NOVEL, HIGH-LINEARITY ATOMIC-FORCE-SPECTROMETER APPARATUS FOR QUANTITATIVE MEASUREMENT OF PROTEIN DYNAMICS

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ABSTRACT We describe a configuration of commercially-available single molecule force spectrometer that makes use of a faster and more linear actuator than previously possible. The linearity, bandwidth and comparative absence of hysteretic behavior for this multi-axis assembly ensure previously unattained resolution. Combined with a robust, simple, high-end design, this new generation of single molecule force-clamp instrumentation is ideally suited to capture the individual force-induced unfolding and folding trajectories of single protein molecules. Initial applications and results are reviewed.

1. A new era in the design of piezoelectric actuators.

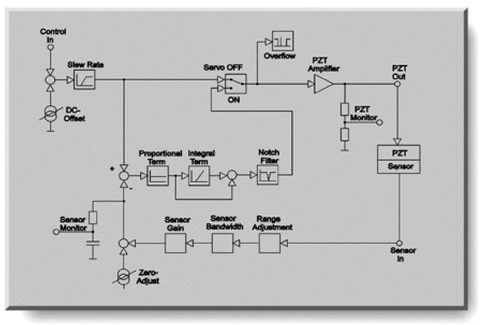


Figure 1. Conventional closed-loop piezoelectric actuator servo principle. Piezoelectric actuation has been a mainstay of atomic-force microscopy and spectroscopy from the earliest applications. The hysteretic and nonlinear characteristics of conventional piezo-ceramic actuation have largely been tamed by the integration of position sensors and servo-controls into nanopositioning stages. In parallel kinematic configurations where a single motion platform is actuated in multiple degrees of freedom by several actuators, parasitic (orthogonal) errors are also compensated automatically by the feedback mechanism.

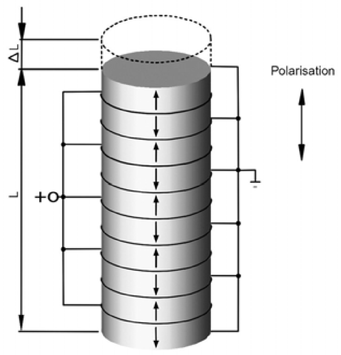


Figure 2. The design of a conventional piezoelectric actuator. The In recent years a multitude of piezoelectrically-actuated mechanisms have become available in single- and multi-axis configurations. The trend has been towards longer travels, and today the market is well-supplied with models offering many hundreds of microns of travel, and very few suitable for single-molecule force-clamp spectroscopy. Long-travel mechanisms present drawbacks for single-molecule force-clamp spectroscopy and similar applications, which benefit from highly exacting, high-bandwidth and bidirectionally accurate actuation over distances typically smaller than 1 micron. Inconveniently for these applications, the resolution, positional noise and inaccuracy of a nanopositioning mechanism scale roughly with travel, and its stiffness scales inversely with the lever ratio of the flexure amplification elements used for providing long travels in practical package sizes.

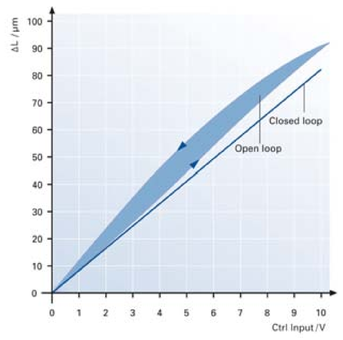


Figure 3. Conventional piezoelectric actuation: hysteretic and non-linear. Until recently, the native hysteresis and nonlinearity of commercially-available piezoelectric materials necessitated the feedback and servo elements of the nanopositioning system design (Fig. 1). These elements reduce system bandwidth; operating a stage in open-loop always provides higher responsiveness. If it were possible to eliminate the sensor and servo entirely while providing adequate linearity and hysteresis, the system would be much snappier— of benefit to single-molecule force clamp applications. Crucially, the cost would also be reduced. Recent advancements in piezoceramic engineering have yielded a novel material which is linear and non-hysteretic in open-loop actuation over distances characteristic of these applications. This linear material can be assembled into multi-axis configurations which directly actuate the workpiece in X, Y and Z with sub-nanoscale precision and accuracy.

