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Adhesion Force Measurements Using an Atomic Force Microscope Upgraded with a Linear Position Sensitive Detector

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Abstract

The atomic force microscope (AFM), in addition to providing images on an atomic scale, can be used to measure the forces between surfaces and the AFM probe. The potential uses of mapping the adhesive forces on the surface include a spatial determination of surface energy and a direct identification of surface proteins through specific protein–ligand binding interactions. The capabilities of the AFM to measure adhesive forces can be extended by replacing the four-quadrant photodiode detection sensor with an external linear position sensitive detector and by utilizing a dedicated user-programmable signal generator and acquisition system. Such an upgrade enables the microscope to measure in the larger dynamic range of adhesion forces, improves the sensitivity and linearity of the measurement, and eliminates the problems inherent to the multiple repetitious contacts between the AFM probe and the specimen surface.

Introduction

The atomic force microscope (AFM)¹ is an imaging device capable of atomic resolution.² The AFM has proven to be a valuable tool in the imaging of a number of different biological samples, including globular protein.^{3–6} The AFM utilizes a small probe on a cantilever, which deflects in response to the intermolecular attractive and repulsive forces found in proximity to the sample.^{1,7,8} In addition to providing an image of the sample, the AFM can measure these intermolecular force.^{7–12}

A major drawback of the AFM is its inability to chemically identify the molecules of the specimen. One of the possible approaches to solving this problem is to analyze the experimentally measurable forces acting between the AFM probe and the surface of the specimen. These forces can be mapped during approach and retraction between the AFM probe and the specimen surface. The adhesive contact forces between the AFM probe and polymer surfaces have already been reported in literature.^{13,14} Such adhesive forces are nonspecific and can be directly related to the interfacial energy between the AFM probe and the specimen.¹³

In contrast to nonspecific forces, biological molecules primarily interact through specific molecular forces. The molecular recognition forces between a ligand and surface-bound

receptor have been measured recently using the surface force apparatus (SFA)¹⁵ and other techniques, including AFM.^{16–21} One expects that the forces required to separate a ligand from its specific binding site should be different from the forces needed to remove a nonspecifically bound ligand. Since the AFM can measure these differences of the specific adhesion forces in a spatially resolved manner, this “specific adhesion force contrast” can be used to identify binding molecules and determine their distribution on the surface of the specimen.

The dynamic range of forces that can be measured using commercially available atomic force microscopes is rather limited for several reasons. One cause of a limited range of force measurements is the actual cantilever position sensing system. In commercial AFM instruments the deflection of the AFM cantilever is measured by an optical lever technique utilizing a four-quadrant photodiode as a position sensor.²² The photodiode receives the laser beam as it reflects from the upper cantilever surface. While this optical lever technique is very appropriate for the “feedback” mode of scanning over the surface of a specimen, any large deflection of the cantilevers is likely to cause an erratic response of the four-quadrant photodiode detection system. As a consequence the system has a limited detection range of cantilever deflections, a drawback that is ever more important when the spring constant of the cantilever is low and the forces are large, as in the case of adhesive forces. If a larger object, such as a spherical silica bead, is used as an AFM probe, the problem becomes acute because the ensuing forces scale with the radius of the spherical probe. In some cases, the four-quadrant photodiode gives an erroneous response because the laser beam profile reflected from the AFM cantilever has a nonsymmetrical intensity profile. Such a nonuniformity is likely to occur when the force measurements are performed in a liquid medium and the reflected laser beam has to refract through a transparent window of an AFM flow cell.

The work in this report describes the modifications made to a commercially available AFM in order to optimize recording force measurements between biological molecules. A novel AFM cantilever deflection sensor and programmable piezodriver were utilized to measure forces in two experimental systems: (a) adhesion forces occurring in dilute electrolyte solution between the silica bead glued onto the AFM cantilever and a silica surface and (b) adhesion forces between biotin immobilized on the silicon nitride cantilever and streptavidin molecules bound to the biotin-coated silicon nitride surface.

