

## Video Article

## Visualization of Recombinant DNA and Protein Complexes Using Atomic Force Microscopy

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DOI: 10.3791/3061

Citation: Murphy P.J.M., Shannon M., Goertz J. (2011). Visualization of Recombinant DNA and Protein Complexes Using Atomic Force Microscopy. *JoVE*. 53. <http://www.jove.com/details.php?id=3061>, doi: 10.3791/3061

## Abstract

Atomic force microscopy (AFM) allows for the visualizing of individual proteins, DNA molecules, protein-protein complexes, and DNA-protein complexes. On the end of the microscope's cantilever is a nano-scale probe, which traverses image areas ranging from nanometers to micrometers, measuring the elevation of macromolecules resting on the substrate surface at any given point. Electrostatic forces cause proteins, lipids, and nucleic acids to loosely attach to the substrate in random orientations and permit imaging. The generated data resemble a topographical map, where the macromolecules resolve as three-dimensional particles of discrete sizes (**Figure 1**)<sup>1,2</sup>. Tapping mode AFM involves the repeated oscillation of the cantilever, which permits imaging of relatively soft biomaterials such as DNA and proteins. One of the notable benefits of AFM over other nanoscale microscopy techniques is its relative adaptability to visualize individual proteins and macromolecular complexes in aqueous buffers, including near-physiologic buffered conditions, in real-time, and without staining or coating the sample to be imaged.

The method presented here describes the imaging of DNA and an immunoadsorbed transcription factor (i.e. the glucocorticoid receptor, GR) in buffered solution (**Figure 2**). Immunoadsorbed proteins and protein complexes can be separated from the immunoadsorbing antibody-bead pellet by competition with the antibody epitope and then imaged (**Figure 2A**). This allows for biochemical manipulation of the biomolecules of interest prior to imaging. Once purified, DNA and proteins can be mixed and the resultant interacting complex can be imaged as well. Binding of DNA to mica requires a divalent cation<sup>3</sup>, such as Ni<sup>2+</sup> or Mg<sup>2+</sup>, which can be added to sample buffers yet maintain protein activity. Using a similar approach, AFM has been utilized to visualize individual enzymes, including RNA polymerase<sup>4</sup> and a repair enzyme<sup>5</sup>, bound to individual DNA strands. These experiments provide significant insight into the protein-protein and DNA-protein biophysical interactions taking place at the molecular level. Imaging individual macromolecular particles with AFM can be useful for determining particle homogeneity and for identifying the physical arrangement of constituent components of the imaged particles. While the present method was developed for visualization of GR-chaperone protein complexes<sup>1,2</sup> and DNA strands to which the GR can bind, it can be applied broadly to imaging DNA and protein samples from a variety of sources.

## Protocol

## 1. Preparing DNA and protein samples to be imaged free of contaminants

1. Isolate biomolecules to be imaged and place in a suitable aqueous buffer. Proteins to be imaged may be purified using liquid chromatography<sup>6</sup>, immunoadsorption followed by removal of antibody and pellet<sup>1,2</sup>, or Profinity eXact purification and tag removal<sup>7</sup> (**Figure 2A**). Isolate DNA molecules to be imaged by miniprep purification (**Figure 2B**).
2. Confirm composition and purity of protein samples to be imaged using SDS-PAGE and western blotting. For DNA, confirm sequence and purity by completing a restriction digest and agarose gel electrophoresis.
3. Incubate samples in an AFM adsorption buffer to promote adherence of the sample to mica. For protein samples, 10 mM HEPES buffer may be used. Additional low-ionic strength salts or other cofactors may be added as well. For DNA samples, include a divalent cation (e.g. Mg<sup>2+</sup> or Ni<sup>2+</sup>) to promote adsorption to the mica substrate. A suitable Mg<sup>2+</sup>-containing buffer for DNA is 10 mM Tris, pH 7.5, 10 mM NaCl, 2 mM MgCl<sub>2</sub><sup>3</sup>. An alternative Ni<sup>2+</sup> buffer is 10 mM HEPES, pH 6.8, 10 mM NiCl<sub>2</sub>.
4. Dilute the sample to a concentration of 5 µg/ml in adsorption buffer. Gently mix. Store on ice while the microscope is being prepared.