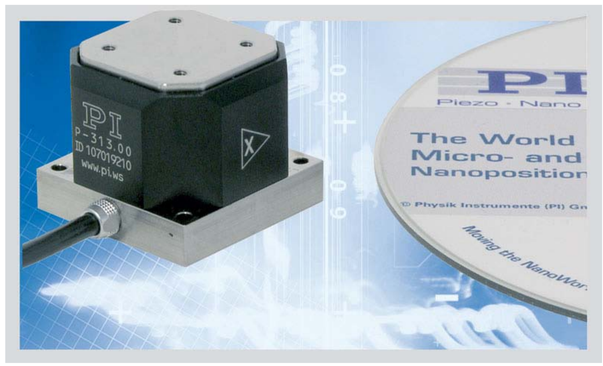


Figure 4. The newly designed P-313.00 piezoelectric actuator from Physik Instrumente. At the heart of this instrument is the integrated XYZ piezo positioner with its new drive principle: A monolithic assembly of three stacked elements of the linear material (with the X and Y elements operating in shear mode and the Z element actuating longitudinally) provides 1x1x0.8 micrometer travel with resolution surpassing 20 picometers. Since the novel piezoelectric material exhibits sub-nanometer hysteresis and a highly monotonic voltage-displacement relationship, no position sensor or servocontroller is needed. Though the 4kHz resonant frequency in X and Y and 11kHz in Z is higher than previous mechanisms', the most significant positioning bandwidth improvement comes from elimination of the sensors and servo loop that would otherwise be necessary. Following-error is thus largely eliminated compared to earlier devices. The resulting combination of sub-nm hysteresis, sub-nm nonlinearity and improved tracking fidelity yields highly repeatable single protein force spectroscopy measurements.

2. The new generation of single molecule atomic force spectrometers

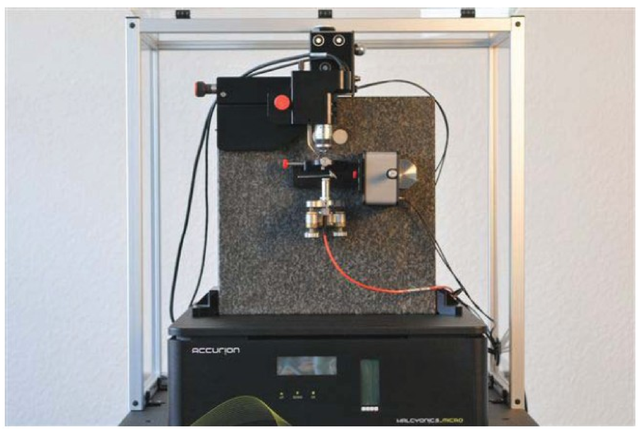


Figure 5. The Atomic-Force-Spectrometer (AFS) commercialized by Luigs & Neumann is an instrument for the study of single proteins placed under a calibrated mechanical load. The AFS is used to understand how mechanical forces, over the full biological spectrum, affect the dynamics and chemistry of proteins. The AFS allows for the picking-up and mechanical manipulation of single recombinant proteins. The AFS instrument was designed to be fully automatic and be able to operate for days, unattended by an operator. Its operating software includes routines that unambiguously identify the mechanical fingerprints of the protein being studied, and thus is able to recognize and store data automatically. At the end of a daylong experiment, it is possible to collect several hundred single-protein traces.

