

Measuring 0.1-nm motion in 1 ms in an optical microscope with differential back-focal-plane detection

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Received May 25, 2004

Back-focal-plane detection of micrometer-sized beads offers subnanometer resolution for single-molecule, optical trapping experiments. However, laser beam-pointing instability and mechanical drift of the microscope limit the resolution of optical-trapping experiments. By combining two infrared lasers with improved differential beam-pointing stability ($<0.05 \mu\text{rad}$), we simultaneously measure and subtract the motion of the microscope stage, leading to a resolution of $<0.1 \text{ nm}$ in 1 ms and stability of 0.5 nm over 60 s. Repeated steps of 0.4 nm at 1 Hz are resolved with a signal-to-noise ratio of 25.

OCIS codes: 170.0180, 180.3170, 170.4520, 170.6920.

Single-molecule experiments are revolutionizing biophysics.^{1–4} Optical-trapping experiments have measured net motion greater than 1 nm, which is sufficient to resolve the 8-nm step of kinesin,¹ and, by averaging over multiple traces, the 5.5-nm step of myosin-II² and the 1.7-nm backsliding of RNA polymerase.³ Single-molecule fluorescence experiments can also resolve motion near 1.5 nm.⁴ Yet a variety of important biological motions occur on even smaller distance scales. In particular, enzymes replicating DNA are expected to take 1-base-pair steps, corresponding to 0.338 nm along the DNA helix.⁵ However, to our knowledge, the measurement of such a step has not been achieved.

In optical-trapping experiments, noise arises from two sources: Brownian motion and instrumental drift. Brownian motion of the bead dominates on short time scales, but Brownian motion has a zero mean, so time averaging reduces the uncertainty in position. In an optical trap with a stiffness of 0.2 pN/nm holding a 200-nm-radius bead, this uncertainty reduces to 0.3 nm in 35 ms. Instrumental noise, however, does not average to zero. Slow drift arises from air currents, temperature variations, and differential motion between the sample and the objective. For optical-trapping experiments, instrumental drift is dominant on the time scale necessary for averaging Brownian motion to subnanometer precision.

For more than a decade it has been known that laser-based measurements theoretically could achieve subpicometer localization of micrometer-sized beads at 1 Hz (Ref. 6) and therefore could resolve subnanometer bead motion. However, instrumental drift at low frequencies limited such measurements.^{6,7} Initially, Denk and Webb introduced a one-dimensional optical-trapping interferometer.⁶ Visscher *et al.* introduced a two-dimensional technique based on the distribution of light in the back focal plane.⁷ Praelle *et al.*, expanding on earlier work,⁸ generalized back-focal-plane detection to three dimensions.⁹ However, real-time measurements of 0.1-nm net motion in an optical microscope have not been reported. Microscopists have long used reference marks, called fiducial marks, embedded

in their specimens. They then measure the distance between the fiducial mark and the object of interest, thereby reducing the effect of sample drift and improving resolution. With video-enhanced differential interference contrast microscopy, Gelles *et al.* used fiducial marks to achieve a resolution of 1–2 nm.¹⁰ Smith *et al.* measured stage motion, using an externally mounted lens and laser, at a resolution of 1.5 nm.¹¹ In this Letter we introduce a local, differential measurement technique that measures 0.1-nm motion with high time resolution (1 ms). Our technique relies on improved beam-pointing stability, which, in turn, facilitates subtraction of stage noise. Most importantly, this technique has sufficient stability that a bead's Brownian motion in an optical trap can be averaged to produce the same spatial resolution.

Our design (Fig. 1) is similar to the original back-focal-plane detection system,⁷ with several enhancements to improve stability. Two temperature-stabilized, optically isolated diode lasers (785 and 850 nm) were passed through single-mode, polarization-maintaining fibers to reduce pointing instability. We further enhanced beam-pointing stability by mechanically stabilizing the fiber immediately before the fiber launch. These laser beams, combined by use of a dichroic mirror, were translated in the imaging plane of the microscope by two two-axis, closed-loop piezoelectric transducer (PZT) mirrors imaged onto

