lateral stress. This also forms a fluid-tight seal between the O-ring and sample. However, some solvents (i.e., nonpolar organic solvents) may dissolve some of the lubricant into the fluid.
- If not using a vertical engage scanner, when positioning the fluid cell over the sample surface, adjust the positioning knob at the base of the optical head to move it slightly forward. This will counter some of the lateral stress on the O-ring resulting from the optical head moving back during engagement.

## 11.5 General Notes on Sample Binding

Samples for AFM imaging should be immobilized on a rigid substrate. Macroscopic samples (biomaterials, crystals, polymer membranes, etc.) can be attached directly to a stainless steel sample disk with an adhesive. Dissolved or suspended samples like cells, proteins, DNA, etc. are usually bound to a flat substrate like mica or glass, for example. Many different sample preparations have been developed and SPM applications articles are an excellent source of

information on sample binding. For a list of articles describing biological applications of AFM, including sample preparation techniques, contact Bruker.

### Binding Specimens to Mica

The following procedures will describe how to bind two different samples to a mica substrate. Mica is commonly used because atomically flat substrates can be simply and inexpensively prepared. In aqueous solutions, the mica cleavage surface becomes negatively charged. Specimen binding is usually accomplished using electrostatic attraction between charges on the specimen and those on the mica surface. Proteins, for example, can usually be made to stick to mica by operating at a pH where they exhibit positively charged domains. DNA, on the other hand, is negatively charged and can be bound either by altering the mica surface charge from negative to positive (using a silanization process) or by dissolving the DNA in a divalent metal counter ion (e.g. $Mg^{++}$, $Ni^{++}$). Both of these techniques are discussed separately below.

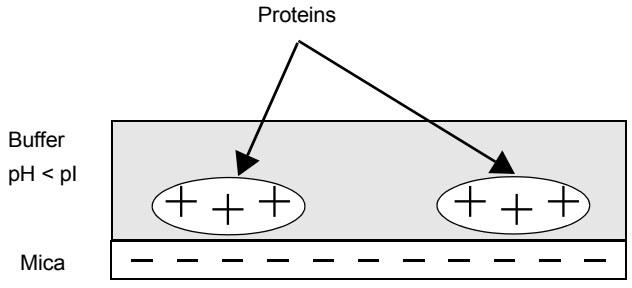
Many other techniques are being developed for chemically modifying mica, glass and other substrates to bind a variety of biological samples. Contact Bruker for a bibliography of references on imaging of biological specimens.

## 11.6 Lysozyme on Mica—A Model Procedure for Protein Binding

### 11.6.1 Protein Binding Theory

All proteins contain free amino groups that become positively charged at sufficiently low pH. If sufficient free amino groups are located on the outside surface of the protein, then the protein will bind to a negatively charged mica surface. Proteins will become positively charged at pH below their isoelectric point and are then able to bind to mica. The protein lysozyme, for example, becomes sufficiently positively charged to bind to mica at pH 6. This is shown schematically below in Figure 11.6a.

**Figure 11.6a** Proteins will typically bind to negatively charged mica when the pH is reduced below the protein's isoelectric point, pI



## 11.6.2 Protein Binding Procedure

The following section gives a detailed procedure for preparing and imaging the protein lysozyme by tapping mode in fluid. The procedure was kindly provided by Monika Fritz at the University of California, Santa Barbara and is described in the following paper:

- Radmacher, M., M. Fritz, H.G. Hansma, P.K. Hansma (1994). "Direct Observation of Enzyme Activity with Atomic Force Microscopy." *Science* **265**, 1577.

1. Obtain the required materials:
   - Deionized water
   - Mica substrates
   - Lysozyme protein L-6876 from Sigma Chemical
   - Phosphate buffer solution, 10 mM $KH_2PO_4$, 150 mM KCl, pH 6 (buffer may be adjusted for other proteins)
   - Tapping Mode Fluid Cell, Model MTFML
   - Cantilevers (Oxide-Sharpened Silicon Nitride tips, Model NP-S, work well)
   - Source of filtered (0.2 µm), compressed air or dry nitrogen
   - UV lamp, high-intensity; Oriel Mod. 6035 pencil-style spectral calibration lamp or equivalent (optional for cantilever cleaning).
   - Syringes: (1) 1 cc; (2) 5 cc; Micropipettes
   - Fluid cell liquid lines (silicone tubing and fittings)
   - Fluid cell o-ring (optional, see **Method 1 (with an O-ring):** Page 198).
   - Tubing clamps (for liquid lines).
   - Filter paper
2. Dissolve the lysozyme in phosphate buffer (PBS) solution to a concentration of 1µg per ml (this concentration provides a convenient coverage for AFM imaging and may be used for a variety of similar size samples). This mixture should be drawn into a clean, 1 cc syringe and capped. Prepare another 5 cc syringe of straight buffer solution.
3. Prepare the fluid cell for tapping mode in fluid operation. Clean the fluid cell and load a cantilever. For best results, clean the cantilever with UV light.
4. Cleave a fresh mica surface by first pressing some adhesive tape against the top mica surfaces, then peeling off the tape.

