Photothermal cantilever actuation for fast single-molecule force spectroscopy

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Photothermal cantilever excitation provides a fast and easy to implement means to control the deflection of standard atomic force microscopy cantilevers. Minute heat pulses yield deflections on the order of several tens of nanometers or when the deflection is kept constant, forces of several hundreds of piconewton can be applied. In our case these pulses resulted in less than 1 K temperature changes at the sample position. Here we present and characterize the implementation of photothermal actuation for single-molecule force-spectroscopy experiments. When molecules are stretched under force-clamp conditions, fast control cycles that re-establish the pulling force after the rupture of molecular domains are essential for detecting the complete unfolding pattern with high precision. By combining the fast response of photothermal cantilever excitation with a conventional piezoactuator, a fast force-clamp with high accuracy and large working distances is reached. Simple feedback mechanisms and standard cantilever geometries lead to step response times of less than 90 μs, which is more than one order of magnitude faster than those of conventional force-clamp systems that are based only on piezo feedback. We demonstrate the fast and accurate performance of the setup by unfolding a protein construct consisting of one green fluorescent protein and eight surrounding immunoglobulin domains at constant force. © *2009 American Institute of Physics.* [DOI: 10.1063/1.3157466]

I. INTRODUCTION

Atomic force microscopy[1] (AFM) has evolved into a versatile technique for mechanical single-molecule experiments. The AFM provides high lateral precision for the one-by-one assembly of single molecules.[2–5] Also, the vertical precision and force sensitivity allows for the investigation of the energy landscape and function of biomolecules in force-spectroscopy experiments. The molecules under investigation are contacted by the tip of a cantilever, which acts as a flat spring, and are stretched, while the applied force and the extension are measured. In constant velocity experiments, the distance of the cantilever from the surface is constantly increased and force-extension traces are recorded. In this way protein domains can be unfolded and, upon approach, refolded again, allowing the examination of the potential width and the rates, which characterize the dynamic mechanical stability.[6] Changes in the unfolding profile can reveal the binding of ligands[7] and force-induced conformational transitions can alter the functional state of an enzyme as in the case of the force-sensor titin kinase.[8] Whereas force-extension experiments are ideal to measure and control the conformation related behavior of biomolecules, the force-clamp mode was developed to gain direct access to the time domain of protein unfolding,[9,10] folding,[11] or chemical reactions.[12] In these experiments the tension applied to the molecule is kept constant by adjusting the distance between cantilever and sample surface in a feedback loop. If, for example, a protein domain unfolds, the contour length of the protein increases and the force suddenly drops. The sample surface is then retracted in the feedback loop to the position at which the force is at the chosen value again.

In order to resolve details of such conformational transitions in feedback-regulated force-spectroscopy modes such as force-clamp or force-ramp, a rapid reaction of the mechanical actuator that adjusts the extension of the molecule is crucial. In most AFMs the actuation is achieved by piezoelectric elements. The response time is determined by the resonance frequency and phase shift, which depend on the stiffness and effective mass of the mechanical elements connecting either sample or cantilever base to these piezo elements. However, the upper limit of the response speed is always determined by the corner frequency of the cantilever itself. Therefore we have developed a technique that uses a cantilever as a fast actuator for force-spectroscopy by taking advantage of its photothermal bending. As depicted in Fig. 1, the conventional piezo AFM actuator design is expanded by a cantilever acting as the sample surface. The deflection of this cantilever is detected in the usual way by beam bounce. A second laser is focused on the base of the cantilever to locally heat and bend it, which is due to the different thermal expansion coefficients of its layer materials such as silicon nitride and metal coatings.