Figure 6. The design of the AFS. The AFS is similar to the Atomic Force Microscope in its basic design, however, the AFS is optimized for force-spectroscopy of short recombinant-proteins. The AFS is fully automatic and operator independent. This is made possible by a set of computer controlled piezo-motors that keep the force-sensor apparatus aligned at all times. Most importantly, a set of piezo-motors constantly optimize the distance between the force-sensing cantilever and the protein layer which is deposited on the surface of a the high resolution piezoelectric actuator beased upon the PI P-313.00 design.

Figure 7. Improved optics system. The improved micro-focusing optics system, together with the USB CCD camera allows focusing the laser spot on the very center of the used cantilever. Achieving such a small laser spot avoids possible light interference with the surface (of particular importance in the case of a gold surface), which is a common source of error in single molecule mechanical experiments.

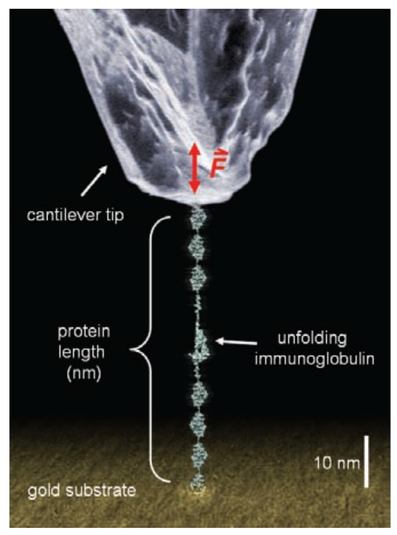


Figure 8. The Atomic-Force-Spectrometer is specifically designed to study the conformational dynamics of individual proteins. The preferred protein sample is arranged as a tandem modular protein. When such polyproteins are extended by an AFS, their force properties are unique mechanical fingerprints that unambiguously distinguish them from the more frequent non-specific events that plague single molecule studies.

The standard method of protein attachment for AFS measurements uses thiol-chemistry which is obtained by layering recombinant proteins containing cysteine anchors at its termini, on a gold-covered glass coverslips. Most effective is the use of HaloTag (Promega) technology combined with thiol-chemistry which readily delivers covalently attached proteins to both; gold covered cantilevers and Halo-ligand covered glass coverslides. Covalently attached proteins can be mechanically manipulated for long periods of time.

2. Single molecule force spectroscopy individual trajectories of protein unfolding and protein folding under force

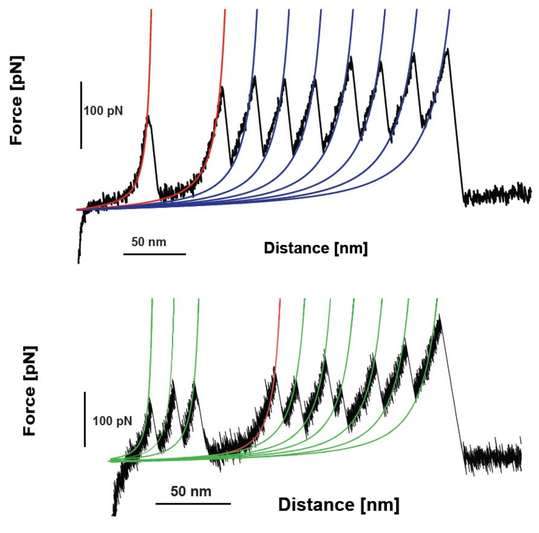
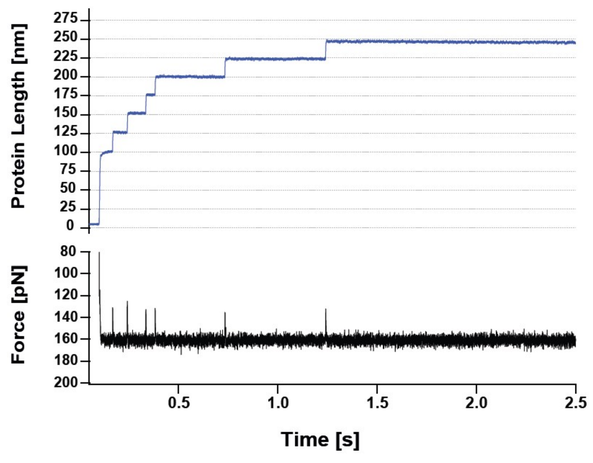


