

Sensing specific molecular interactions with the atomic force microscope

E.-L. Florin, M. Rief, H. Lehmann, M. Ludwig, C. Dornmair, V.T. Moy
& H.E. Gaub

Physikdepartment, Technische Universität München, 85748 Garching, Germany.
Tel: +49 89 3209 2487 Fax: +49 89 3209 2469.

Abstract: One of the unique features of the atomic force microscope (AFM) is its capacity to measure interactions between tip and sample with high sensitivity and unparalleled spatial resolution. Since the development of methods for the functionalization of the tips, the versatility of the AFM has been expanded to experiments where specific molecular interactions are measured. For illustration, we present measurements of the interaction between complementary strands of DNA. A necessary prerequisite for the quantitative analysis of the interaction force is knowledge of the spring constant of the cantilevers. Here, we compare different techniques that allow for the *in situ* measurement of the absolute value of the spring constant of cantilevers.

INTRODUCTION

The evolution of scanning probe microscopes (Binnig & Rohrer, 1987), and in particular, the development of atomic force microscopes (AFM) (Binnig *et al.*, 1986), have resulted in a family of novel instruments that allow for the mapping of local physical and chemical properties of samples with molecular and, in certain cases, even atomic resolution. As such, the tip of a scanning probe microscope may be regarded as a nanoscopic force sensor and the piezo scanner as an actuator with the precision of fractions of an Ångstrom. Furthermore, the AFM and other scanning probe techniques can be operated under ambient conditions in aqueous environments, thus opening up a variety of novel applications in life sciences (Hansma *et al.*, 1988; Engel, 1991; Radmacher *et al.*, 1992; Fritz *et al.*, 1994; Hansma

& Hoh, 1994). Recently, the AFM was applied as a force apparatus to measure specific molecular interactions between individual molecular pairs (Florin *et al.*, 1994; Lee *et al.*, 1994a,b; Moy *et al.*, 1994a,b). As illustrated in the overview in Fig. 1, the AFM joins a growing number of techniques designed to probe the intramolecular and intermolecular forces in biological systems (Kishino & Yanagida, 1988; Evans *et al.*, 1991; Smith *et al.*, 1992; Svoboda *et al.*, 1993; Leckband *et al.*, 1994).

An essential requirement for the quantitative measurement of interaction forces is an accurate method for the calibration of the spring constant of the AFM cantilever. Since the widely used commercially available silicon nitride cantilevers vary significantly in their spring constant, several methods for the calibration of AFM cantilevers have been proposed and are used in different

