Precision Surface-Coupled Optical-Trapping Assay with One-Basepair Resolution

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ABSTRACT The most commonly used optical-trapping assays are coupled to surfaces, yet such assays lack atomic-scale (−0.1 nm) spatial resolution due to drift between the surface and trap. We used active stabilization techniques to minimize surface motion to 0.1 nm in three dimensions and decrease multiple types of trap laser noise (pointing, intensity, mode, and polarization). As a result, we achieved nearly the thermal limit (<0.05 nm) of bead detection over a broad range of trap stiffness (kT = 0.05–0.5 pN/nm) and frequency (∆f = 0.03–100 Hz). We next demonstrated sensitivity to one-basepair (0.34-nm) steps along DNA in a surface-coupled assay at moderate force (6 pN). Moreover, basepair stability was achieved immediately after substantial (3.4 pN) changes in force. Active intensity stabilization also led to enhanced force precision (~0.01%) that resolved 0.1-pN force-induced changes in biomolecular dynamics (1). Surface-coupled trapping assays have measured the one-codon step of the ribosome that, with mechanical amplification, corresponds to a 2.7-nm displacement (2). To resolve smaller motions, such as the one-basepair (0.34-nm) step of RNA polymerase (3), researchers decoupled their experiments from the surface using dual-beam optical-trapping assays (3,4) to reduce surface-induced noise. Yet, the vast majority of single-molecule optical-trapping assays are coupled to surfaces (5–11). What is needed is a general method that permits surface-coupled optical-trapping assays to achieve positional precision at or near the atomic scale.

Measurements of biological motion by optical-trapping nanometry are corrupted by various noise sources (thermal, mechanical, laser, etc.). Thermally driven Brownian motion is dominant on short timescales (<0.01–0.1 s). Such motion, which has a zero mean, can be averaged to atomic-scale dimensions at the expense of temporal resolution and sets a theoretical limit for positional precision (i.e., the thermal limit; see the next section). To achieve this thermal limit, other noise sources must be reduced to maintain atomic-scale instrumental stability over the same (or ideally longer) time period.

Current surface-coupled optical-trapping assays lack such atomic-scale instrumental stability. A surface-coupled assay is physically connected to its local environment (e.g., coverslip or micropipette) and thus is sensitive to mechanical perturbations through this connection. One common application of this class of assays has a DNA molecule attached to a surface at one end while attached to an optically trapped bead at the other (Fig. 1 A). As a first step toward a surface-coupled optical-trapping instrument with atomic-scale sensitivity, we stabilized an optical microscope to 0.1 nm in three dimensions (12). In applying this technique to optical traps, two detection lasers (one monitoring the trapped bead, the other measuring fiducial position) establish a local, differential measurement reference frame (Fig. 1 B). Local detection reduces common noise (e.g., thermal expansion) unmeasured by sensors in closed-loop stages. Differential measurement reduces noise (e.g., air currents, objective drift) common to both lasers (13). The ultimate limit on instrumental stability is set by the differential pointing stability between the detection lasers (<0.065 nm laterally, ∆f = 0.1–50 Hz).

Mechanical stabilization of the surface alone was insufficient to achieve atomic-scale resolution in our optical-trapping apparatus. The trapping laser was also a significant source of instrumental noise. To achieve basepair resolution, dual-beam assays not only decoupled their assay from the surface, they reduced laser pointing instability by either encasing the optics in a helium enclosure (3) or by using a differential measurement (4). In contrast with these passive methods, we adopted an active method (Fig. 1 B). We translated pointing, mode, and polarization noise into intensity noise through the combination of a single-mode, polarization-maintaining fiber and a polarizing beam splitter. The resulting intensity was sampled and actively stabilized with a feedback loop to an acousto-optic modulator (AOM) positioned before the fiber. Minimization of intensity was motivated, in part, by our analysis that shows that trap intensity fluctuations of 1% induce apparent motion of 1 bp for a 1-μm-long DNA molecule (see the next section).