Materials and Materials

AFM Cantilever Deflection Sensor and Electronics

The Nanoscope II atomic force microscope (Digital Instruments, Inc.) was modified as shown in Figure 1. The AFM stage, the AFM head including the laser diode, and the stepping motor which allowed the AFM head to be lowered onto the specimen surface were used from the original Nanoscope system. The *z*-position of the piezocrystal carrying the specimen was controlled by a separate computer-based function generator residing on a custom-built PC-interface card. The computer provided for a flexibility in programming of the timing, the speed, and the shape of the signal driving the piezocrystal. The D/A converter output (± 10 V) was fed into a custom-built high-voltage amplifier (± 150 V). The

four-quadrant photodiode supplied as an integral part of the optical level system was moved out of the optical path of the reflected laser beam. The laser beam fell onto a linear position sensitive detector (PSD; Model 1L30, SiTek ElectroOptics) placed at an optical distance of approximately 150 mm from the cantilever. According to the manufacturer, the nominal nonlinearity of the PSD was $\pm 0.05\%$ of the detector length (30 mm). The detector signal was amplified and filtered by a low-pass filter (EGG PARC, Model 113) and sent to one of the two synchronized A/D converters (12-bit conversion, 12-ps sampling speed). The output of the high-voltage amplifier was attenuated by a custom-built differential attenuator and monitored by the second A/D converter. In this way the two signals, (1) z -position of the piezocrystal and (2) PSD response, were simultaneously recorded with an adjustable sampling time. By use of simultaneous sampling, any phase shifts between the signals received by the two A/D converters were avoided.

Determination of Cantilever Displacement Limits for the Four-Quadrant Photodiode Detection System

A cantilever with an ultrasharp tip (Digital Instruments, Inc.) was lowered in contact with the surface of a cleaned glass coverslip in air sitting on the piezocrystal driven by the computer-generated piezodriving signal. The four-quadrant photodiode response ("A – B" signal) was fed into the modified AFM electronics, so that the signal was amplified and recorded by the same electronics as the PSD. The piezocrystal was moved upward a total of 1794 nm, before returning to its original starting position while the four-quadrant photodiode response was recorded as a function of piezocrystal movement.

PSD Linearity Measurements

To determine whether the PSD will respond linearly at ranges of cantilever deflection greater than those obtained with the four-quadrant photodiode detector system, an ultrasharp cantilever (Digital Instruments, Inc.) was engaged into contact with a cleaned glass microscope coverslip inside the fluid cell filled with 70% ethanol. The Nanoscope II stepping motor was used to further displace the cantilever upward in increments of 0.2, 0.4, or 0.8 μm . Using the stepping motor one avoids the hysteresis and drift associated with the piezocrystal and the undesired effect they would have on a linearity measurement.

Adhesion Force Measurements

The silicon nitride cantilevers without the integral pyramidal AFM tip were obtained from Park Scientific Instruments. A narrower rectangular cantilever (10- μm width, 100- μm length, 0.6- μm thickness), with a nominal spring constant of 0.08 N/m was used. The upper side of the cantilever was coated with gold to improve the reflectivity of the laser beam. In the experiment where a silica bead was used as a probe, the thin triangular cantilever was used (13- μm width, 100- μm , 0.6- μm thickness, 0.21 N/m nominal spring constant). The silica bead (Duke Scientific, approximately 20- μm diameter) was cleaned in chromic acid (80 °C) and glued (Speedbonder 325 Structural Adhesive, Loctite Corp.) to the underside of the cantilever by use of the optical microscope equipped with a micromanipulator. No attempts were made to measure the spring constant of the cantilever after the bead was glued.

