

Fast imaging and fast force spectroscopy of single biopolymers with a new atomic force microscope designed for small cantilevers

M. B. Viani,^{a)} T. E. Schäffer, G. T. Palocz, L. I. Pietrasanta, B. L. Smith, J. B. Thompson, and M. Richter

Department of Physics, University of California at Santa Barbara, Santa Barbara, California 93106

M. Rief and H. E. Gaub

Lehrstuhl für Angewandte Physik, Ludwig-Maximilians-Universität, 80799 München, Germany

K. W. Plaxco

Department of Chemistry, University of California at Santa Barbara, Santa Barbara, California 93106

A. N. Cleland, H. G. Hansma, and P. K. Hansma

Department of Physics, University of California at Santa Barbara, Santa Barbara, California 93106

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Small cantilevers allow for faster imaging and faster force spectroscopy of single biopolymers than previously possible because they have higher resonant frequencies and lower coefficients of viscous damping. We have used a new prototype atomic force microscope with small cantilevers to produce stable tapping-mode images ($1\ \mu\text{m} \times 1\ \mu\text{m}$) in liquid of DNA adsorbed onto mica in as little as 1.7 s per image. We have also used these cantilevers to observe the forced unfolding of individual titin molecules on a time scale an order of magnitude faster than previously reported. These experiments demonstrate that a new generation of atomic force microscopes using small cantilevers will enable us to study biological processes with greater time resolution. Furthermore, these instruments allow us to narrow the gap in time between results from force spectroscopy experiments and molecular dynamics calculations. © 1999 American Institute of Physics.

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I. INTRODUCTION

The atomic force microscope (AFM) has proven to be a versatile tool for studying biological systems. For example, the AFM has been used in liquid tapping mode¹ to image dynamic biological processes such as transcription² and polymerization of fibrin.³ More recently, AFM has been used, in a completely different type of experiment, to perform force spectroscopy of single biomolecules.⁴⁻⁶ Force spectroscopy experiments provide a powerful method for characterizing the mechanical properties of single molecules.⁷⁻⁹

Both of these methods, dynamic imaging and force spectroscopy, are limited by their respective time scales. In the case of imaging, commercially available AFMs typically require a minute or more to acquire a single image. Since many interesting biological processes occur in times shorter than a minute, there is much to be gained by increasing the image acquisition rate. In the case of force spectroscopy experiments, it is possible to fit phenomenological models to force spectroscopy data taken over greatly varying time scales and obtain quantitative information about the energy landscape of the probed single molecules.⁹⁻¹¹ Increasing the speed at which these experiments are performed should make new features in the energy landscapes of single molecules accessible to experimental detection. Furthermore, faster measurements will narrow the large gap between the time scales of experiment and the time scales of molecular dy-

namics calculations, thereby allowing for more confident extrapolations between theory and experiment.

We report here the ability to take repeated (>20) liquid tapping-mode images of DNA adsorbed onto mica with an image acquisition time of 1.7 s. These short image acquisition times are possible because small cantilevers have both high-resonant frequencies in liquids (>100 kHz), which allow for high-tapping frequencies, and low-spring constants (<0.5 N/m), which minimize sample damage. We also report results of force spectroscopy experiments carried out on the multidomain protein titin,^{7,8,12} in which we probe the force dependence of unfolding at significantly higher rates than those previously reported.⁶

II. EXPERIMENTAL METHODS

All imaging and force spectroscopy experiments were performed in the prototype AFM that is depicted in Fig. 1. This microscope is an improved version of an earlier prototype AFM designed for small cantilevers.¹³ As in the prior version, this microscope uses optical beam deflection¹⁴ to detect the cantilever motion. The optics were designed using a ray-tracing program¹⁵ and have a calculated effective numerical aperture of 0.3. The focused spot on the cantilever has a measured $1/e^2$ diameter (in air) of $3.4\ \mu\text{m}$, which is about twice what we would expect for a diffraction-limited spot. Note that two of the lenses are adjustable in order to allow the user to refocus the laser onto the cantilever when operating in different media (such as air or water).