The photothermal effect has already proven to be fast

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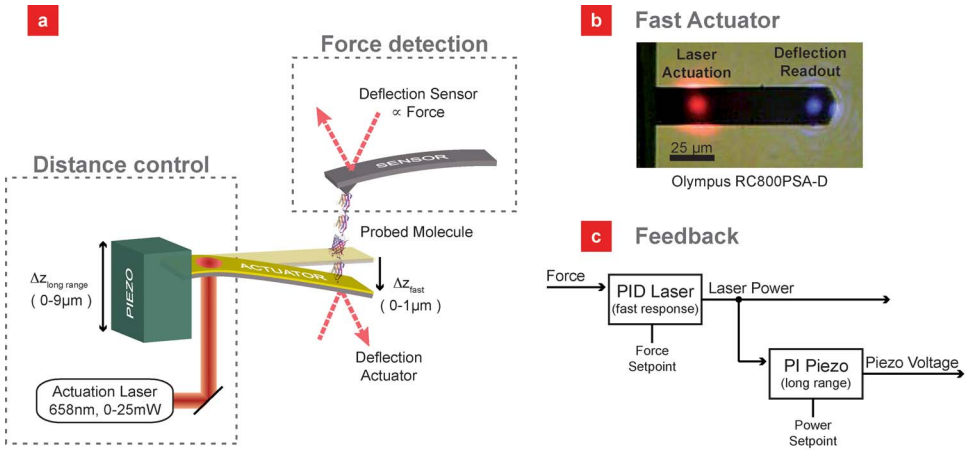

FIG. 1. (Color online) (a) Schematic drawing of the demonstrated setup. Two AFM heads are combined such that their cantilevers can get in contact. One of them serves as a force sensor like in traditional applications, the other one is used together with a piezo element as an actuator for distance control. Its deflection can be regulated by the intensity of a power-modulated laser due to the photothermal effect. The molecule of interest is probed between the two cantilevers. (b) Image of the actuator cantilever with the deflection readout and the regulating lasers focused on it. (c) Block diagram of the combined feedback loops. The actuator is regulated by the force signal from the cantilever and provides fast response to the unfolding of protein domains. The piezo adjusts the distance of the AFM head such that the feedback laser is held at an optimal operating point.

and accurate for high speed scanning AFM. It outperforms even sophisticated piezo feedbacks.[13] But its limited deflection range with standard AFM cantilevers and moderate laser power is not sufficient for force-spectroscopy applications with the goal of complete unfolding of large molecules. However, combining cantilever actuation with a slower piezo-based feedback results in both fast response and wide actuation amplitude.

Here we demonstrate and characterize for the first time a fast force-clamp AFM setup based on fast photothermal cantilever actuation combined with a long range piezo feedback. We demonstrate the performance of the setup by unfolding a fusion protein consisting of one green fluorescent protein (GFP) and eight immunoglobulin (Ig) domains[14] at a constant force.

II. EXPERIMENTS

A. Photothermal cantilever actuation

The laser illumination of the cantilever leads to a local heating of the cantilever material due to energy absorption. The resulting dynamic temperature profile depends on the material properties like thermal conductivity, density, specific heat capacity, and heat transfer coefficient to the ambient medium, as well as on the lever geometry and the illumination position. It is well described with a one-dimensional heat diffusion equation.[15] When a cantilever with layered materials is used, as is the case for the majority of standard cantilevers with a reflection enhancing metal coating, differences in the thermal expansion coefficients result in bending that releases mechanical stress. This bending principle is the same as in commonly used bimetal switches and thermometers.

For static illumination the cantilever deflection is proportional to the applied power (Fig. 2). For dynamic signals the efficiency of actuation drops with increasing frequency. Figure 3 shows the spectral amplitude response of the cantilever induced by sinusoidal laser excitation power in an aqueous solution. It is a convolution of the mechanical response of the cantilever spring (harmonic oscillator with resonant frequency f_R and quality factor Q) and the photothermal response, which decreases as the modulation frequency increases. This is because the temperature variation in the illuminated position and the thermal diffusion length decrease with higher frequency.[15] In order to obtain a precise and fast actuation response, a well-tuned controller is needed that modulates the laser power such that the deflection stays close to its set point over a wide frequency bandwidth.