For studying the dynamics of molecular motors and nucleic acid structures, we sought to achieve atomic-scale
stability immediately after substantial (3.4 pN) changes in applied force. Previous work has only reported basepair stability at constant force, presumably after any thermal perturbations caused by changes in the trap laser power have subsided. Minimization of laser noise during changes in laser power was not straightforward. Large changes in the requested laser power led to a gain inversion in our feedback system and unpredictable results. The root cause was a thermal transient in the AOM induced by a change in the radio-frequency power used to modulate the laser power. Such transients took tens of seconds to ~10 min to subside. Understanding the origin of this pointing error allowed us to add extra optics to minimize its effect. With this enhanced active stabilization of the trapping laser in conjunction with active stabilization of the stage, we demonstrated sensitivity to 1-bp steps along DNA at moderate force and 1-bp stability immediately after a substantial change in force as well as sensitivity to 0.1-pN force-induced changes in DNA hairpin-unfolding dynamics.

NOISE IN OPTICAL-TRAPPING ASSAYS

Thermal noise limit

Brownian motion of a trapped bead has a zero mean, so time-averaging the bead position \( \langle x_{bd} \rangle \) reduces the uncertainty in position as long as instrumental drift is negligible over the time span averaged \( (\tau_{avg}) \). On short timescales \( (<1/f_0; f_0 \) is the roll-off frequency of the trapped bead motion), bead motion is correlated and thus, not statistically independent. However, by averaging \( N \) independent data points spaced at the correlation time \( (1/f_0) \), the standard deviation of the data \( \sigma = \sqrt{k_B T/k_T} \); \( k_B T \) is thermal energy and \( k_T \) is the trap stiffness \( (14) \) decreases by \( \sqrt{N} \) to achieve the thermal limit of detection. The uncertainty in \( x_{bd} \) after averaging over \( \tau_{avg} \) is given by the standard error of the mean \( (\sigma_{SEM}) \), i.e.,

\[
\sigma_{SEM} = \frac{\sigma}{\sqrt{N}} = \sqrt{\frac{k_B T}{k_T} \frac{1}{\tau_{avg} f_0}} = \sqrt{\frac{k_B T}{(k_T)^2} \frac{12 \pi^2 \eta r_{bd}^2}{\tau_{avg}}},
\]

where \( k_T = 12 \pi^2 \eta r_{bd} f_0 \), \( \eta \) is the viscosity of the liquid, and \( r_{bd} \) is the radius of the bead. We note that this calculation is for an isolated trapped bead removed from the surface. Theoretically, in an optical trap with a high trap stiffness of \( k_T = 0.53 \) pN/nm and \( r_{bd} = 165 \) nm, this uncertainty reduces to 1 bp in 2.5 ms. At a moderate \( k_T \) of 0.086 pN/nm, the time to average to \( \sigma_{SEM} = 1 \) bp increases to 95 ms. Experimentally, we achieved one-basepair resolution in 2.5 ms and 103 ms, at the respective \( k_T \). Thus, averaging of thermal noise can quantitatively match the theoretically predicted results, achieving basepair precision in \( x_{bd} \) at reasonable bandwidths.

Intensity fluctuations affect measurements of DNA length

Multiple types of laser noise degrade trap performance and therefore instrumental stability. Laser pointing noise causes motion of the trap relative to the detection laser and thus erroneous measurements in \( x_{bd} \). Historically, methods to reduce laser noise have primarily focused on laser pointing instability (3,4), because such motion has a 1:1 coupling with bead motion. However, under load, fluctuations in
intensity lead to fluctuations in bead position since the bead is offset from the trap center (Fig. 2 A). Here we calculate the effect intensity fluctuations have on both \( x_{\text{bd}} \) and the contour length \( L \) in a DNA-tethered particle assay (Fig. 2 B).