## 2. Mounting AFM probe

1. Place the liquid cell probe holder onto the corresponding cantilever installation docking station.
2. Locate the long, thick cantilever (referred to as the 'B' cantilever) of the sharp nitride lever (SNL) probe to be used for imaging. Carefully transfer it to the liquid cell probe holder, ensuring that the tip remains upright. Technical note: Extreme care must be paid while the probe is in transit, as dropping it from any height may damage or destroy the cantilever or tip. A microlever SNL (MSNL) probe may be used as an alternative to the SNL probe.
3. Remove the probe holder from the docking station and examine the cantilever under a light microscope to ensure it is intact and properly seated in the liquid cell probe holder.
4. Carefully remove the AFM head from the instrument dovetail assembly by tightening the knurled head clamp screw. Technical note: Extreme care must be paid while manipulating the AFM head, as dropping it from even a short distance (e.g. if not seated correctly in the dovetail assembly) may cause substantial damage.
5. Invert the AFM head and firmly push the liquid cell probe holder onto the four pins at the base of the AFM head. Carefully return the AFM head into the instrument dovetail assembly. Release the knurled head clamp screw to fasten the AFM head into the instrument.

### 3. Locating cantilever tip, aligning laser, and adjusting photodetector

1. Using the AFM instrument software, locate the cantilever tip by moving the knobs of the onboard light microscope. Adjust the illumination of the microscope if necessary to improve tip identification. Bring the end of the cantilever tip into close proximity of the crosshairs displayed on the instrument software.
2. Bring the cantilever tip into focus by adjusting the up and down arrows of the optics controller on the software. Use slow (S) or medium (M) speed while adjusting the optics.
3. Align the laser to the cantilever tip using the laser adjustment knobs. Move the adjustment knobs until the red laser dot is inside the white illumination spot. Trace along the cantilever arm using a zig-zag pattern until the laser is positioned on the cantilever tip. When properly aligned, a strong reflection spot will appear in the front window of the AFM head.
4. Center the photodetector by adjusting the photodetector knobs on the AFM head. This will align the laser dot in the crosshairs of the photodetector display of the instrument software. The observed signal sum should be approximately 4-6.

### 4. Positioning AFM head and tuning cantilever

1. Place a freshly cleaved sheet of mica attached to a metal AFM specimen disc on the magnetized sample holder atop the AFM holder plate. This setup will be used to position the AFM head prior to imaging the DNA or protein sample prepared in Step 1.
  1. Technical note: The magnetized sample holder and liquid cell probe holder are thicker than other sample fastening mechanisms and probe holders. If there is currently insufficient clearance between the mica-sample holder assembly and AFM head, move the AFM head up by selecting the withdraw icon on the instrument software several times.
2. Rotate the AFM holder plate so the specimen disc is positioned for the initial imaging. Ensure the mica substrate is centered under the AFM head.
3. Move the AFM head down toward the surface of the mica-specimen disc using the focus surface control of the instrument software. Select the medium (M) speed on the Z motor, and continue towards the surface until the AFM head is approximately 2 mm above the mica. At this time, switch to using the slow (S) Z motor speed in order to avoid abrupt contact between the probe and the surface (referred to as crashing the tip).
4. Proceed to move the probe closer to the mica substrate surface until distinct features on the mica surface or the tip reflection are in focus. Toggle between surface and tip reflection focal planes using the "focus on:" icon from the instrument software.
5. Select the tune icon from the instrument software and tune the cantilever. The resonance frequency of the recommended SNL and MSNL cantilever probes is 20-60 kHz. Select a 5% peak offset. Technical note: Adequate cantilever tuning is essential for adequate sample imaging.

### 5. Imaging mica sample surface

1. Engage the probe onto the mica surface. Set initial scan size to 10  $\mu\text{m}$  and scan rate to 1 Hz. Integral gain and proportional gain can be initially set to 0.2 and 0.4, respectively. Adjust gains as needed in order to have trace and retrace scans roughly overlap. Decreasing the amplitude setpoint will increase the interaction between the probe and the mica and ensure engagement.
2. Capture complete scan images of the 10  $\mu\text{m}$  field. Decrease scan size to 5, 1, and 0.5  $\mu\text{m}$  and capture complete images of each field. Adjust gains and scan rate as necessary. Capturing images of the mica surface provides a baseline for comparison to the DNA- and protein-containing samples.
3. Disengage the probe with a single click on the disengage icon on the instrument software.