^{a)}Corresponding author; electronic mail: viani@physics.ucsb.edu

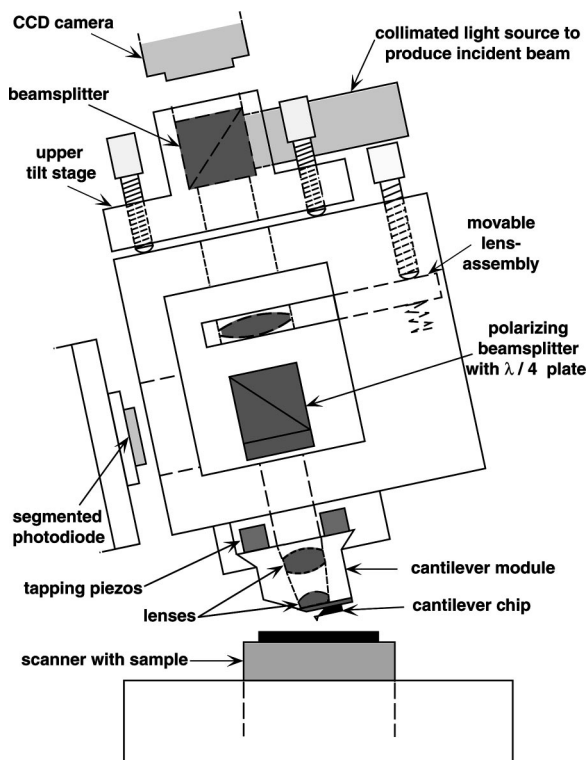


FIG. 1. Schematic of a new prototype AFM designed for small cantilevers. Cantilever motion is detected via optical beam deflection. This is done by using high numerical aperture optics to focus a laser beam in a tight spot onto the cantilever.

The cantilevers used for both imaging and force spectroscopy are nominally 100 nm thick, 3–5 μm wide, and 10–14 μm long. These cantilevers have measured resonant frequencies in water ranging between 100 and 200 kHz and estimated spring constants of 0.1–0.2 N/m. Figure 2 shows an array of cantilevers that are similar to those used in the experiments. The cantilevers were made out of low-stress silicon nitride using standard micromachining techniques¹⁶ and the tips were grown via electron-beam deposition.¹⁷

All images were taken in liquids via tapping mode. A

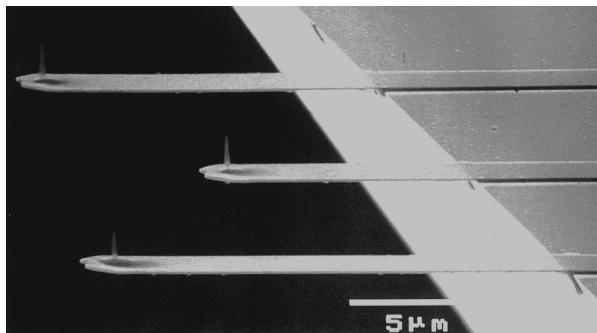


FIG. 2. An array of small cantilevers fabricated using micromachining techniques. The cantilevers are made from low-stress silicon nitride and are 100 nm thick, 5 μm wide, and 14–24 μm long. The tips are grown by electron-beam deposition. Small cantilevers have much higher resonant frequencies than large cantilevers making them useful for large-bandwidth applications. For example, a 10- μm -long cantilever with a measured spring constant of 62 mN/m has a resonant frequency in water of 130 kHz. In contrast, a 200- μm -long commercially available cantilever with a similar spring constant has a resonant frequency in water of only 4 kHz.