B. Apparatus

The introduction of a second power-modulated laser in the AFM head adds a fast actuation element in the z direction in addition to the piezo. But the induced bending of a cantilever by photothermal actuation cancels the direct relation between the deflection and the external force applied to the stretched molecule. Therefore, one either has to calculate the photothermal effect when using a sole cantilever as sensor and actuator or introduce two distinct cantilevers that serve

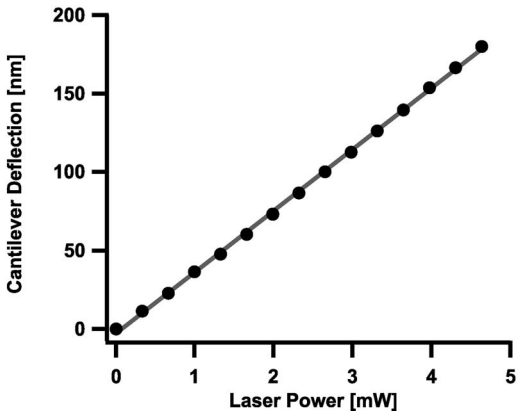

FIG. 2. Linearity of the photothermal bending of an Olympus RC800PSA-D lever. The gray line is a line fit to the measured deflection values at different laser diode powers with a slope of approximately 40 nm/mW.

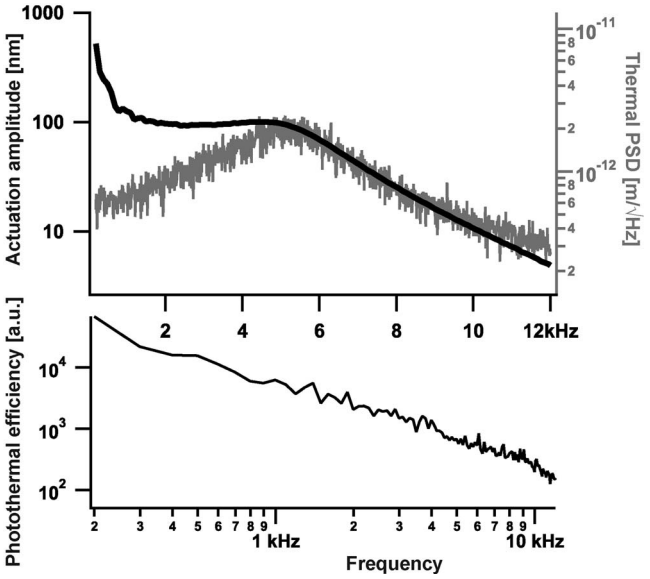

FIG. 3. Top: Frequency dependant drive efficiency of the actuator. The cantilever is driven with a sinusoidal excitation signal of about 11 mW amplitude. The resulting cantilever oscillation amplitude at the driving frequency (black) is a convolution of the photothermal excitation efficiency, decaying with increasing frequency, and the mechanical excitability of the cantilever, which is visible in the thermal noise power spectral density (thermal PSD, gray). Bottom: The photothermal excitation efficiency is the ratio of the photothermal driving amplitude and the thermal PSD.

as sensor and actuator, respectively. In the demonstrated setup we chose the latter strategy, since it allows for clearer interpretation of the data and provides a direct method for demonstrating the performance of the combined feedback system. Nevertheless, it is, in principle, possible to implement this photothermal actuation in a setup with only one cantilever if the dynamics of motion is well characterized as we will discuss later.