We use a simplified one-dimensional model where the forces on the bead are balanced. The force exerted by the DNA \( F_{\text{DNA}} \) is equal to the force exerted by the trap \( F_T \), i.e.,

\[
F_{\text{DNA}} = F_T = k_T x_{\text{bd}}. \tag{2}
\]

Initially, \( F_{\text{DNA}} \) is equal to \( F_{\text{DNA}}^0 = k_T^0 x_{\text{bd}}^0 \), where \( F_{\text{DNA}}^0 \) is the initial force, \( x_{\text{bd}}^0 \) is the initial bead position, and \( k_T^0 \) is the initial trap stiffness. If the intensity of the trap \( I \) fluctuates by \( \delta I \), there will be a fluctuation in the trap stiffness \( \delta k_T = \delta f k_T \) that moves the bead \( \delta x_{\text{bd}} \) and changes \( F_{\text{DNA}} \). To calculate the current \( F_{\text{DNA}} \), we use a Taylor series to expand around the initial extension of the DNA \( (x_{\text{DNA}}^0) \), i.e.,

\[
F_{\text{DNA}} = F_{\text{DNA}}^0 - k_{\text{DNA}}^0 \delta x_{\text{bd}}, \tag{3}
\]

where we define the initial DNA stiffness as \( k_{\text{DNA}}^0 = \left. \frac{\partial F_{\text{DNA}}}{\partial x_{\text{DNA}}} \right|_{x_{\text{DNA}} = x_{\text{DNA}}^0} \). Thus, after the intensity fluctuation, the balance of forces in Eq. 2 can now be rewritten as

\[
F_{\text{DNA}}^0 - k_{\text{DNA}}^0 \delta x_{\text{bd}} = (k_T^0 + \delta k_T)(x_{\text{bd}}^0 + \delta x_{\text{bd}}). \tag{4}
\]

Solving for \( \delta x_{\text{bd}} \) gives

\[
\delta x_{\text{bd}} = -x_{\text{bd}}^0 \delta k_T / (k_T^0 + k_{\text{DNA}}^0 + \delta k_T). \tag{5}
\]

After calculating \( \delta x_{\text{bd}} \), we calculate the apparent change in the contour length, \( \delta L \). We determine the fractional extension of the DNA \( f = x_{\text{DNA}} / L \), which is the ratio of the DNA extension to the contour length. To determine \( f \), we use a simple inverse formula valid at moderate force 1 pN \(< F < 10 \) pN (15) where enthalpic stretching is not significant (16),

\[
f = 1 - \left( \frac{1}{\sqrt{4 k_T x_{\text{bd}} / k_B T}} \right)
\]

Here \( k_B T \) is thermal energy (4.1 pN-nm), and \( p \) is the persistence length of the DNA (40 nm) at our ionic conditions and contour length (1000 nm) (17). Then, we can use \( f \) to find \( L \)

\[
L = x_{\text{DNA}} / f = (x_{\text{bd}} - x_{\text{bd}} - r_{\text{bd}}) / f,
\]

where \( x_{\text{stage}} \) is the distance between the trap center and the tether point of the DNA. Given Eqs. 6 and 7, \( \delta L \) can be found by calculating the initial contour length and subtracting it from the current contour length:

\[
\delta L = \frac{(x_{\text{stage}} - x_{\text{bd}} - \delta x_{\text{bd}} - r_{\text{bd}})}{1 - 1 / \sqrt{4 p k_T (k_{\text{bd}}^0 + \delta x_{\text{bd}}) / k_B T}} - \frac{(x_{\text{stage}} - x_{\text{bd}} - r_{\text{bd}})}{1 - 1 / \sqrt{4 p k_T x_{\text{bd}} / k_B T}}.
\]

As expected in a first-order approximation, we find that \( \delta L \propto \delta I \) (Fig. 2 C) and \( \delta L \propto L \) (Fig. 2 D). We note that at constant force, \( \delta L \) is independent of \( k_T \) (Fig. 2 E). Given the parameters.
A 1% fluctuation in intensity creates a partially. The gain inversion was caused by transient heating of the AOM when the output intensity of the AOM was changed substantially. Our success was disabling the fan in the head unit and replacing it with a ducted, 18.6-L/s fan to reduce transient heating of the Nd:YVO4 crystal.