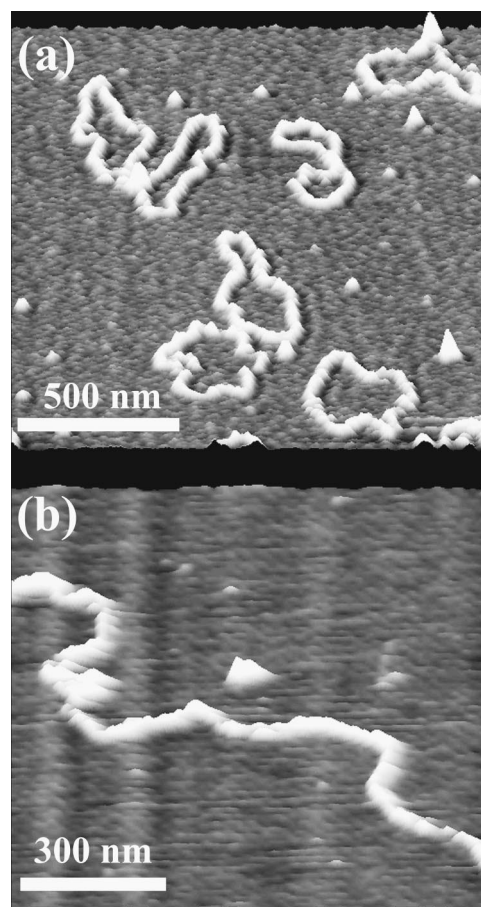


FIG. 3. Tapping-mode images (taken with small cantilevers) of DNA on mica in buffer solution. (a) This image (256 \times 256 pixels) of pBluescript was taken in 5.6 s, corresponding to a scan rate of 30.5 lines/s. The tapping frequency was 130 kHz. (b) This image (128 \times 128 pixels) of Lambda BstE II Digest was taken in 1.7 s, corresponding to a scan rate of 73.2 lines/s. The tapping frequency was 191 kHz. In both of these images the width of the DNA appears large: 30–50 nm. These large widths are a result of blunt tips.

Nanoscope III controller and software¹⁸ was used to run the microscope during imaging. However, in order to increase the imaging bandwidth the deflection signal was bandpass filtered around the tapping frequency, and fed to an external rms-to-dc converter with a user set bandwidth of 5–10 kHz. A setpoint was subtracted from the output of the external rms-to-dc converter and this signal was fed back into the AFM as the error signal. This technique has been described in detail elsewhere.¹⁹

Figure 3 shows two tapping-mode images of DNA adsorbed onto mica in a buffer solution.²⁰ Figure 3(a) is a tapping-mode height image (256 \times 256 pixels) of circular DNA molecules²¹ and was acquired with a tapping frequency of 129 kHz in a time of 5.6 s. The cantilever used had a measured resonant frequency of 163 kHz in water. Figure 3(b) is a tapping-mode height image (128 \times 128 pixels) of linear DNA molecules²² and was taken with a tapping frequency of 191 kHz in 1.7 s. The cantilever used had a measured resonant frequency of 227 kHz in water.

Force spectroscopy data were taken using macros that we wrote in IGOR PRO.²³ The software interfaced to the AFM via a data acquisition board and allowed the user to apply a time-dependent voltage signal to control the Z motion of the

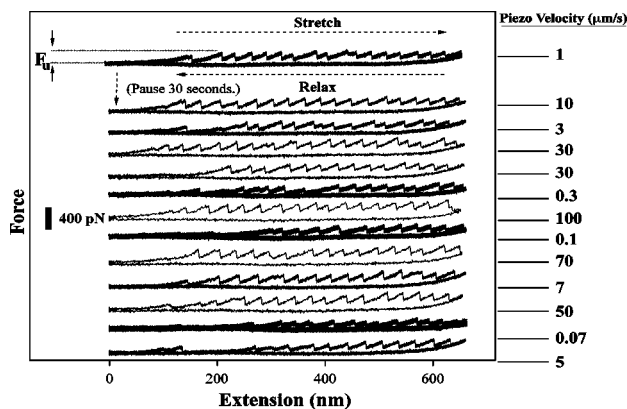


FIG. 4. Consecutive stretch-relaxation cycles of a single titin molecule. Note the sawtooth pattern; as the molecule is stretched the force increases until it is reduced by the unfolding of a domain. Each “tooth” corresponds to the successive unfolding of individual domains. After relaxing the molecule to zero extension, it is possible to restretch the molecule and obtain another sawtooth pattern. Here, we show a set of 13 consecutive stretch-relaxation cycles of a single titin molecule in which the piezo velocity v_p is varied for each cycle, as indicated, over three orders of magnitude. We waited for approximately 30 s after the end of one cycle before beginning the next one so that the molecule was able to fully refold. Note that F_U is defined as the unfolding force for an individual domain.

piezo while simultaneously monitoring the cantilever deflection and actual voltage at the piezo. Both channels were sampled at a rate of 100 kHz.