To measure adhesion forces using a biotin–streptavidin recognition model, biotin was chemically bonded to the surface of the cantilever. The cantilevers were cleaned in an oxygen plasma (200 mmHg, 50 W) for 5 min. The cleaned cantilevers were immediately placed into a 2% solution of (mercaptopropyl)- dimethylethoxysilane (MPDMS) (Hüls) in toluene (EM Science), protected from light, and allowed to incubate for at least 12 h at room temperature. After successive washes in toluene, acetone, and ethanol, the cantilevers were placed in a solution of 50 mM Tris, pH 8.3, 5 mM EDTA. Iodoacetyl-LC-biotin (0.2 mM, Pierce Chemical) was reacted with the sulfhydryl group of MPDMS-coated cantilevers overnight in the dark at room temperature. The cantilevers were then washed with borate-buffered saline (BBS) and stored in BBS until used for a force measurement. The surface coverage of biotin on the cantilevers was not determined.

For the experimental system using silica bead glued to cantilevers, the surface chosen to generate adhesion forces was an oxidized silicon wafer (Si/SiO₂ surface, p-type, (100) orientation, highly polished with a roughness of 1 nm, HEDCO Microengineering Laboratories, University of Utah). The silicon wafer surface was cleaned in ethanol and oxidized in an oxygen plasma (200 mmHg, 50 W) for 2 min.

To measure adhesive forces using the streptavidin–biotin experimental system, silica wafers with a 25–30 nm CVD silicon nitride coating were utilized (Si/Si₃N₄ surface, HEDCO Microengineering Laboratories). Biotin was covalently bonded to the surface of the silicon nitride film by following the same method used to modify the cantilevers. After the attachment of biotin to the Si/Si₃N₄ surface, the sample was incubated in a solution of 1 μ M immunopurified streptavidin (Pierce Chemical) in BBS for 1.5 h at room temperature. The wafers were then washed with BBS and stored in BBS until used for a force measurement.

The cantilever with the silica bead was placed into a holder of an AFM fluid cell. The fluid cell was filled with a 0.01% (w/v) solution of F108 Pluronic surfactant (BASF) in 10 mM NaCl, pH 2.6, and the laser beam reflected from the cantilever was adjusted to fall onto the middle part of the position-sensitive detector. By use of the stepping motor, the AFM head was lowered until contact between the AFM probe and the Si/SiO₂ surface was established, i.e., until the position detector indicated a slight deflection of the cantilever due to its contact with the surface. The silica bead and the Si/SiO₂ surface were allowed to remain in contact for 30 s before the computer generated a movement of the piezocrystal driving the Si/SiO₂ surface 0.897 μ m away from the AFM probe in the *z*-direction and back to its original position. The speed of the movement was 0.45 μ m/s in both directions. The resulting signals were stored in a numerical form in the computer before further analysis.

Figure 2 shows the schematics of the measurements for the thin rectangular cantilever in the biotin–streptavidin system. The biotinylated Si/Si₃N₄ surface, with bound streptavidin, was positioned onto the piezocrystal and the flow cell assembled. The flow cell was filled with BBS and the laser beam adjusted to reflect off the back of the selected cantilever onto the middle part of the position-sensitive detector. The cantilever was lowered onto the surface by use of the stepping motor until the position detector indicated a slight deflection of the cantilever due to its contact with the surface. The measurements proceeded by a computer-generated movement of the piezocrystal in the *z*-direction, 0.897 μ m away from the AFM

probe and back to its starting position. The speed of movement was $0.45 \mu\text{m/s}$ in both directions.

Results

The adhesive forces generated between the AFM probe and surface can potentially lead to cantilever displacements which are larger than what the detection system utilizing the four-quadrant photodiode can measure. In this instance, the electronics and/or the photodiode can become saturated, and an erroneous force–displacement curve is recorded even though the actual relationship between the measured force and the relative surface separation may be quite different. To examine how limited the range of cantilever displacement measurements is in an unmodified AFM system, the four-quadrant photodiode was incorporated into the modified AFM electronics, and its response was recorded for various ranges of piezodisplacements when a relatively stiff cantilever was in contact with the rigid sample. As shown in Figure 3, the four-quadrant photodiode could only measure the range of cantilever displacements equal to or smaller than 986 nm .²³ For a $100\text{-}\mu\text{m}$ -long cantilever, this displacement amounts to an angular cantilever deflection of approximately 0.6° .