A gain inversion (Fig. 1A) motivated us to minimize intensity noise in combination with pointing noise to improve long-term instrumental stability.

MATERIALS AND METHODS

Optical trapping microscope

Our highly stable optical-trapping system, based on earlier work (12,13), consisted of a high-powered 1064-nm laser for trapping, a 785-nm laser for detecting bead motion, and an 850-nm laser for detecting stage drift (Fig. 1B). We used both passive and active methods to improve the stability of the apparatus.

Passive stabilization of the instrument reduced mechanical, thermal, and laser noise. We improved the mechanical stability of the microscope (TE-2000; Nikon, Tokyo, Japan) by stiffening the condenser pillar with an aluminum trapezoid (12.5-mm thick). In addition, the objective and stage were mounted with custom-built monolithic parts, and the whole apparatus was mounted on a research-grade optical table. We reduced thermal noise by coupling the lamp to the microscope with a liquid light guide (3-mm diameter; Rofin, Dingley Victoria, Australia). To minimize laser noise, optics external to the microscope were enclosed in a box, and the beam path was further enclosed in 25-mm diameter tubing where possible. Measurements were made in an acoustically quiet (NC30), temperature-regulated room (±0.2°C).

Active stabilization was used to minimize the instrumental drift of the lasers and the sample. We actively stabilized the sample by measuring a fiducial mark on the surface (a low-index glass post (12) or a silicon disk (18)). We then used this position in a feedback loop to a piezo-electric (PZT) stage (PS17.3CD; Physik Instrumente, Karlsruhe, Germany) to keep the surface position constant relative to the detection laser. We actively stabilized the detection lasers, as reported earlier (12).

We detected bead and fiducial marks using back-focal-plane detection (19–21); detection electronics were the same as in previous work (12). Trap stiffness was controlled by a computer-generated voltage to the trap intensity servo. Trap stiffness, at 10 different laser intensities, was calibrated using the equipartition theorem at a trap height of ~400 nm from the surface and verified to within 10% using power spectral analysis.

Active reduction of trap laser noise

To reduce trap laser noise, we also used active stabilization. Specifically, we minimized multiple types of laser noise by transforming pointing, mode, and polarization noise into intensity noise using the combination of a single-mode, polarization-maintaining fiber and a polarizing beam splitter, as was previously done for the detection lasers (12). We then sampled 10% of the light onto a photodiode (YAG-444-A; PerkinElmer Optoelectronics, Fremont, CA). The photodiode signal was converted to voltage using an analog transimpedance amplifier with a resistance of 10 kΩ (see 22) and was analyzed using custom-built electronics. Specifically, we used a low-delay, proportional-integral analog servo controller with a 200 kHz bandwidth (24) that output a voltage signal to the AOM (1205C, 1205, California) to keep the surface position constant relative to the detection laser. We actively stabilized the detection lasers, as reported earlier (12).

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Sample preparation

We fabricated fiducial marks (low-index glass posts (12), radius = 500 nm; silicon disks (18), radius = 500 nm) onto cover slips for use in active stabilization. To enhance usability in single-molecule assays, the coverslips were cleaned with a 10-min Piranha etch (100 mL H2SO4 and 15 drops H2O2 at 80°C) after nanofabrication. We constructed epoxy-rigidified flow chambers using double-stick tape (3M, St. Paul, MN) as a spacer and 5-min epoxy (Devcon, Danvers, MA) for rigidity. The epoxy was essential to both stiffening the flow chamber and maintaining its structural integrity over ~12 h while exposed to aqueous solution. For experiments with only a trapped bead, we used 10-μM polyethylene beads (330-nm diameter; Interfacial Dynamics, Eugene, OR) in wash buffer (25 mM Tris acetate, pH 7.5; 1 mM Mg(OAc)2; 1 mM NaCl; 1 mM dithiothreitol; 0.4% Tween-20 (Bio-Rad, Hercules, CA); and 3 mg/mL bovine serum albumin (concentration cited is that before filtration through a 0.2-μm filter)).