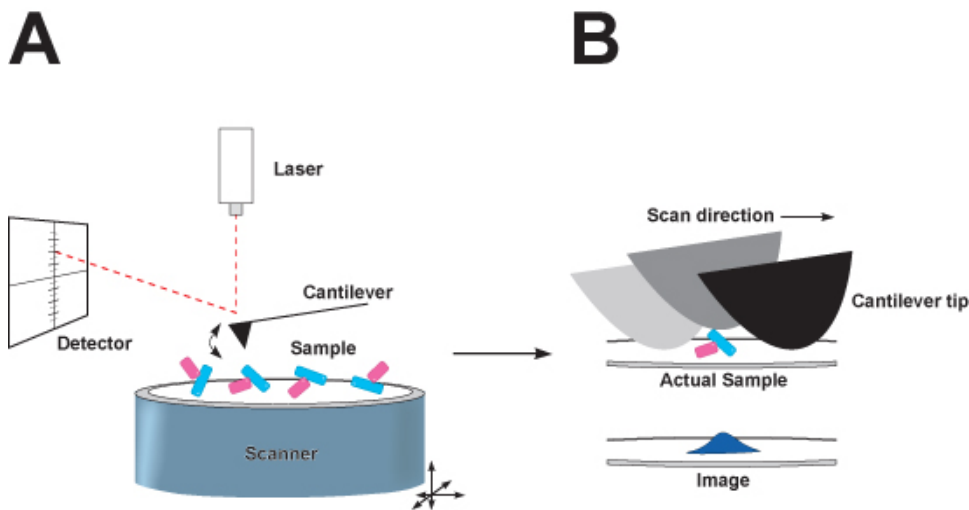
### 6. Imaging biomolecules of interest

1. Gently mix the DNA or protein sample prepared in Step 1 and prepare to load it onto the imaged mica surface. Mix 5  $\mu\text{l}$  of the prepared sample with 45  $\mu\text{l}$  of fresh adsorption buffer (final sample concentration = 0.5  $\mu\text{g/ml}$ ).
2. Carefully add 50  $\mu\text{l}$  of the 0.5  $\mu\text{g/ml}$  biomolecule-containing solution directly onto the mica surface. Do this without moving the probe or AFM head, and ensure the sample solution (but not pipet tip) makes contact with the probe.
3. Pause 5 minutes to allow the biomolecules in the sample to adhere to the mica surface.
4. At the conclusion of 5 minutes, pipet an additional 50-100  $\mu\text{l}$  of adsorption buffer into the liquid cell probe holder being careful to not touch the probe, AFM head, or mica with the pipet tip. This will form a meniscus and allow for AFM tapping mode imaging in fluid. **Figure 3** shows the final arrangement of the fluid cell, cantilever, sample, sample holder, and meniscus.
5. Readjust the photodetector and realign the laser to the cantilever as necessary. Technical note: viewing the probe tip and laser alignment is complicated due to the diffraction pattern caused by adding sample buffer into the probe holder. Manually retune the cantilever, if needed.
6. Re-engage the probe using the instrument software. Adjust vertical deflection, integral gain, and proportional gain as necessary. This will begin the process of re-imaging the section of mica scanned immediately prior to adding sample and capture images.
7. Increase scan size (500 nm-10  $\mu\text{m}$ ) to identify regions of interest. Zoom-in and reduce scan rate to 0.5 Hz to improve image resolution.
8. Once imaging is complete, disengage the probe by selecting withdraw icon on the instrument software several times. Ensure sufficient clearance between the AFM head and specimen disc before disassembly of the liquid cell or specimen disc.
9. Thoroughly rinse the liquid cell probe holder and specimen disc with distilled water to prevent salt crystals from forming as residual adsorption buffer evaporates. Dry with compressed air.