The force spectroscopy experiments were carried out as follows. First, native titin was diluted by adding 25–50 μl of phosphate buffered saline (pH 7.4) to 2.5–5 μl of titin (0.5 mg/ml) on a freshly cleaved mica surface. The titin was allowed to adsorb onto the mica for approximately 30 min and then the sample was thoroughly rinsed to remove unbound molecules. Next, the sample was mounted in our prototype AFM and more buffer was added. The tip of the cantilever was brought into contact with the sample for 1–60 s. In this process, much like fly fishing, in a few percent of the attempts the tip “catches” the amino acid chain of a single protein molecule. The molecule can then be stretched by moving the sample away from the cantilever and relaxed by moving the sample back towards the cantilever.

In Fig. 4, the measured force-versus-extension curves for 13 consecutive stretch-relaxation cycles are shown for a single titin molecule. We waited for 30 s between cycles in order to let the molecule fully refold. The stretch-relaxation cycles were performed with piezo velocities varying over three orders of magnitude (0.07–95 $\mu\text{m/s}$).

III. ANALYSIS AND DISCUSSION

To a first approximation, the pixel frequency f_p of an image should be less than 1 over the time necessary for the cantilever to come to equilibrium. More specifically, this means $f_p < f_c/Q$ where f_c is the cantilever resonant frequency and Q is its quality factor. Since the quality factor of small cantilevers in liquid is of order 1 (usually in the range of 1.1–1.5), then the above inequality requires the pixel frequency to be less than the cantilever’s resonant frequency. It is also necessary that the tip actually touch the sample in the time necessary to acquire one pixel; therefore, the pixel fre-

quency should also be less than the tapping frequency. For stable imaging it is usually necessary to keep the tapping frequency f_T less than or equal to the resonant frequency of the cantilever. Therefore, an order of magnitude estimate on the upper limit of the pixel frequency is $f_p < f_T \leq f_c$. Since small cantilevers have a much higher resonant-frequency-to-spring-constant ratio than their larger counterparts, they can be used to image faster without introducing extra sample damage from increased cantilever stiffness.

In the images presented in Fig. 3, the pixel frequency [23.4 kHz in Fig. 3(a) and 18.8 kHz in Fig. 3(b)] is significantly less than the tapping frequency. The cantilever tapped the sample an average of 5.5 times per pixel for the image in Fig. 3(a) and 12 times per pixel for the image in Fig. 3(b). Therefore, for both of these images, the cantilever was not the limiting factor in the imaging speed. However, the pixel frequency of these images are larger than the resonant frequency of the piezotube in the z direction (~ 15 kHz). In order to image faster, the bandwidth of the feedback loop needs to be increased. Even though the feedback was not optimal, we were still able to obtain multiple (>50) images in a single scan session, at fast imaging speeds (<5 s per image), without substantial sample damage.

The speed with which force spectroscopy data can be taken is limited by the cantilever’s resonant frequency and coefficient of viscous damping. The resonant frequency sets the usable bandwidth of the cantilever. In the ideal measurement, the cantilever’s resonant frequency should be much greater than the frequency range of the measured forces. In this case, the cantilever’s amplitude response function can be considered frequency independent and the deflection signal of the cantilever is proportional to the force being applied to the cantilever. However, in the case where the resonant frequency of the cantilever is comparable to the frequency of