For experiments with DNA, we prepared double-stranded DNA by polymerase chain reaction (GeneAmp XL PCR Kit; Applied Biosystems, Foster City, CA) using one digoxigenin-labeled and one biotin-labeled primer (Integrated DNA Technologies, Coralville, IA). These labels enabled us to attach a DNA molecule (L = 556 or 1007 nm) at one end to a streptavidin-coated bead (320-nm diameter; Spherotech, Lake Forest, IL) and at the
other end to an antidigoxigenin-coated coverslip. Coated coverslips were made by incubating 20 mg/mL antidigoxigenin antibody (Roche, Indianapolis, IN) suspended in 0.1 M sodium phosphate buffer for 1 h in flow chambers. Bead-DNA complexes were made by incubating streptavidin-coated beads (900 pM) at a 9:1 molar ratio with labeled DNA at room temperature. After washing the flow chamber with wash buffer, the bead-DNA mixture was flowed into the chamber and allowed to incubate for 1 h before washing again. Preparation of the 20TS06/T4 hairpin followed published protocols (27), and tethers (L = 1020 nm) were made using a protocol similar to the one above. Coverslips were reused (>20 times) by soaking the flow chamber in boiling water for 1 h (to remove the coverslip) and then cleaning the coverslip with a 10-min Piranha etch.

DNA stretching under active stabilization

For a bead-DNA complex, we first determined the vertical location of the surface by monitoring the sum signal as a tethered bead was brought into contact with the surface (28). We then lowered the stage 300 nm. We found the lateral tether point position by performing a two-dimensional elasticity-centering procedure, which also returned the persistence length. Bead-DNA complexes anchored by multiple DNA molecules (determined by a low persistence length) were not studied. We next centered the 850-nm laser on a nearby fiducial mark using a PZT mirror. Finally, we stretched the DNA along the x axis using the PZT stage to a specified force (with a corresponding move of the stage-tracking 850-nm laser). After stretching the tether, we actively stabilized the sample using a simple software-based feedback loop with a 100-Hz update rate and a proportional gain of 0.05. Concurrent with measuring fiducial mark position, the software also measured the trapped bead position.

Contour length was derived using the well-established method of Wang et al., which takes into account both the vertical and horizontal motion of the bead in the trap (16). For this analysis, one needs Δrvert, Δrhor, r0, the ratio of horizontal to vertical trap stiffness (5.2), and ztrap, the height from the coverslip surface to the trap center. As stated above, trap stiffness was calibrated at 10 different stiffness values (up to 0.4 pN/nm) using both the equipartition theorem and power spectral analysis. We also established the linearity of the trap (F = kT Δrhor) using hydrodynamic drag, with a <2% deviation from linearity at Δrhor ~ 70 nm.

For measuring DNA hairpin dynamics under constant force, we implemented a force-clamp in parallel with the above stage stabilization. We modulated kT at 100 Hz such that the force (F = kT Δrhor) was maintained to ~0.01%. Since we aperiodically modulated kT by ~20% to maintain constant F, substantial low-frequency intensity noise would be expected without active stabilization in laser intensity (Fig. 3B). The benefit of modulating kT over moving the stage is a faster response time and the ability to have two independent servo loops controlling sample position and force.