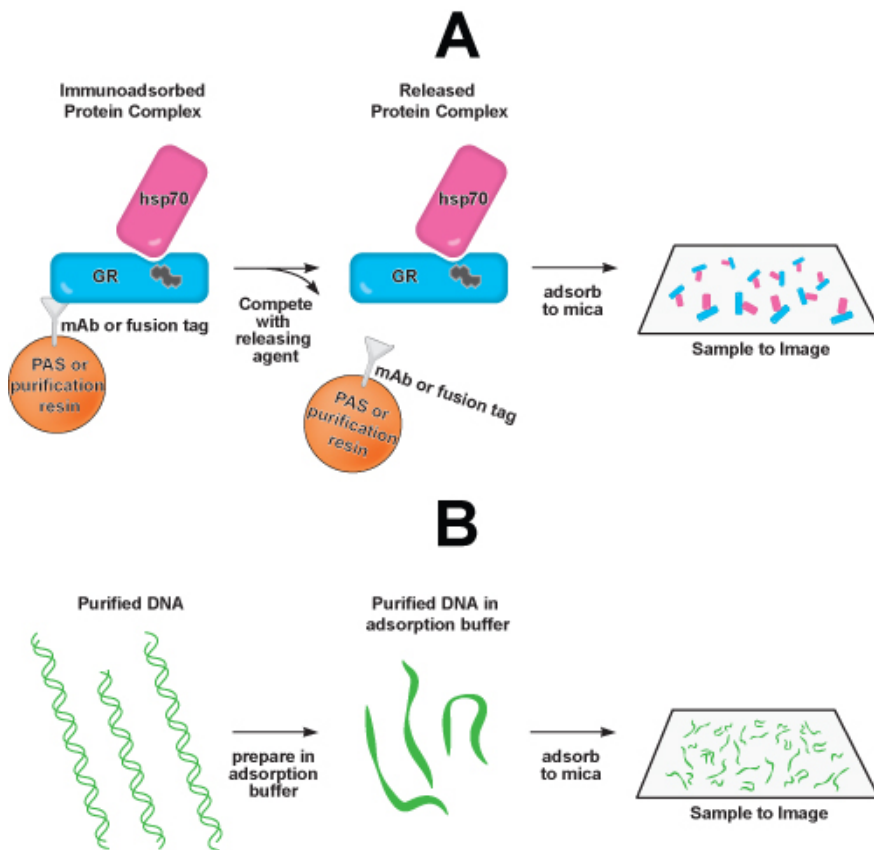
### 7. Representative Results:

Examples of AFM images are presented in **Figure 4**. Mica substrate (A) provides a molecularly flat surface onto which DNA and protein can adsorb. Imaging mica prior to biomolecule samples provides a negative control and assessment of imaging noise. It also provides a level of assurance that the cantilever is properly tuned and subsequent sample imaging will be successful. Double-stranded plasmid DNA (B, D), presumptively supercoiled, is readily identified by its asymmetric appearance and uniform depositing on the previously unremarkable mica substrate. Protein complexes of discreet particle sizes (C, E) are also uniquely distinguishable from the mica substrate. Particle size differences indicate sample heterogeneity, and can be useful to approximating protein complex stoichiometry or biochemical activity. The general diagonal shape and consistent orientation of the protein particles observed in Panel C is an imaging artifact, as proteins would expectedly be oriented on the mica substrate in a random fashion. Possible causes of the observed artifact is a physical tip abnormality or AFM imaging at too rapid of a scan rate. While tip convolution (illustrated in **Figure 1B**) prevents absolute length measurement calculations in the x and y axes, height

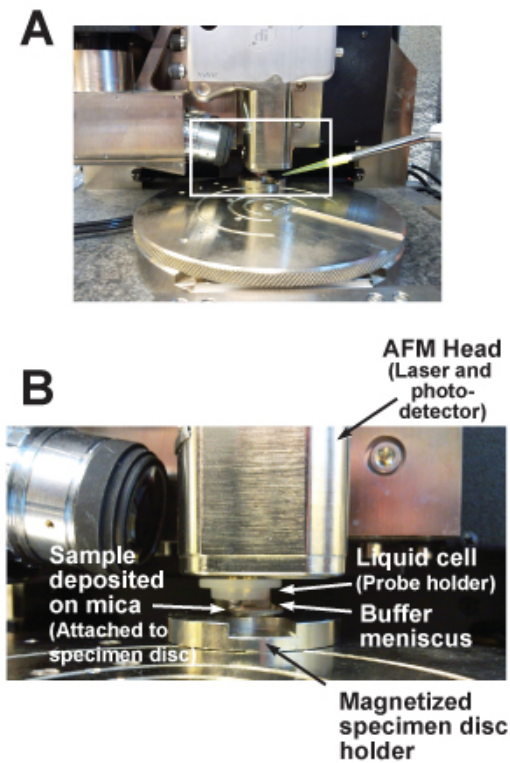
measurement (z axis) and relative x and y measurements may be nonetheless useful for estimating biophysical properties of the imaged biomolecules.