For the proof of principle experiments two custom-built AFM heads of the same design[16] were combined such that the two cantilevers can be brought into contact (see Fig. 1). Because they were built to work with optical microscopes, their vertical piezos are integrated in the head and such each AFM fills only one hemisphere. The two AFM controllers (MFP-3D controller, Asylum Research, Santa Barbara, CA, USA) were synchronized by a trigger channel such that the time delay for recorded data is lower than 100 μs. Cantilever positions are read out by optical beam deflection of infrared, superluminescent laser diodes. One cantilever (Olympus RC800PSA) served as actuator. Its deflection depends linearly on the power of an adjustable laser (658 nm, P_{max} =25 mW, Blue Sky Research, USA) focused on the lever near its fixed end. By mounting the lever upside down, the gold coating, which serves as a support for nonspecific protein adsorption, is oriented toward the other cantilever, which is used for force detection. Furthermore, the strength of the photothermal effect is increased roughly by a factor of 3 in this geometry since more power is absorbed by the lever when the laser hits the silicon nitride first. Infrared filters and different incident angles assure that no light from the actuation laser interferes with the deflection detection. Short biolevers (Olympus RC150VB-A) were used as force sensors, because they combine fast response [f_R=7.9 kHz in 1x phosphate-buffered saline (PBS)] with a low spring constant (approximately 30 pN/nm) and a low photothermal response. During photothermal deflection of the actuator thermal bending of the sensor may occur due to heating of the surrounding fluid, which leads to errors in the force signal. For the given sensor/actuator combination the sensor deflection is less than 4% of the actuator deflection at constant illumination and thermal equilibrium. This corresponds to less than 1.3 pN/nm actuator deflection. During the experiment, only short laser pulses are applied and there is a time lag until equilibrium is reached, making the actual error much smaller. For typical illumination times shorter than 10 ms these values drop by one order of magnitude so that the deflection "crosstalk" amounts less than 0.4%. This effect could be completely suppressed by using uncoated cantilevers as sensors or by thoroughly adjusting the coating thickness such that the photothermal response of the sensor is minimized. Furthermore for the one-cantilever setup, which is proposed subsequently in this paper, this limiting effect is removed.

The excitation feedback used for keeping the pulling force constant consists of a combination of fast photothermal cantilever actuation with a small working range (<1 μm) and a slower piezo feedback with the full z-direction working rage of the AFM (approximately 9 μm). The deflection signal of the sensor is directly fed to a fast analog proportional-integral-derivative controller (Stanford Research Systems, SIM960, 100 kHz) that controls the current of the actuation laser. The current serves as input for the piezo PI feedback, which keeps the laser power at an optimal working point. A block diagram of the feedback system is depicted in Fig. 1(c).

The force sensing cantilever was calibrated by pushing it to a hard surface to determine the deflection sensitivity of the photodiode (nm/V) and by fitting a single harmonic oscillator response function to its thermal noise spectrum.[17] However, this method is not adequate to determine the deflection sensitivity of the actuator cantilever since the photothermally induced deflection depends on the heating spot position, and differs from the free cantilever response. Instead both cantilevers were brought into contact with the z-piezo while the photothermal force feedback was activated with a set point of about 50 pN. If the actuator is now moved further in the direction of the sensor with its piezo, the feedback compensates this movement by bending the actuator in the other direction. Since this bending equals the distance moved by the piezo, recording the actuator deflection versus the piezo position yields the actuator deflection sensitivity.

C. Combined feedback

The basic performance of the combined feedback on the actuator with input from the force sensing cantilever was tested under pushing conditions with feedback parameters keeping the cantilevers in contact at a force set point of 500 pN. The system was disturbed by applying step functions of different heights to the z-piezo of the sensor AFM head, which is not used for the position feedback and thus provides an easy means to check the regulatory behavior of the feed-

