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Atomic force microscopy with sub-picoNewton force stability for biological applications

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ABSTRACT

Atomic force microscopy (AFM) is widely used in the biological sciences. Despite 25 years of technical developments, two popular modes of bioAFM, imaging and single molecule force spectroscopy, remain hindered by relatively poor force precision and stability. Recently, we achieved both sub-pN force precision and stability under biologically useful conditions (in liquid at room temperature). Importantly, this sub-pN level of performance is routinely accessible using a commercial cantilever on a commercial instrument. The two critical results are that (*i*) force precision and stability were limited by the gold coating on the cantilevers, and (*ii*) smaller yet stiffer cantilevers did not lead to better force precision on time scales longer than 25 ms. These new findings complement our previous work that addressed tip-sample stability. In this review, we detail the methods needed to achieve this sub-pN force stability and demonstrate improvements in force spectroscopy and imaging when using uncoated cantilevers. With this improved cantilever performance, the widespread use of nonspecific biomolecular attachments becomes a limiting factor in high-precision studies. Thus, we conclude by briefly reviewing site-specific covalent-immobilization protocols for linking a biomolecule to the substrate and to the AFM tip.

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1. Introduction

Drift, in position and force, is a long-standing problem that limits the application of atomic force microscopy (AFM) in biology [1,2]. Poor long-term force stability hinders AFM-based single-molecule force spectroscopy experiments (SMFS), particularly those occurring over longer (>1 s) time frames. For example, state-of-the-art AFMs can be used to study the equilibrium folding and unfolding of proteins only over a few seconds [3], rather than the hundreds of seconds achieved with optical traps [4]. In addition, lack of force control hinders AFM imaging – the force set point during long scans often needs to be manually updated [1]. To quantify the scale of this problem, a force precision and stability of 5–10 pN is typical for commercial instruments, with custom instruments achieving 2 pN [3]. A related problem is the extended periods of time, often hours or even overnight [3], required for the AFM to "settle" after loading an AFM tip. Hence, routine and timely sub-picoNewton (pN) force precision and stability would accelerate a wide range of AFM-based biophysical assays, particularly if it could be achieved with commercial cantilevers on commercial instruments.

Recently, we found that the cantilever itself is the major source of force drift [5]. To understand this result, we need to review an underlying assumption in how force (F) is measured in AFM (Fig. 1A). It is assumed that changes in tip deflection (Δz) arise only from changes in the applied force. The force is then determined using $F = -k\Delta z = -k(z_{tip} - z_0)$ where k is the cantilever stiffness and z_{tip} the instantaneous deflection of the cantilever. Implicit in this assumption is that the zero-force position of the cantilever (z_0) does not depend on time. Contrary to this expectation, a simple test showed that z_0 is not constant, but drifts significantly for a popular class of soft silicon nitride cantilevers (BioLevers, Olympus) [5]. Specifically, the cantilever deflection laser measured an 800-fold higher drift rate when focused onto the cantilever than onto the base of the chip on which the cantilever was mounted (Fig. 1B, inset). This test unambiguously shows that the cantilever is the primary source of force drift (Fig. 1B), rather than external opto-mechanical sources (*i.e.*, laser pointing noise). As we will dis-

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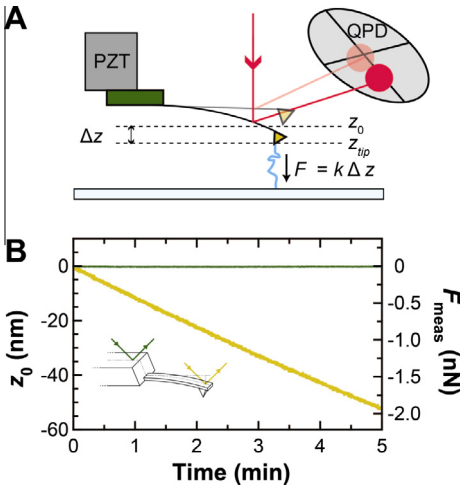

Fig. 1. Source of force drift in an atomic force microscope (AFM). (A) Schematic of a force spectroscopy experiment. The AFM tip is attached to a molecule and retracted from the surface. Force is determined by the deflection ($z_{tip} - z_0$), as measured on a quadrant photodiode (QPD). (B) With no molecule attached, the zero force position z_0 (*gold*) of a cantilever (short BioLever) was measured as a function of time 2 h after wetting. A similar record (*green*) was measured after repositioning the detection laser onto the chip holding the cantilever. These measurements were scaled using the cantilever's sensitivity ($S = 0.043$ V/nm) and stiffness ($k = 37$ pN/nm). This comparison demonstrates that the primary source of force drift is the cantilever. PZT: piezoelectric stage. This figure is reprinted from [5] with permission from the American Chemical Society.

cuss in detail below, the primary cause of this cantilever drift is its gold coating (Fig. 2).