RESULTS AND DISCUSSION

Atomic-scale sensitivity to trapped bead motion

After reducing both surface perturbations and trap laser noise, we first demonstrated the excellent positional stability of the trap laser relative to the detection laser. One useful metric is to plot the power spectral density (PSD) of a trapped
bead (Fig. 4 A, inset), which shows the noise as a function of frequency. Another useful metric for calculating noise is to integrate the PSD within a specified bandwidth to produce the integrated noise. This integrated noise (0.17 nm) was a fraction of a basepair over a broadly useful bandwidth ($\Delta f = 0.02$–100 Hz) for a 330-nm diameter bead trapped at a stiffness of 0.53 pN/nm (Fig. 4 A). This noise level was within 0.05 nm of the thermal noise limit and was maintained over a wide range of trap stiffness (Fig. 4 B). Interestingly, our integrated trap noise was only ~0.05 nm higher than the best reported trap stability (3), though our method used a fourfold lower stiffness and did not require the encasement of optics in helium.

Next, we achieved atomic-scale sensitivity to bead motion. Using a PZT mirror, we generated 0.4-nm trap motion (limited by 1 bit changes in a control voltage). The resulting steps were well resolved (0.43 ± 0.08 nm (peak ± half-width at half maximum, HWHM)) with a signal/noise ratio of 5 in a trace filtered to 5 Hz using a Savitzky-Golay window (Fig. 4 C). Thus, by reducing mechanical perturbations and excess trapped-bead noise, we achieved sufficient stability and precision to measure atomic-scale bead motion.

1-bp sensitivity in a surface-coupled assay

We next achieved 1-bp stability in a surface-coupled DNA assay (Fig. 1 A) over tens of seconds. Measurements of DNA contour length ($L = 556$ nm) were taken at a moderate force of 6 pN with and without active stabilization of both the surface and trap (Fig. 5 A). Active stabilization resulted in a fourfold decrease in the integrated noise to 0.15 nm at 0.1 Hz (Fig. 5 B). Additionally, we maintained a 1-bp (0.34-nm) positional precision over a useful frequency range ($\Delta f = 0.03$–2 Hz). The stability for a 10-fold longer time period (Fig. 5 C) was slightly larger at 0.48 nm ($\Delta f = 0.003$–2 Hz). Such long-term traces contain some rapid changes in $L$ of ≥1 bp. At this noise level, only ~10% of identified steps would be erroneous for a molecular motor stepping in 1-bp increments at the rate of 0.2 s$^{-1}$. As expected, the spatial precision increased with increasing force (Fig. 5 D) over a broad bandwidth ($\Delta f = 0.1$–10 Hz), but is currently limited by the residual error in stage stabilization.

Ideally, we seek to perform high-resolution studies of force-sensitive states. For example, the Escherichia coli RecBCD helicase experiences a force-induced backward slip if exposed to $F > 6$ pN after a short time (~1–10 s) (26). To measure RecBCD’s motion with basepair resolution during such short-lived states, one would make a step change in the force from less than to greater than 6 pN and take data within this sub-10 s time window—a period much shorter than the thermal equilibrium time of the AOM (~10–1000 s). Such force jumps have also been used to characterize RNA structures (29). With active stabilization of the lasers and the microscope, we attained 1-bp stability after dynamic force changes generated by modulating $k_T$. Specifically, the spatial precision in the 60 s after a force change of 3.4 pN (Fig. 5 E) yielded a stability of 0.31 nm (RMS) over $\Delta f = 0.1$–10 Hz, in quantitative agreement with steady-state measurements (Fig. 5 D). A small (~2 nm), systematic offset in $L$ accompanied the change in force. Such offsets have been seen previously (26) and are due, in part, to the difficulty in correctly modeling the elasticity of short DNA molecules (17).

Finally, we demonstrated sensitivity to steps along DNA by moving the stage in a series of 0.34-nm increments every 5 s (Fig. 6 A). Conceptually, this stage motion is the signal input (Fig. 6 A, blue). The deduced contour length, calculated without incorporating the stage motion, is the signal corrupted by thermal and mechanical noise. Quantification of steps in single-molecule experiments can be done by a step-fitting algorithm (6). Such fitting of our data recapitulated the input
motion over both a few (0.33 ± 0.08 nm (mean ± SD; N = 6)) and many steps (0.38 ± 0.13 nm (mean ± SD; N = 52)). Another metric to demonstrate step detection is a pairwise-distance distribution that revealed a peak at 0.31 ± 0.09 nm (peak ± HWHM) (Fig. 6 B). Experimentally, the signal/noise ratio of the data is ~4, within the signal/noise threshold for accurate determination of steps (30). Thus, we resolved the smallest known biological step in the widely used surface-coupled assay.

