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Warhammers for Peaceful Times

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ABSTRACT The Perkins group has recently developed a number of improved atomic force microscopy cantilevers using the focused ion beam technology. They compared the performance of these cantilevers in "real-life" biophysical single-molecule force spectroscopy measurements on protein unfolding, and the results of this comparison are reported in this issue of *Biophysical Journal*.

Single-molecule force spectroscopy (SMFS) captures the relationship between molecules' extension and tension and allows for the examination of their conformational states near or very far from equilibrium conditions (e.g., (1,2). and references cited therein). This direct mechanical manipulation of the examined molecules or molecular pairs separates SMFS from other spectroscopies and structural techniques that operate only near equilibrium (e.g., infrared, circular dichroism, electron paramagnetic resonance, NMR, and x-ray crystallography) and for these reasons, SMFS offers unique insights into molecular behavior and structures in their transient states, such as those that occur during ligand-receptor adhesive interactions (3), the operation of molecular motors (4), or during protein unfolding and refolding reactions (5,6). Of the three main SMFS platforms that include optical tweezers (OT), magnetic tweezers, and atomic force microscopy (AFM) (7), AFM-based SMFS is the most popular thanks to a large number of commercial and home-made AFM instruments and the (relative) ease of AFM measurements. However, AFM-based SMFS traditionally offers lower force precision and resolution (~5–10 pN within the 1 kHz bandwidth) compared with optical tweezers and magnetic tweezers, which can easily resolve piconewton and subpiconewton forces that are of great biological significance. Also, compared with the other two platforms, AFM instruments typically suffer greater drifts during force measurements and thus offer lower force stability over long measurements.

Within the last 5 years or so, the Perkins group at the JILA/NIST and the University of Colorado Boulder focused on identifying the physical reasons underlying these AFM weaknesses and limitations, and set out to circumvent them to bring the performance of AFM-based SMFS to bridge that of optical tweezers while maintaining the advantages unique to AFM (ease of operation and measurements, the ability to apply not only small but large forces, e.g., exceeding 100 pN, and performing quick SMFS measurements with high loading rates). The Perkins group unequivocally demonstrated that large force drifts in AFM are caused by the AFM cantilevers themselves, and specifically identified the gold coating that reflects the laser light to be detected at the photodiode as the cause of the problem. By simply stripping the reflective gold layer from the back of AFM cantilevers using quick chemical etching, they practically eliminated (or greatly reduced) cantilever drifting and significantly improved force stability over long periods of time (e.g., 100 s) (8). However, these gold-stripped cantilevers still suffered a somewhat low signal/noise ratio and relatively low force precision, which was not on a par with optical tweezers. Their temporal resolution was also not too great. The next significant improvement by the Perkins group involved a combination of gold stripping (albeit now in a limited manner, as preserving a high reflectivity pad at the free end of the cantilever proved to be beneficial for generating strong force signals) with a very significant reduction of the size (and thus the mass) of the cantilever, which had been aimed at reducing its hydrodynamic drag coefficient. To achieve the latter goal, the group used focused ion beam (FIB) technology, which exploits highly energetic ions (e.g., gadolinium) to cut through the silicon nitride body of the cantilever to remove various sections from the cantilever, and thins it by removing a layer of the material from the back side of the cantilever (9). This elegant approach produced a number of cantilevers with various geometries and sizes all equipped with a reflective mirror pad at the cantilever-free end for a robust force signal upon

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cantilever bending in SMFS measurements. Some cantilevers had windows cut out with FIB in their bodies to reduce their mass and drag, whereas others were trimmed to small, single-beam shafts with a larger head. These cantilevers were subjected to rigorous testing of their force stability, force precision, and temporal resolution. The results of these comparative measurements and the evaluation of the "real-life" performance of these cantilevers in biophysical SMFS measurements on protein unfolding is now reported by the Perkins group in a Biophysical Letter in this issue of *Biophysical Journal* (10). Among the four different types of AFM cantilevers produced by the group using the FIB approach, the "Warhammer" cantilever, whose shaft is reduced to a mere 2–3 μm in width and a larger tip section (hammer head) is preserved for light reflectivity, proved to be the overall best-performing cantilever. The amazing AFM-based SMFS performance of the Warhammer cantilever was demonstrated by executing pulling experiments on mechanically weak proteins, such as calmodulin and α3D, and the measurements on calmodulin captured unfolding intermediates previously detected only by optical tweezers but unresolved by AFM. These cantilevers also showed a fantastic force precision and stability over slow pulling measurements, together with great temporal resolution. This is a spectacular achievement that should capture the attention of SMFS practitioners and the single-molecule biophysics community, as it creates an opportunity not only for experienced users, but also for potential new users who may consider acquiring a commercial AFM or building one for themselves now that this development promises an order of magnitude improvement of AFM performance for the types of measurements that previously were within the exclusive domain of optical tweezers. At this point, the embracement of this technology is to some extent dependent on the availability of FIB instruments to AFM users, and it is likely that the "army" of SMFS biophysicists equipped with Warhammers would grow very quickly if Warhammer cantilevers became available commercially. There is also a small issue related to possible difficulties when focusing the light on the tiny head of the Warhammer, when using older commercial instruments. Also, the appearance of a light interference pattern in the force signal due to the very small size of the cantilever may be considered as a nuisance, although the latter issue may be solved relatively easily via the method suggested by the authors in the Supporting Material that accompanies their letter. Congratulations are due to the Perkins group for such a significant improvement within the SMFS field.

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