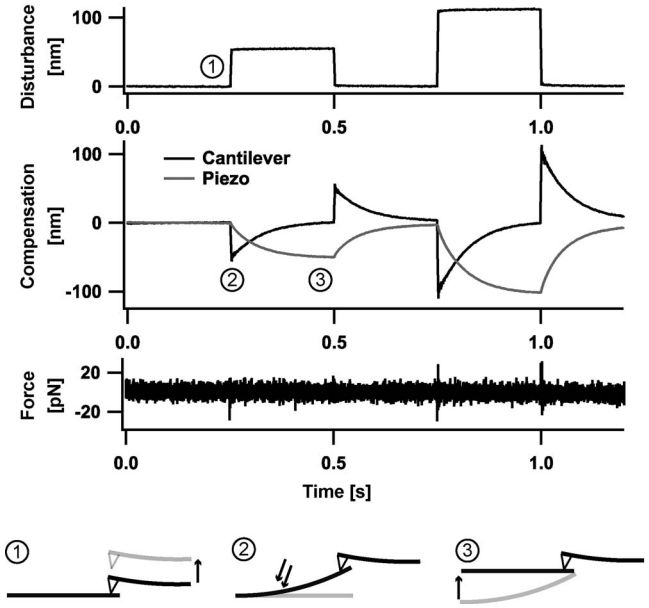

FIG. 4. Demonstration of the working principle of the combined actuator feedback. The force setpoint was chosen such that both AFM cantilevers are held together with contact force of 500 pN. If the system is disturbed with the sensor AFM piezo (top graph, step 1) the contact force is held constant (bottom graph) by the feedback loops. As a fast response the actuator is deflected photothermally (step 2). On a longer timescale the piezo feedback corrects for this deflection to assure that the actuator is held at an optimal operational point (step 3). The graph shows that the system is easily able to follow the maximum actuation speed of the disturbing piezo which nicely demonstrates that photothermal actuation is outperforming piezo actuation. The full performance of the photothermal feedback with the chosen cantilevers can be seen in Fig. 6.

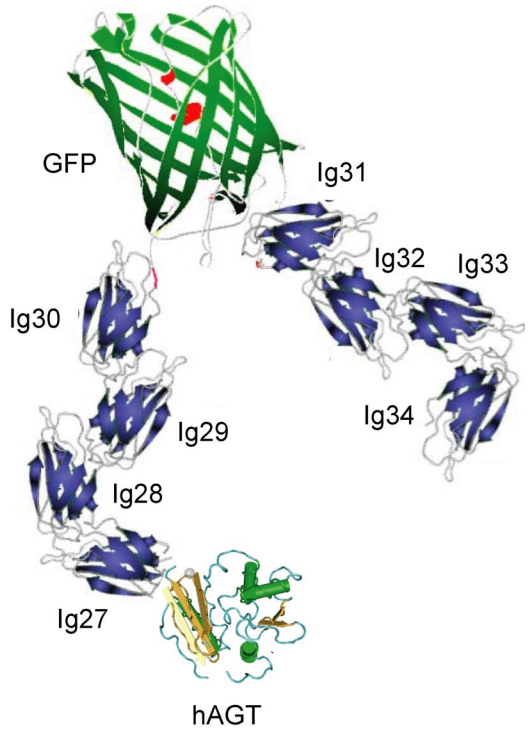

FIG. 5. (Color online) The Ig-GFP fusion protein that is used for the demonstrated force-clamp traces. It consists of eight Ig, one GFP, and one hAGT domain.

back loops. A sample trace is shown in Fig. 4. The disturbing movement is compensated with the two actuators. As a fast response, the actuation cantilever is deflected by the laser in the same direction as the disturbance. The feedback loop acting on the actuator AFM piezo recognizes the actuator deflection and moves the piezo with a slower time constant to assure that the laser power is held at a good operational point for full actuation amplitude of further steps. This proves that the combined feedback is easily able to follow the maximum response speed of the disturbing piezo (approximately 50 μm/s initial slope) and shows that photothermal actuation is outperforming a sole movement of the piezo. Under single-molecule force-clamp conditions, the response of the feedback was further increased and the cantilever and piezo response was even faster.