**Precise force control**

The precise control of force developed here is beneficial to experiments beyond resolving steps of molecular motors. Biological structures are highly sensitive to changes in the applied force (8,27). To illustrate the importance of steady-state force control, we studied a previously characterized DNA hairpin, 20TS06/T4 (27), and measured its unfolding dynamics (Fig. 7). We obtained an unfolding distance (∆x) of 19.7 nm and a force at which the hairpin has a 50% probability of being unfolded (∆F 1/2) of 12.3 pN, in quantitative agreement with previous results (27). Small force changes (∆F) of 0.1 pN in either direction substantially shifted the probability of being unfolded (Fig. 7). More quantitatively, a force change of <1% (∆F/∆F 1/2) shifted the probability of being folded by ~20%. In such cases where the biological molecule under study is exquisitely sensitive to the applied force, precise force control must be maintained. Yet, commercial lasers rarely specify intensity stability to >1%. Hence, our reduction in intensity noise to ~0.01% (RMS) provides a biologically useful increase in precision for force measurements.
CONCLUSIONS

We used active stabilization of both the surface and trap to achieve 1-bp positional precision in a surface-coupled optical-trapping assay. By actively stabilizing multiple forms of laser noise, we tracked bead position to within 0.05 nm of the thermal limit and measured 0.1-pN force-induced changes in DNA hairpin unfolding dynamics. Our current short-term (~1 s) positional precision is limited by the residual error (0.1 nm) in surface stabilization. We expect that it is this noise that prevents us from reaching the theoretical limit under these conditions (30). Further improvements to increase surface stabilization could include a feedback loop with a field-programmable gate array coupled with a stiffer stage to increase the loop closure time (31). To improve long-term stability, differential laser motion could be minimized with a more compact optical design and by launching all three lasers from one fiber. This improved the differential laser stability for two lasers, in a different application, from ~65 pm laterally to 19 pm (Δf = 0.1–50 Hz) (18).

In comparison with existing high-resolution dual-beam methods (3,4), we achieved 1-bp positional precision at a factor of 1.2–3 lower force with a surface-coupled assay and without the encasement of optics in helium. Dual-beam assays with passive force-clamps enable enhanced measurements, in part, by eliminating the need for a compliance correction (32). Dual-beam assays with differential detection offer a small (√2), but significant, increase in time resolution (4). In contrast, our active stabilization method increases force sensitivity, stabilizes the geometry in all three dimensions, and is surface-coupled for rapid adoption to a wide variety of existing assays (5–11). Dual-beam assays would also benefit from increased force precision. Future assays could combine single-molecule force and fluorescence assays using total internal reflection fluorescence, a surface-based technique (9), with the enhancements presented here.

The authors thank Marileen Dogterom for the step-finding algorithm, Wayne Halsey for DNA preparation, Lora Nugent-Glandorf and Amanda Carpenter for help in the early development of the apparatus, and the JILA shops for custom machining and electronics.

This work was supported by a Burroughs Wellcome Fund Career Award in the Biomedical Sciences (to T.T.P.); a grant from the Optical Science and Engineering Program (National Science Foundation—Interdisciplinary Graduate Education Research Traineeship) (to A.R.C.); a National Physical Science Consortium fellowship (to A.R.C.); grants from the National Science Foundation (Nos. Phy-0404286 and Phy-1551010); a W.M. Keck Initiative in the RNA Sciences; and the National Institute of Standards and Technology (NIST). Mention of commercial products is for information only; it does not imply NIST’s recommendation or endorsement. T.T.P. is a staff member of NIST’s Quantum Physics Division.
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