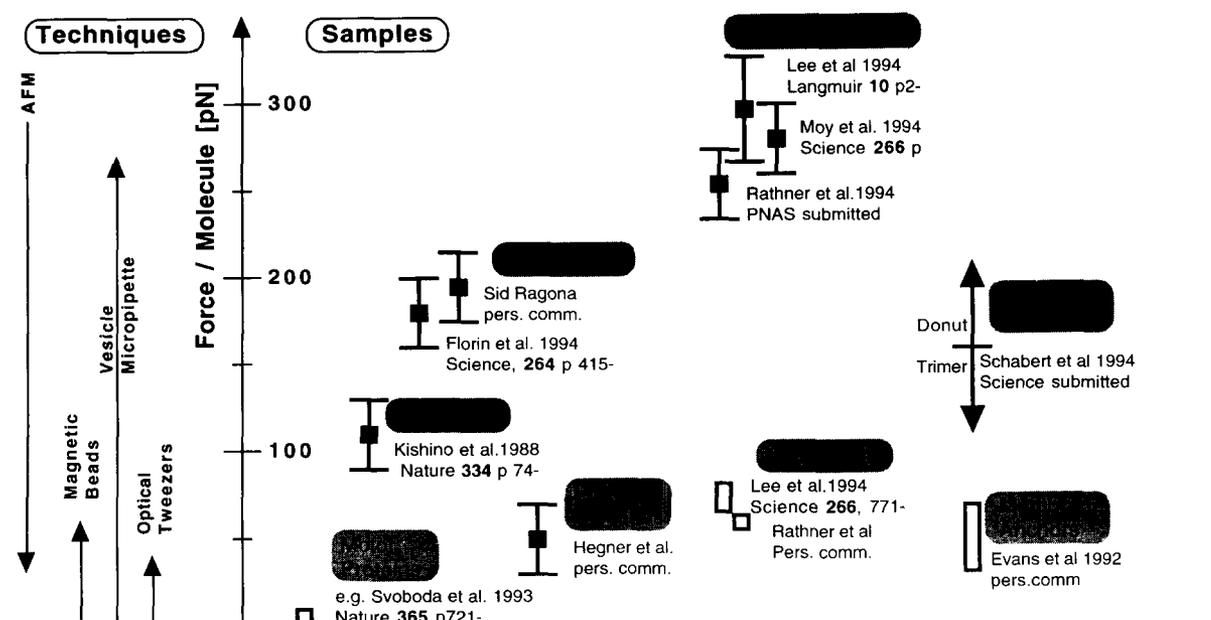


Fig. 1. Literature overview on measurements of intramolecular forces and specific binding forces between individual molecules.

groups. However, to best of our knowledge, no comprehensive study of these different methods has been carried out to determine the consistency of the different approaches. In this report, we compare results from three commonly used calibration protocols. We have found that although the different methods provide comparable values, the calibration method based on the thermal fluctuation of the cantilever is by far the most convenient to use.

MEASURING FORCE CONSTANTS OF AFM CANTILEVERS

There exist at least five different methods, that are used in various laboratories, for the calibration of AFM cantilevers (Butt *et al.*, 1993; Cleveland *et al.*, 1993; Li *et al.*, 1993; Hutter & Bechhoefer, 1994; Senden & Ducker, 1994). Although each method is based on sound theoretical principles, to our knowledge there has been no direct confirmation of any of them by cross-examination. In light of the fact that the AFM is now used more frequently as a technique to measure surface forces, it has become more critical that exact values are reported. It has been shown that measured values for the spring constants differ significantly from the manufacturers' speci-

cations (Cleveland *et al.*, 1993). It seems that each cantilever must be calibrated independently, and that it is negligent to assume that all cantilevers from one batch are alike. We have calibrated AFM cantilevers using three of the more commonly accepted methods: (i) measurement of the cantilever resonance as a function of added masses; (ii) measurement of the bend ratio when pushed against a reference cantilever; (iii) absolute measurement of the thermal excitation spectrum.

Experimental set-up

The atomic force microscope used for these investigations was a scanned stylus AFM that was built in our laboratory (for a detailed description see Florin *et al.*, 1994b). In this kind of AFM the cantilever is positioned in all three directions using a peizo tube while the sample is stationary. Cantilever deflection is detected optically by the beam-bounce technique. Our instrument is controlled by an Apple Macintosh computer with a Mac Adios ADDA interface. The corresponding software was developed in our laboratory.

Thermal fluctuation analysis

The equipartition theorem assigns the same thermal energy to each degree of freedom of a thermodynamic system. Each eigenmode of the cantilever thus gets excited with the same thermal energy, $1/2k_B T$. Since the higher-order vibration modes of the cantilever do not significantly contribute to the deflection of the end of the cantilever (Meyer & Heinzelmann, 1992), where any deflection is detected, the time average of the thermally excited vibration amplitude is directly related to the effective spring constant at a given temperature in the following way:

$$\frac{1}{2}k_B T = \frac{1}{2} C \langle x^2 \rangle$$

where C and $\langle x^2 \rangle$ denote the spring constant and the time-average square of the thermal fluctuation of the cantilever, respectively. In order to separate the contribution due to thermal excitation from the overall measured fluctuation, we examined the data in the frequency domain. According to Parseval's theorem, the integral of the power spectral density over the frequency domain equals the time domain integral of $x(t)^2$:

$$\int |x(t)|^2 dt = \int |\bar{x}(\omega)|^2 d\omega$$

The dominant contributions to the power density stem from the resonance of the cantilever, so the frequency average may be restricted to the resonance range. Since the resonances of our instrument are negligible in the resonance range of the cantilever, the thermal contribution to $\langle x^2 \rangle$ can be calculated by integrating over the power density spectrum. Using this approach, the spring constants of cantilevers can be calibrated in either air (Hutter & Bechhoefer, 1994) or solution (Howard & Hudspeth, 1988). Figure 2 shows a power density spectrum of cantilever fluctuation recorded in water. According to the Nyquist theorem, the sampling rate of data collection was at twice the maximum frequency of the desired spectrum. In our measurement 4096 data points were taken at a sampling rate of 20 kHz. To obtain the power spectral density from the fluctuation data, a fast Fourier transform (FFT) algorithm was applied (Press *et al.*, 1981). The spectrum presented in Fig. 2 is the average of ten subsequently obtained spectra.

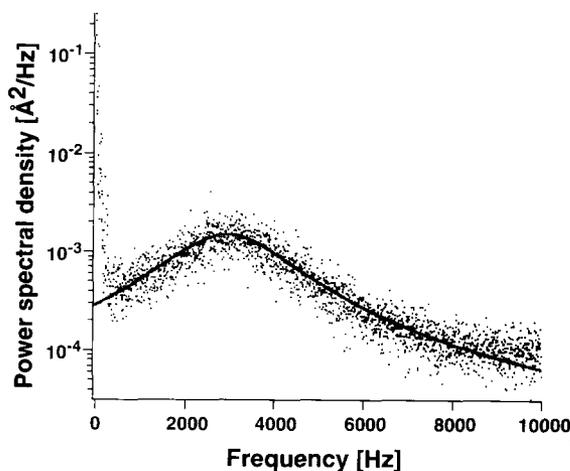


Fig. 2. Thermally excited amplitude power spectrum of a cantilever. The solid line is a Lorentzian resonance curve fitted to the spectrum. The low frequency response attributed to mechanical oscillation was marginalized.

Resonance shift measurements

We have confirmed the validity of the calibration procedure described above in comparison to other established calibration techniques. In the method introduced by Cleveland *et al.* (1993), the V-shaped cantilever is assumed to have harmonic behavior. Hence the resonance frequency of the cantilever is given by

$$\nu = \frac{1}{2\pi} \sqrt{\frac{k}{m}}$$

where k and m are the spring constant and the effective end mass of the beam, respectively. A defined weight (tungsten spheres, 5–10 μm diameter, GTE Sylvania, Towanda, PA) attached to the end of the cantilever adds to the end mass and lowers the resonant frequency of the system. Based on the relationship given above, this provides a means to determine the spring constant of the cantilever.

Reference spring method

The third commonly used calibration method relies on the precision of a previously calibrated standard spring, either a second AFM cantilever of known stiffness or a macroscopic lever with a comparable spring constant. The spring constant of the cantilever to be tested is obtained from the slopes of the cantilever deflection signal versus the piezo displacement curve measured

on a rigid surface and on the standard spring. For the calibrations presented here, we used a polymer lever of about 35 mm in length, 2 mm in width and 0.1 mm thickness that was cut from a sheet of overhead-transparency film (AGFA Transparex T787, Belgium). This standard lever was calibrated by measuring the deflection as a function of applied weights. It had a spring constant of $80 \pm 5 \text{ mN m}^{-1}$.

Piezo calibration

All of the methods listed above also require calibration of the detection system of the cantilever deflection. We have performed this calibration as follows: the cantilever was lowered with the piezo against a hard sample (glass cover slip) and the beam deflection detector signal was recorded as a function of the applied voltage. Then the piezo elongation was calibrated by measuring the interference fringes of the laser between the cantilever holder at the end of the piezo and the glass cover slip, again as a function of the applied voltage. From these curves the dependence of the detector signal as a function of the displacement of the base of the cantilever was calculated.