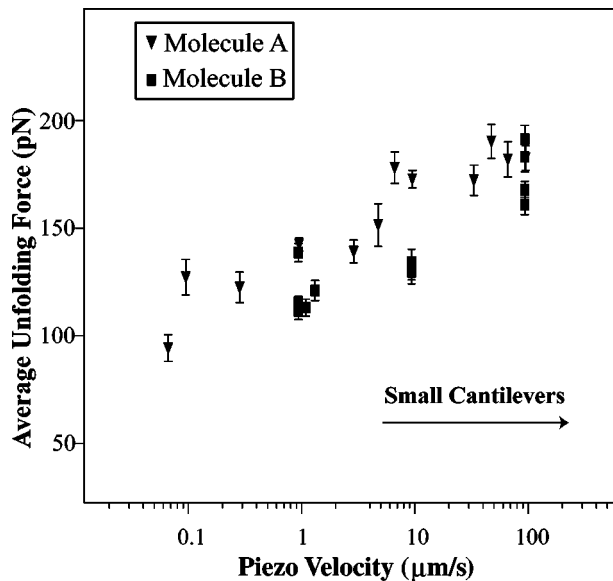


FIG. 5. Average force of unfolding plotted as a function of the piezo velocity. Each data set shown corresponds to a single molecule, which was repeatedly stretched at different rates. The average unfolding force $\langle F_U \rangle$ is calculated by averaging F_U for all unfolding events within a single stretch-relaxation cycle of the molecule. The data set labeled “molecule A” corresponds to the raw data shown in Fig. 4.

the force being measured, the cantilever deflection signal will be dependent on both the magnitudes of the force and the cantilever's frequency-dependent amplitude response function. In the data presented here the fastest stretch-relaxation cycle was performed at $95 \mu\text{m/s}$, which is 30 times faster than has been previously reported.⁶ At this piezo velocity the measured "sawtooth" force signal exerted by the molecule on the cantilever had a frequency of 3 kHz, which is well below the resonant frequency of the cantilever used (110 kHz). The cantilever's coefficient of viscous damping also becomes important at very fast piezo velocities because viscous forces on the cantilever can become comparable to the forces of interest, i.e., the forces being exerted by the molecule on the cantilever. In the data presented in Fig. 4, the effect of viscous forces on the cantilever is negligible except for the very fastest cycles where the magnitude of the viscous force becomes comparable to the noise.²⁴ Since commercially available cantilevers have coefficients of viscous damping one order of magnitude larger than small cantilevers, this experiment is only possible with small cantilevers.¹⁶

Figure 5 shows the average unfolding force $\langle F_u \rangle$ plotted as a function of piezo velocity for two data sets. The unfolding force has a logarithmic dependence on the piezo velocity, which has been previously reported for receptor-ligand bonds¹¹ as well as for titin unfolding.⁶ The logarithmic relationship arises from the contribution of thermal fluctuations, which provide the additional energy necessary to unfold the protein when the external force is too small to overcome the energy barrier that prevents unfolding. If the force is applied relatively slowly to the bond, there will be more time for thermal fluctuations to provide this additional energy, thus the unfolding will occur at a relatively low force. In contrast, if the force is applied at a higher rate, there will be less time for thermal fluctuations and the unfolding will occur at a relatively higher force.

Recent force spectroscopy experiments performed on receptor-ligand bonds have shown that it is possible to learn about specific features in the energy landscape of single molecules by performing the experiments over many orders of magnitude in speed.¹¹ Increasing the speed with which force spectroscopy experiments can be performed opens the door for learning about new features in single molecule energy landscapes. Furthermore, molecular dynamics simulations of force spectroscopy experiments are performed at pulling speeds over six orders of magnitude faster than experiment.²⁵

Decreasing the gap between experimental and theoretical time scales will allow for more confident comparisons between results.

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¹⁹D. A. Walters *et al.*, *Proc. SPIE* **3009**, 43 (1997).

²⁰Samples were prepared by putting $30 \mu\text{l}$ of DNA solution (0.5–1 $\mu\text{g/ml}$ in 10 mM HEPES (pH-7.5)/1 mM NiCl_2) onto freshly cleaved mica.

²¹pBluescript purchased from Stratagene, La Jolla, CA 92037.

²²Lambda BstE II Digest purchased from Sigma, Saint Louis, MO 63101.

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²⁴The magnitude of the viscous forces can be seen in the stretch-relaxation cycles near zero extension as force hysteresis.

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