5. Deposit 50µl of protein solution on the freshly cleaved mica.

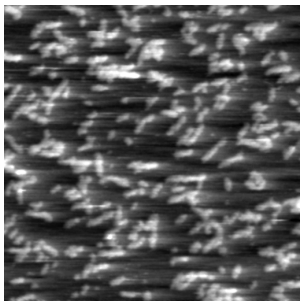
6. Allow 20-30 minutes for the protein solution to bind to the mica substrate. Binding time may vary with different samples. For longer binding times, put the mica in a covered dish with a wet piece of filter paper to keep the liquid from evaporating.

7. Rinse the sample with a large quantity of buffer to remove unbound protein. Leave a drop of buffer on the mica.

8. Mount the sample on the scanner end cap. Seal the fluid cell and fill with buffer.

9. After the fluid cell has been flushed with buffer solution, reclamp the drain line. This is important for low-noise, low drift imaging. The sample is now ready for tapping mode imaging. A good tapping mode image of lysozyme protein on mica is shown in Figure 11.6b

**Figure 11.6b** Tapping mode image of lysozyme in buffer solution using above sample preparation (Scan size = 500nm).



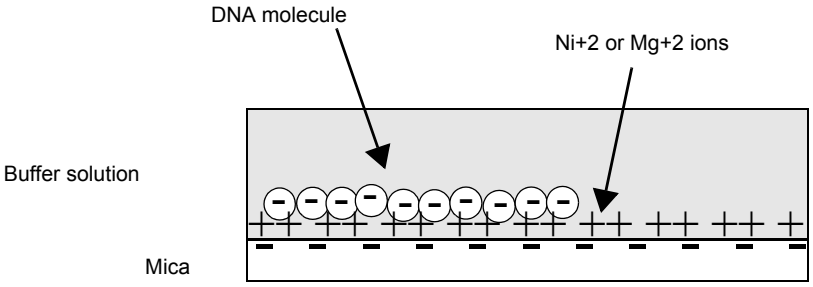
**Note:** It is also possible to prepare samples inside the fluid cell by flowing the protein solution through the fluid cell. In this case, it may be helpful to engage the tip in contact mode with a zero **Scan size** to protect proteins from binding to the tip.

## 11.7 Binding DNA to Mica

### 11.7.1 DNA Binding Theory

DNA and mica are both negatively charged, and so it is necessary to modify the mica surface or the DNA counter ion to allow binding. The counterion method is done by adsorbing the DNA onto the mica in the presence of a divalent (+2 charged) ion, like $Ni^{+2}$. The divalent ion will serve as a counterion on the negatively charged DNA backbone and will also provide additional charge to bind the mica. This is shown schematically in Figure 11.7a.

**Figure 11.7a** Negatively charged DNA may be bound to negatively charged mica in the presence of divalent counterions, such as $Ni^{+2}$.




## 11.7.2 DNA Binding Procedure

The following procedure is adapted from these sources:

- Dunlap, D.D., A. Maggi, M.R. Soria & L. Monaco (1997) "Nanoscopic Structure of DNA Condensed for Gene Delivery." *Nucl. Acids Res.* **25**, 3095.
- Kasas, S., N.H. Thomson, B.L. Smith, H.G. Hasma, X. Zhu, M. Guthold, C. Bustamante, E.T. Kool, M. Kashlev & P.K. Hasma (1997) "*Escherichia coli* RNA polymerase activity observed using atomic force microscopy." *Biochemistry* **36**, 461.
- Lyubchenko, Y.L. & L.S. Shlyakhtenko (1997) "Direct Visualization of Supercoiled DNA *in situ* with Atomic Force Microscopy." *Proc. Natl. Acad. Sci. USA* **94**, 496.

Many other references regarding DNA imaging are listed in the Biological Applications Bibliography; call Bruker for a copy.

1. Obtain the required materials:
   - Mica substrates
   - DNA: BlueScript II SK9(+) double stranded plasmid DNA, 2961 base pairs, 1mg/ml in 10mM Tris, 1mM ethylenediaminetetraacetic acid (EDTA) from Stratagene, La Jolla, CA.
   - Buffer solution: 10 mM HEPES and 5 mM $NiCl_2$ pH 7.6 (for loose binding and air imaging), or $NiCl_2$ (for tight binding and fluid imaging)
2. Dilute DNA in buffer solution to a final concentration of 2.5 ng/µl.
3. Glue a piece of mica to a metal support as described in **Section 11.2.6** on page 198. Cleave the mica substrate with a piece of adhesive tape. Place 30µl of the DNA solution in the center of the mica disk. The DNA will bind to the mica within 1 minute.

4. Load the prepared sample onto the AFM scanner and assemble the fluid cell, as previously described in **Section 11.2.6** on page 198. It may be helpful to wait for the temperature of the buffer to stabilize (20 minutes or more) before imaging.

The sample is now ready for tapping mode imaging.