Comparison of the different techniques

We have calibrated a panel of 11 cantilevers using the three methods described above. The results from these measurements are listed in Table 1. Two wafers of cantilevers were obtained

from Digital Instruments (Santa Barbara, CA). Calibration was carried out on V-shaped Si_3N_4 cantilevers 200 μm long and 20 μm wide. The manufacturer's specification provides a value for the spring constant of 60 mN m^{-1} for these cantilevers. The cantilevers from wafer No. 1 were calibrated by all three techniques within a few days of each other. Cantilevers from wafer No. 2 had been used in previous force measurements involving different electrolytic environments and had been stored for several weeks before they were calibrated. As shown in Table 1, the values of the spring constants for a given cantilever were similar for the different methods of calibration. The measured values for wafer No. 1 vary by about 15% for a given cantilever using the different methods. On wafer No. 2, however, differences between the different techniques for a given cantilever were up to 20% and stiffnesses between cantilevers measured with the same technique differed by a factor of 1.8. On this wafer the method based on thermal fluctuations provided systematically lower numbers than the other two techniques. This may stem from the fact that the thermally driven fluctuations are smaller on stiffer cantilevers and their contribution to the measured spectral power density may be over-estimated. For this reason the thermal fluctuation method seems particularly suitable for soft cantilevers. The simplicity of this calibration procedure allows implementation in the form of a software module in the AFM. It then provides an *in situ* calibration of the cantilever even during an experiment.

TABLE 1 Comparison of the spring constants of various cantilevers measured by different methods.

Cantilever	Charge	Resonance shift (mN m^{-1})	Reference spring (mN m^{-1})	Thermal noise (mN m^{-1})
1	1	29.8		
2	1	32.8		
3	1	30.0		
4	1	31.2	29.6	
5	1	32.9	26.3	
6	1	32.7	28.9	30.6
7	1	27.8	23	26.6
8	1	28.6	35.9	26
9	2	38.5	37	29
10	2	56.4	43	37.6
11	2	56.6	61.8	53.8

MEASURING SPECIFIC BINDING FORCES

Since its invention, the AFM has been used extensively to investigate the properties of DNA (Hansma *et al.*, 1992). Many of these studies have been motivated by attempts to develop an alternative method for DNA sequencing. Recently, chemical functionalization of the AFM tip has provided the means specifically to probe the chemical properties of a sample (Moy *et al.*, 1994a,c). In the current study, we have focused our investigation on the interaction between single strands of DNA. The same AFM was used to measure the interaction between active groups immobilized on the tip of the cantilever and molecules on an opposing substrate. The adhesive force between the functionalized surfaces was measured via the maximum cantilever deflection during retraction. Cantilevers were calibrated by different methods, as discussed above. Figure 3 depicts the construction of the oligonucleotide functionalized AFM tips.

Functionalization of the tips

The functionalization of AFM tips has been described in greater detail elsewhere (Moy *et al.*, 1994a). Biotin-labeled bovine serum albumin (BSA) was adsorbed overnight onto the surface of the AFM tips. The cantilevers were incubated in 50 μl solution (1 mg biotin-labeled BSA per ml of phosphate buffer saline (PBS); 20 mM PO_4^{2-} , 150 mM NaCl, Milli-Q H_2O , pH 7.2) of biotin-labeled BSA at 37°C in a humidified

chamber. Then the cantilever chips were rinsed with PBS, followed by the incubation in a 50 μl solution of avidin (1 mg ml^{-1} PBS). After 5 min incubation, biotin-labeled oligonucleotide (50 μl at 5 $\mu\text{g ml}^{-1}$ in water) was added as the final step in the procedure.

DNA synthesis

The biotin-labeled oligonucleotide 5'-dT^{Biot}-d(T)₂₀ was synthesized by the method of solid phase synthesis on an Applied Biosystem synthesizer 394-08 (Applied Biosystems, Foster City, CA) using dimethoxytrityl-labeled Biotin Amidite (Applied Biosystems) coupled to the 5'-thymidine residue. The labeled oligonucleotide was cleaved from the support and deprotected by overnight incubation in ammonia at 55°C. After ethanol precipitation, 5'-dT^{Biot}-d(T)₂₀ was purified on a OPC cartridge (Applied Biosystems) according to the instructions provided by the manufacturer. Poly d(A) derivatized and poly d(C) derivatized agarose beads were obtained from Sigma (Deisenhofen, Germany). The beads were rinsed four times with TE buffer (10 mM Tris-Cl, 1 mM EDTA, 0.1 M NaCl; pH 8) before measurements were taken. Force measurements were carried out in TE buffer at room temperature.