Gold coatings are added to cantilevers to enhance their reflectivity and are traditionally seen as critical to improving the signal-to-noise ratio in AFM experiments [6]. Coating a cantilever only on its back side leads to a substantial thermally induced force drift because of the bimetallic effect [7,8]. Hence, many cantilevers, including the ones we studied, are coated on both sides to minimize such temperature-induced drift. Even with cantilevers coated on both sides, drift due to the gold coating has been previously reported [9,10]. The novel result from our work is not that the gold is associated with drift [7–11]. Rather, the key insight arises from a pair of results that are contrary to the conventional wisdom in AFM: (*i*) removing a cantilever's gold coating does not sacrifice the signal-to-noise ratio over relevant bandwidths (0.001–10,000 Hz) and (*ii*) smaller cantilevers do not always lead to better force precision. These results led to the unexpected insight that uncoated long BioLevers outperform uncoated BioLever Minis on time scales longer than 25 ms.

In this paper, we first discuss the current state of high-precision AFM, focusing on recent work on calmodulin that highlights the need for greater precision and stability in AFM. We then discuss our recent demonstration that removing the gold coating from cantilevers significantly increases the force precision and force stability [5]. We extend this result with related new results on the use of uncoated cantilevers in bio-imaging. With these advancements, the force stability during a typical AFM-based SMFS experiment is now limited by the use of nonspecific biomolecular attachments. We thus conclude by briefly reviewing site-specific covalent-immobilization protocols for linking biomolecules to the substrate and to the AFM tip.

1.1. Recent advances in sub-pN force resolution

The rates of folding and unfolding of biomolecules under constant force are sensitive to sub-pN changes in the applied load [12,13]. Hence, both sub-pN force precision and stability are critical to studying macromolecular folding at equilibrium conditions. As a result, most AFM-based force spectroscopy experiments have focused on non-equilibrium stretching protocols where the molecule under study is stretched at a relatively high rate (50–5000 nm/s). Such fast stretching protocols minimize the effect of positional and mechanical drift. If the whole experiment is over before there is significant drift, then the drift does not affect the results. In contrast, state-of-the-art optical traps can study equilibrium folding and unfolding because sub-pN force precision and stability are readily accessible. Clearly, the range of experiments that could be performed with AFM as well as their experimental precision would be enhanced if AFM could achieve a comparable level of instrumental performance.

Sub-pN force precision in a limited bandwidth has been observed during AFM-based SMFS experiments using lock-in amplification [14]. In this study, a 5-nm oscillation was applied to the tip at 20 Hz. These improvements allowed the folding pathway of an immunoglobulin to be more carefully examined. Overall, lock-in detection improves force precision in a specified bandwidth but can complicate interpretation.

A more general approach is to minimize drift and improve precision of the instrument. For example, the Rief group developed a custom-built, low-drift AFM. The stability of this instrument enabled them to investigate the conformational fluctuations of the calcium-sensing protein calmodulin at the single-molecule level [3] with high force precision (~2 pN) due, in part, to their extraordinarily slow pulling velocity (1 nm/s). At this stretching rate, they observed equilibrium hopping between two folding sub-states over 1–2 s. They went on to show how the kinetics of this hopping depended on the Ca^{2+} concentration. This example shows how a unique low-drift AFM facilitated partial reconstruction of calmodulin's folding/unfolding kinetic pathway.

Two years later, the same group elucidated the full kinetic pathway for calmodulin folding and unfolding using an ultrastable dual-beam optical trap [4]. The motivation for changing measurement platform was better force stability and precision. The

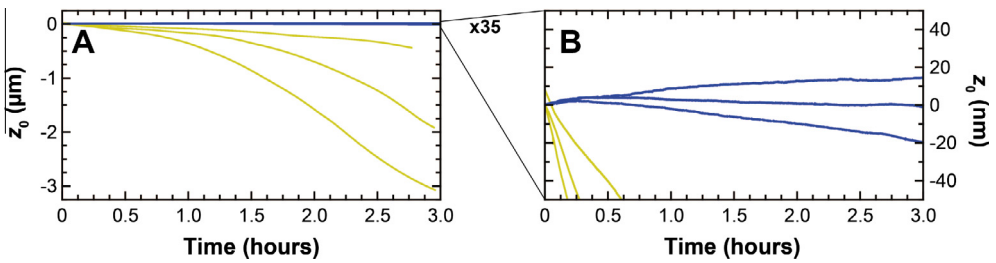

Fig. 2. Drift in the zero force position (z_0) on gold-coated (*gold*) versus uncoated (*blue*) BioLever Mini cantilevers. (A) Three-hour-long traces of z_0 show that gold-coated BioLever Mini cantilevers drift significantly more than uncoated ones. (B) Scale expanded by a factor of 35 shows minimal residual drift on uncoated BioLever Mini cantilevers.

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