D. Ig-GFP fusion protein unfolding

As a model system, we used an Ig-GFP fusion protein consisting of eight titin Ig domains, one GFP-domain, and one human O^6-alkylguanine-DNA-alkyltransferase (hAGT) domain, which may be immobilized to the surface. It is depicted in Fig. 5 and described in detail in Ref. 14. The Ig domains unfold with a contour length increment of 29.0 ± 0.5 nm (Ref. 18) and the GFP in up to two substeps with a total increment of 76.6 ± 0.6 nm.[19] Proteins were allowed to adsorb to the actuator cantilever from a 300 μg/ml solution in PBS (*p*H 7.4, 150 mM NaCl). After 10 min of incubation, the sample was rinsed with PBS and measurements were started.

Figure 6 shows sample traces, which clearly reveal the stepwise unfolding of several protein domains with increments of 49.1, 11.2, 4.6, 26.0, 25.7, and $\pm$ 0.6 nm, respectively. The first three steps together are equal to 85% of the contour length increment in GFP and the last two correspond to 90% and 89% of the contour length increment in individual Ig domains. Due to the entropic elasticity of polymer chains, the observed step sizes in force-clamp experiments are smaller than the characteristic contour length increments and depend on the pulling force.[9] The measured values match the expectations very well[20] even though the trace plateaus are not completely stable but exhibit a drift on the order of 2 nm. This drift can be due to several effects. First, the AFM heads are not temperature stabilized and thus already small changes in the environment temperature may lead to position nonconformance of the piezo sensor in the nanometer regime. Second, there is a slight decay in the tip-sample distance that correlates with the displacement of the actuating cantilever. This might either be caused by uncertainties in the inverse cantilever sensitivity or by the thermal crosstalk with the sensing cantilever that was discussed above.

As mentioned in the introduction, the fast re-establishment of the pulling force after the rupture of a domain is crucial for the proper detection of unfolding substeps and for the interpretation of force-clamp data in terms of (un)folding. The response time for force re-establishment can be determined by fitting the force trace with a single exponential function. For the given 200 μm long actuator with a