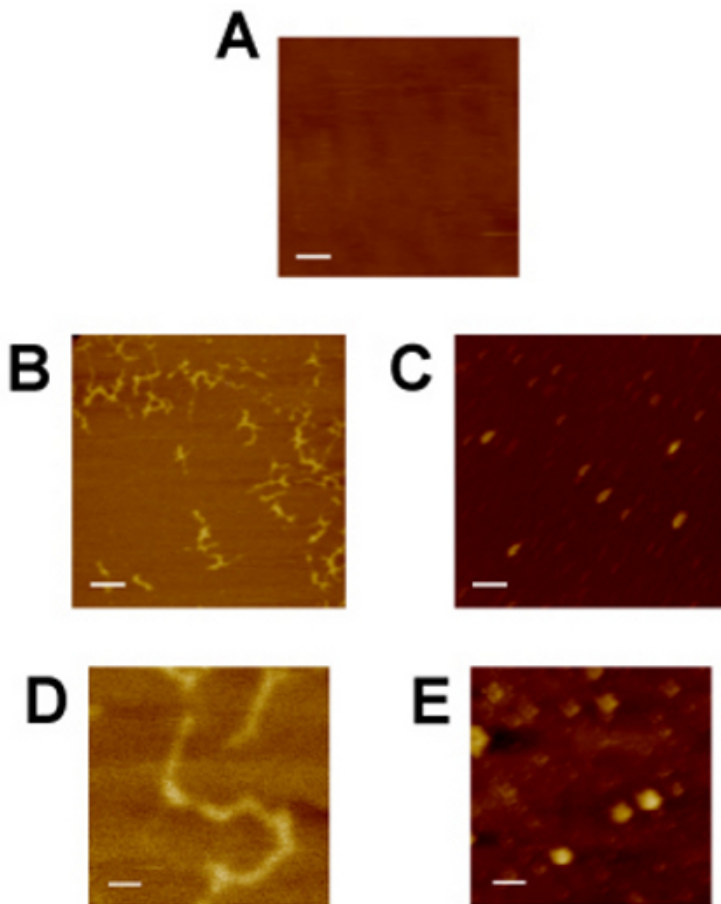
**Figure 1:** Schematic presentation of tapping mode atomic force microscopy (AFM). A, the microscope. At the end of the cantilever is a sharp tip that oscillates up and down as it scans over the surface of a mica substrate to which biomolecular complexes adsorb. As the scanner moves in the x and y directions, a laser beam is reflected from the back of the cantilever onto a position-sensitive photodiode detector to map the vertical (z) distance the tip moves as it passes over the biomolecular complexes seated on the mica. B. Distortion of the image in the x and y directions caused by tip convolution. The nominal radius of a conventional silicon nitride tip is larger than the particles to be imaged, and the edge of the tip contacts the sample as it traverses the surface. Tip convolution results in the imaged particles appearing larger in the x and y directions, but not the z direction. This effect can be minimized by the use of tips with a smaller nominal tip radius.



**Figure 2:** Illustration of immunoabsorbed protein and DNA sample preparation. A, Release of immunoabsorbed GR•hsp70 protein complex from the monoclonal antibody (mAb)-protein A-Sepharose (PAS) pellet. Incubating the immunopellet with a peptide containing the mAb epitope will facilitate the release of the GR•hsp70 protein complex from the pellet, allowing the complex to be collected from the supernatant and visualized by AFM. B, Preparation of DNA for AFM imaging requires the use of a divalent cation adsorption buffer. The divalent cation increases the affinity of DNA for the mica substrate.