RESULTS AND DISCUSSION

A series of experiments was performed in which ssDNA molecules on tip and sample were allowed to bind for a given time before they were separated again while the adhesion force between tip and sample was recorded. The maximum adhesion force was taken to be the rupture force. In this series of measurements, AFM tips were functionalized with a 21-thymidine residue oligomer and were used to probe agarose beads functionalized with either adenine or cytosine. Figure 4 plots the rupture force between the interacting surfaces as a function of the duration of time for which the surfaces remained in contact. At short contact duration (30 s), the T₂₁ functionalized tips showed low adhesion to polyadenine agarose beads and no significant adhesion to polycytosine beads. The adhesion reached its maximum after 2 min for the adenine-thymidine interaction. A measurable but clearly much lower interaction was observed between thymidine and cytosine only after an

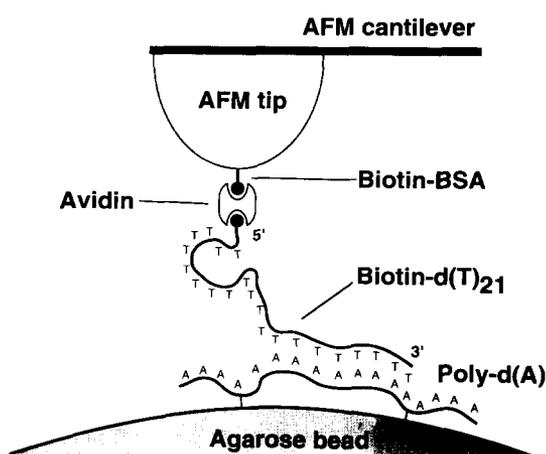


Fig. 3. Schematics of the surface chemistry used in measurements of the specific interaction between complementary DNA strands with the AFM.

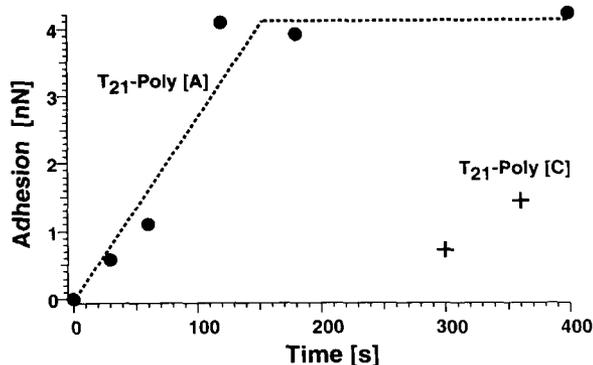


Fig. 4. Dependence of the unbinding force of DNA strands on the duration of surface contact.

incubation time of 5 min. These forced unbinding experiments demonstrate that the AFM may be used to detect specific interactions between complementary ssDNA by measuring the force that is required to separate the hybridized strands. Because of the multiplicity of bonds as well as the unknown attachment geometry of the molecular partners, the absolute value of the unbinding force in this case, in contrast to single-molecule experiments, cannot easily be interpreted. Furthermore we have to assume that in cases where the complementary strands had annealed throughout their full length in a geometry where the load is applied symmetrically to the 5' ends, the biotin-streptavidin bond yields rather than the base-pair bonds (Florin *et al.*, 1994a; Lee *et al.*, 1994b). The time development of the adhesion strength, however, is a novel and unique feature that has not been observed previously in force measurements of receptor ligand interactions (Florin *et al.*, 1994a; Moy *et al.*, 1994a,c). This incubation-time dependence may be interpreted as slow reorientation of the partially hybridized ssDNA fragments into more stable structures. Such time-dependent measurements may provide new information on the dynamics of the double-helix annealing process.

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