Figure 9. Individual constant velocity unfolding trajectories. Once a single protein is picked up, the AFS can be operated in two modes; constant velocity or force-clamp. Under constant velocity, a single protein is extended at a set-rate of up to 4,000 nm/s, while the extension forces are recorded with a resolution of 5 pN. The extension of a polyprotein at constant velocity results in sawtooth pattern traces of unfolding, which can be used to study protein unfolding and folding. The AFS has software that automatically detects and saves sawtooth pattern fingerprints. Upper trace: Unfolding trajectory of an HaloTag-$I27_8$ polyprotein. Fits to the Worm-Like Chain model (WLC) of polymer elasticity reveals a first unfodling peak (red) that occurs at ~ 120 pN of force, eliciting ~66 nm of length, which is the signature of HaloTag unfolding. Subsequently, the 8 force peaks occurring at ~200 pN, with an associated contour length of ~28.6 nm, correspond to the sequential unfolding of each I27 module in the polyprotein chain. Lower trace: Similarly, in the HaloTag-ProteinL_8 construct, the entire polyprotein unfolds upon application of force. In this case, the green peaks (~ 160 pN) correspond to the sequential unfolding of each individual Protein L module, with an associated contour length increase of ~19 nm. As before, the red peak corresponds to the unfolding of the HaloTag moiety.



Proportional-Integral-Differential (PID) feedback circuit adjusts the piezoelectric actuator that controls the length of a protein, such as to keep the force equal to a set value with a time constant of 0.5-5 ms, depending on the type of cantilever used. Loading a polyprotein at constant force produces staircases of unfolding steps that can be measured with sub-nanometer resolution. Operator designed force-protocols can be used to measure the dynamics of protein unfolding and protein folding (1), and protein chemical reactions (2,3). In this particular case, an $(I27)_8$ polyprotein was picked from a gold coated coverslide surface. Upon the application of a constant force of 160 pN for 2.5 seconds, the protein stochastically unfolds in steps of 25 nm of length.

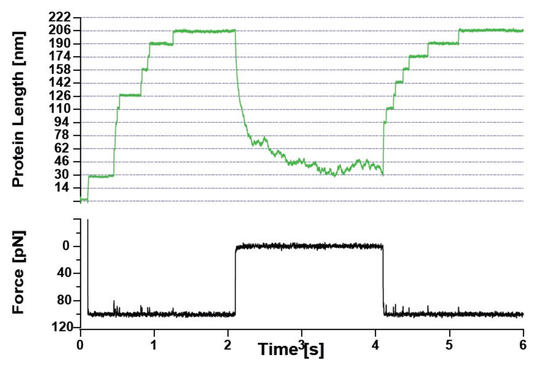


Figure 11. Individual folding trajectory of a HaloTag-ProteinL8 polyprotein. The AFS allows to design force-protocols to probe mechanical protein folding, at the single molecule level. Using the force-quench protocol, a first pulse of 100 pN unfolds the protein to almost its contour length, as observed by the 16-nm increase in length, corresponding to the sequential unfolding of each individual Protein L monomer in the chain. After 2 seconds, the force is released (0 pN) in order to trigger folding. After a 2 seconds, the force is raised again back at 100 pN. The concomitant extension, the 'test pulse' probes the folding status of the protein. In this case, we observe that the test pulse is composed of well-defined 16 nm steps. The recovery of the mechanical stability of the protein is a direct, unambiguous signature of sucessful protein folding.

References:

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3.-Jorge Alegre-Cebollada, Pallav Kosuri, Jaime A. Rivas-Pardo, Julio M. Fernandez (2011) Direct observation of disulfide isomerization in a single protein Nature Chemistry. 3:882-887