When a position-sensitive detector system is used to measure forces where large deflections of the cantilever are expected and/or large movements of the piezocrystal are used, its response has to be linear at these extremes in order to accurately record the experiment. Figure 4 shows the signal of the linear PSD when the cantilever displacement of known magnitude was recorded for a total displacement range of $8 \mu\text{m}$. The cantilever movements were made in the increments of 0.2 , 0.4 , or $0.8 \mu\text{m}$ in panels a–c of Figure 4, respectively. The correlation coefficient, R^2 , very nearly approached unity in every set of measurements, indicating that the PSD electronics provided an equivalent and linear response to all cantilever deflections in the displacement range as large as $8 \mu\text{m}$.

Adhesive force measurements made with the silica bead–silica surface experimental system in the absence of protein were measured with our modified microscope with the external linear PSD and the custom-designed signal acquisition system. A representative force–piezodisplacement plot is shown in the lower panel of Figure 5. The force axis was calibrated by measuring the deflection of the cantilever as it traveled a known distance while in contact with the surface and by multiplying the distance, d , with the manufacturer-provided cantilever spring constant, k , i.e., $F = k \cdot d$. The x -axis is the relative displacement of the piezocrystal generated by the user-programmable signal generator. The resultant adhesive force peak has a “right” triangle shape, with an abrupt cantilever release from the surface. The upper panel of Figure 5 shows the force–piezodisplacement plot recorded in the identical system with the four-quadrant detector and the Nanoscope II electronics.

Five successive measurements using the biotin–streptavidin–biotin experimental system were made in the same location on the sample surface with the narrow rectangular cantilever (Figure 6a–e). The first measurement resulted in an adhesive force peak with a rounded, dome appearance, indicating that after the adhesive contact was apparently broken, the cantilever was slowly being released back to its resting position. The force–distance traces having adhesive force peaks with a rounded appearance in our system and the systems of

others^{17,19} are characteristic of measurements of the specific interactions of proteins. With each successive measurement (Figure 6b–e), the adhesive force increases and the adhesive peak began to slowly take on the triangle shape, so that by the fifth measurement, the resultant peak was almost a perfect right triangle; resembling a force–displacement trace obtained with the proteinless silica bead–silica surface experimental system (Figure 5).

Discussion

The upgrade of the AFM with an external linear position sensitive detector (Figure 1) and a dedicated user-programmable function generator and data acquisition system provides several distinct advantages over the commercially available AFM instruments for use in adhesive force measurements. The four-quadrant photodiode accurately measures the displacement of the cantilever only when the reflected laser beam has a symmetrical cross section.²² An asymmetrically shaped laser beam produces an error in the cantilever displacement measurement. The external position-sensitive detector is not sensitive to the shape of the reflected laser beam providing that the beam profile does not change during the measurement.

A larger movement of the reflected laser beam on the four-quadrant photodiode can result in the saturation of the detection electronics during measurements. The larger area of the PSD allows detection of an increased range of cantilever deflections, as illustrated by the comparison between Figures 3 and 4. The PSD response remains linear at these greater ranges of cantilever deflections. Having this greater range of linear responses in the sensing electronics allows for a complete force measurement to be recorded accurately regardless of the amount of cantilever engagement or the magnitude of the resulting adhesive force (Figure 4).