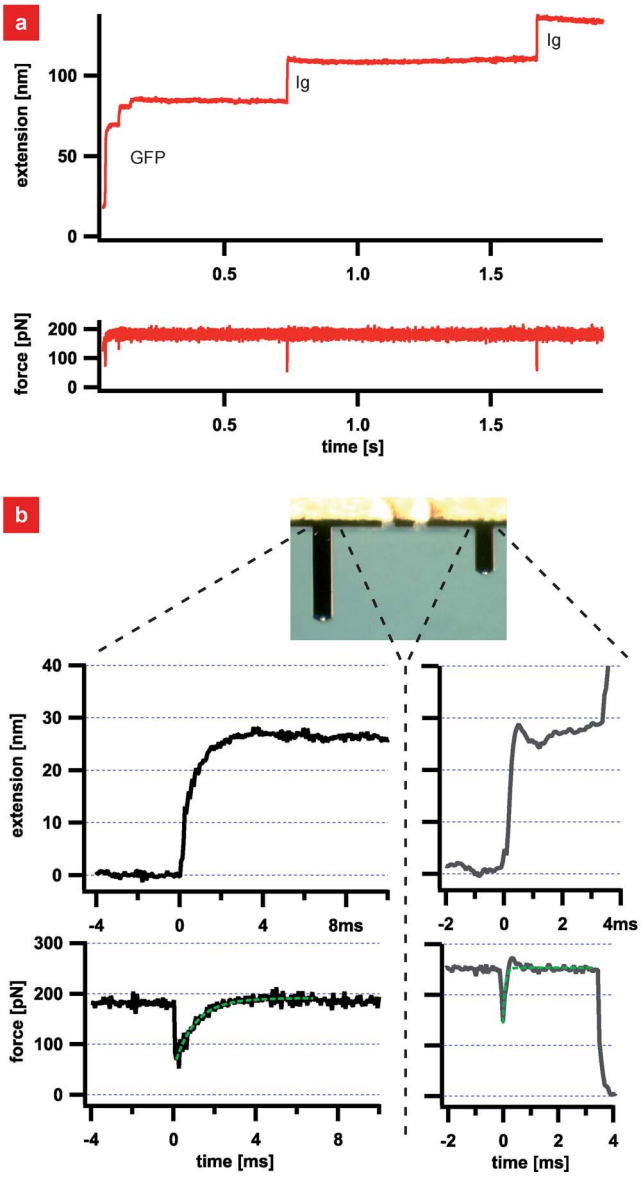

FIG. 6. (Color online) Unfolding traces of the Ig-GFP fusion protein under force-clamp conditions. (a) Unfolding of GFP and two Ig domains with an Olympus RC800PSA-D cantilever at 180 pN pulling force. Substeps of GFP unfolding are observable. (b) Black: zoom in the unfolding of the first Ig domain. The force is re-established with a time constant of 930 μs (fitted line). Gray: unfolding of an Ig domain with a faster actuator (Olympus RC800PSA-B) and higher feedback gains at 250 pN pulling force. The characteristic time constant gets reduced to 80 μs. At $t=4$ ms the unspecific binding to the cantilever ruptures and the force drops to zero.

resonance frequency of $f_R=5.2$ kHz (RC800PSA-D), the time constant of the rise was around 1 ms [Fig. 6(b), black]. Since small cantilevers provide advantages in AFM,[21] we also used a shorter actuator with a fivefold higher resonance frequency and higher feedback gains. The measured response time of only 80 μs during protein unfolding [Fig. 6(b), gray] is about one order of magnitude faster than conventional piezo based AFMs and nicely shows that the limitation is so far only given by the mechanical and photothermal response of the actuator.

One may argue that the temperature rise of the cantilever and its surroundings induced by the modulation laser modifies the ambient conditions of the protein or even denatures it. However, measuring the cantilever deflection while changing the buffer temperature gave a photothermal sensitivity of 92 ± 5 nm/K for the RC800PSA-D cantilever. This is the sensitivity for a uniform heat profile over the whole cantilever length. During photothermal bending the temperature profile has its maximum close to the rear end so the impact on the protein is even smaller. Given that typical domain lengths are shorter than 100 nm and that the piezo feedback assures that the laser power is only shortly raised during domain rupture, the temperature differences applied to the protein are below 1 K and thus negligible.

III. DISCUSSION

We have presented a novel technique that allows fast control of the cantilever position in single-molecule force-spectroscopy experiments by combining photothermal cantilever actuation with a traditional piezo actuator. As the photothermal effect acts on the cantilever itself, actuation is performed as directly as possible and response times are in principle much faster than for piezo-based feedbacks, which have to move at least the mass of the sample and its holder. The combination of the photothermal actuation with a piezo allows a wide working range. With the simple feedback mechanism and standard cantilevers, the response time for the force re-establishment after unfolding of titin Ig domains was only 80 μs, corresponding to an actuation bandwidth of 12.5 kHz. By compensating the frequency dependence of the photothermal effect electronically and by optimizing the cantilever geometries, much faster actuation could be achieved. In the AFM imaging application, for example, Yamashita *et al.*[13] reached feedback bandwidths on the order of 100 kHz for photothermal cantilever actuation. Further improvement of the feedback can be reached by electronically damping the quality factor of the first eigenmode (Q control) and by different controller types such as field programmable gate arrays.[22]

The measurement setup that we presented consists of two cantilevers, one serving as a force sensor, and the other as a displacement actuator. The separation of sensing and actuating gives a more direct insight into the dynamic variables but in principle it is possible to use the same cantilever for both. This requires a thorough characterization of the actuation dynamics and programmable sets of filters[22] in order to obtain the applied force from the deflection signal and the dynamic laser power. Such a setup would allow very fast actuation even for AFM heads that are designed to work with optical microscopes.[16] They so far have been suffering from slower dynamics since the complete head or at least a large part of it has to be moved by the piezo.

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