**Figure 3:** Final arrangement of the AFM head, liquid cell probe holder, sample, specimen holder, and buffer meniscus. A, Care must be taken when depositing sample and additional buffer onto the mica substrate to avoid physical contact between the pipet tip and AFM head, liquid cell, and mica substrate. B, Magnification of A. The buffer meniscus spans from the specimen disc to the liquid cell probe holder.



**Figure 4:** Atomic force micrographs of mica substrate (A), 2xGal4-2xGRE-luciferase plasmid DNA <sup>8</sup> (B, D), and GR•hsp70 protein complexes (C, E) in buffered solution. Mica serves as a control image to compare with the visualizations of DNA and protein. GR•hsp70 protein complexes were prepared by immunoadsorption of GR primed with hsp70 and then released from the antibody-bead pellet using a mAb competing antibody (illustrated in Figure 2A). DNA was prepared by conventional plasmid miniprep. Panels A, B, and C are of an equivalent magnification (scale bars = 200 nm), as are Panels D and E (scale bars = 40 nm).

## Discussion

AFM provides a unique microscopic technique capable of imaging individual uncoated biomolecules in aqueous and near-physiologic buffered solutions in real-time. This allows for the visualization of individual proteins and DNA molecules, as well as multiprotein complexes and protein-DNA complexes. Imaging macromolecular particles with AFM can be useful for assessing sample homogeneity and for identifying the physical arrangement of the constituent components of the observed particles. This approach of observing individual macromolecular particles can be a useful adjunct to conventional biochemical techniques, such as immunoprecipitation assays, polyacrylamide gel electrophoresis, and size exclusion column chromatography, which provide significant summative data representing the  $10^3$ - $10^{11}$  individual biomolecular complexes of interest present in a sample.

Research into multiprotein complex stoichiometry, biomolecular interactions, and cofactor requirements can all be investigated using AFM. The method presented here can be adapted to accommodate specific biophysical questions of interest. For example, using a liquid cell probe holder capable of exchanging buffer, it is possible to assay cofactor requirements for protein complex formation. DNA-protein assemblies can be formed and dissociated in near-real-time and even while being imaged. DNA can be generated with specific sequences of interest (e.g. a presumptive response elements or novel protein binding sequence), mixed with the hypothesized binding protein, and imaged to provide direct evidence of intermolecular interactions.

Tapping mode AFM imaging is substantially less complicated if dry samples are utilized rather than samples in aqueous solutions, and linear DNA stands and closed-circle DNA plasmids have both been imaged in this way<sup>3,9</sup>. The method presented here utilizes samples in buffered solutions in order to provide a more physiological imaging environment. Other noteworthy methods of imaging proteins should also be considered, including near field infrared microscopy<sup>10</sup>. The complementary use of immunoabsorption in consort with AFM provides an opportunity to prepare a myriad of protein complexes, using well-established biochemical techniques, and then visualize them following release from the immunoabsorbing antibody-bead pellet. For example, immunoabsorbed GR has been assayed for its association with the molecular chaperone protein hsp70<sup>1</sup> as well as the dynein motor protein<sup>2</sup> using this approach in order to estimate complex size and stoichiometries. Particle size and biophysical features (e.g. rigidity) have been useful in ascertaining the identity of the biomolecules observed in AFM imaged samples<sup>11,12</sup>. It is also possible to confirm biomolecule identity by the addition of an interacting biomolecule (e.g. a ligand or monoclonal antibody), that would bind to its target and cause a particle size increase if the target biomolecule is present.

## Disclosures

No conflicts of interest declared.

## Acknowledgements

This work was funded by National Institutes of Health Grant GM086822. The authors would like to thank Drs. Alec Pakhomov & Paul Wallace (Univ. of Washington Nanotechnology User Facility (NTUF)) and Andrea Slade (Bruker AXS) for their expert technical assistance. DNA AFM imaging was conducted at the Univ. of Washington NTUF, a member of the National Nanotechnology Infrastructure Network. The 2xGal4-2xGRE-luciferase plasmid was kindly provided by the laboratory of Dr. Keith Yamamoto (Univ. of California, San Francisco).

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