The force–displacement measurement in commercial AFM instruments is based on a repetitious up–down cycling of the piezocrystal. For nearly all commercial AFM instruments, the force–displacement measurements cannot be executed before the AFM probe engages in contact with the specimen surface. The uncontrolled engagement into contact often leads to development of an excessive pressure acting on the specimen surface. When biological macromolecules, such as proteins, are present on the surface of the specimen, the excessive pressure can destroy their structure and make them biologically dysfunctional. An example of the cumulative effect of probe pressure on proteins can be seen in this work (Figure 6). Successive measurements made in the same location on the sample eventually resulted in force–displacement response indicative of the nonspecific adhesion (Figure 5). Therefore, an unmodified AFM could potentially destroy the protein during the process of engaging, before any force–displacement measurements are made. In the modified instrument, however, the cantilever with the AFM probe is lowered onto the surface of the specimen by means of the stepping motor while the piezocrystal is at rest. The occurrence of the contact is monitored by the position detector.

Although the speed of the repetitious up–down movement of the piezocrystal in commercial AFMs can be adjusted, the control over the length of the binding time between the molecules on the AFM probe and the specimen surface is not available. In our modified

AFM, upon establishment of contact, the binding can occur for any desired period of time, after which the movement of the piezocrystal in the z-direction is initiated by a user-programmable signal generator. The use of a dedicated signal generation system provides for flexibility in programming of the timing, speed, and shape of the voltage function driving the piezocrystal and the sampling speed of the acquisition system. In this way, the modified AFM can be optimized for a particular force–displacement measurement.

Improved sensitivity of the modified AFM may be another advantage. Small movements in the cantilever will result in larger movements of the laser beam the further away from the microscope the beam is intercepted. The beam will move a greater distance on the external PSD than the four-quadrant photodiode because the PSD can be placed a greater distance from the microscope.

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23. According to the manufacturer (Digital Instrument, Inc.), the dynamic range of the four-quadrant photodiode detector has been extended in the latest version of Nanoscope III. In addition, a “false engagement” possibility is provided which enables the user to record the force–displacement curve doing the first up–down cycle.

Figure 1.

Schematics of the modification of Nanoscope II AFM (Digital Instruments) for the force–displacement measurements. The z -position of the piezocrystal was controlled by a separate computer-based signal generator residing on a custom-built PC-interface card. The D/A converter output (± 10 V) was fed into a custom-built high-voltage amplifier (± 150 V). The laser beam reflected from the cantilever fell onto a linear position sensitive detector (PSD; Model 1L30, SiTek ElectroOptics) placed at an optical distance of approx. 150 mm from the cantilever. The detector signal was amplified and filtered by a low-pass filter (EGG PARC, model 113) and sent to one of the two synchronized A/D converters (12-bits conversion, 12 μ s sampling speed) residing on the custom-built PC-interface card. The output of the high voltage amplifier was attenuated by a custom-built differential attenuator and monitored by the second A/D converter.

Figure 2.

Schematics of the specific adhesion force measurements between the biotinylated rectangular cantilever and the biotinylated Si/Si₃N₄ surface with bound streptavidin. Objects are not drawn to the same scale.

Figure 3. Range of the detectable cantilever displacement using the four-quadrant photodiode A–B signal and the modified AFM electronics. The arrows indicate the dynamic range of the displacement measurements.

Figure 4.

Linearity of the PSD response in the 8-μm range of the cantilever displacements in (a) 0.2-, (b) 0.4-, and (c) 0.8-μm displacement increments. The straight line, its equation and R^2 , is calculated by the linear regression.

Figure 5.

Comparison between two force–displacement measurements in an identical experimental system using two different position-sensitive detection systems: (upper panel) force–displacement output of the Nanoscope II instrument; (lower panel) force–displacement measurement recorded by the external linear position sensitive detector and custom-built electronics (Figure 2).

Figure 6.

Comparison between five successive force–displacement measurements (a–e) in the biotin–streptavidin–biotin system performed on the same location of the sample surface using the PSD and the custom-built electronics (Figure 2). The contact time between the biotin–streptavidin surface and the biotinylated narrow rectangular cantilever was 30 s between each measurement.