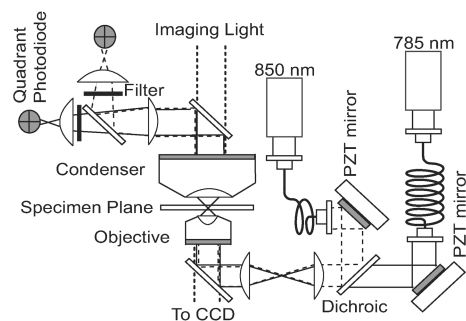


Fig. 1. Optical layout for differential back-focal-plane detection by use of two diode lasers. Shaded components are in optically conjugate planes.

the back focal plane of the objective. To reduce air currents, optics external to the microscope were enclosed in a box. These experiments were performed in a temperature-controlled ($\pm 0.2^\circ\text{C}$), acoustically quiet clean room.

An inverted microscope was mounted onto an air-damped optical table. We replaced the original microscope stage with a monolithic aluminum stage designed to hold a three-axis, closed-loop piezoelectric stage. The objective (PlanAPO-100X-IR; N.A. 1.4) was held in a custom-designed monolithic mount. We supported the condenser optics directly off the vertical condenser pillar.⁷ However, we further stiffened this pillar with a supplementary aluminum trapezoid (12.5 mm thick). The condenser's back focal plane was imaged by a pair of lenses onto quadrant photodiodes (QPDs), which were held in a 60-mm square-cage assembly along with the necessary dichroics and narrowband blocking filters. We determined the normalized intensity difference of the light incident upon the QPDs along the X and Y axes by using custom-built electronics. The normalized differences as well as the sum signal were then digitized at 16-bit resolution.

We demonstrate our technique by using beads stuck to a coverslip, as stage motion is the dominant noise source and our differential detector system will work in conjunction with conventional surface-coupled optical-trapping assays.^{1,2,12} Beads to be used as fiducial marks must be rigidly stuck to the coverslip. We affixed polystyrene beads ($r = 200$ nm) to the coverslip glass by first incubating 2-pM beads in 50 mM of Mg^{2+} for 10 min in an $\sim 15\text{-}\mu\text{l}$ epoxy-stabilized flow chamber¹² and then heating them to 100°C for 20 min. Buffered solution was then reintroduced into the sample chamber.

At the start of each measurement, a stuck bead was crudely centered within the 785-nm detector beam focus ($I \cong 40 \mu\text{W}$ at the QPD). Next we moved the stage separately along each axis (-400 to $+400$ nm) while monitoring the QPD output (V^{QPD}). This translated the bead through the stationary beam, yielding a voltage-versus-distance curve, $V^{\text{QPD}}(x_{\text{stage}})$, with the functional form of the derivative of a Gaussian,^{7,8} thus allowing the bead's center to be determined. We then centered the 850-nm laser on the same or a nearby ($<4 \mu\text{m}$ away) bead using the PZT mirror. To center the beads vertically we monitored the change in the sum signal for 850-nm light scattered from the bead onto the QPD.⁹ The entire process was done twice, first as a coarse alignment and then as a fine alignment.

We calibrated the sensitivity (volts per nanometer) of individual bead–laser pairs during each measurement by moving stuck beads through both detector beams simultaneously over a smaller range (-200 to $+200$ nm). This yielded a single-valued $V^{\text{QPD}}(x_{\text{stage}})$ curve for each laser. Next the resultant voltages were digitized at 1 kHz, converted to position, and then smoothed and decimated as indicated.

Both lasers measured essentially the same motion ($\sim 0.1\text{-nm/s}$ drift plus additional high-frequency noise) from two different beads stuck to the same microscope

slide [Fig. 2(a)]. These data demonstrate that the common-mode signal of stage drift dominated the laser-pointing instability. Our pointing stability allows us to use one bead as the fiducial mark for the other,¹⁰ thereby subtracting out the stage drift. The difference (Δx) showed $>10\times$ increased stability (<0.5 nm over 60 s) and suppression of the higher-frequency noise. By tracking the rapid 0.5–0.8-nm motion of the stuck beads [Fig. 2(b)], we demonstrated our ability to resolve motion via the differential signal to 0.1 nm in 1 ms. This corresponds to a joint, differential angular noise of $0.05 \mu\text{rad}$ between the lasers when $\Delta x = f_{\text{obj}}\Delta\theta$, where f_{obj} is the focal length of the objective (2 mm).

Molecular motors take steps.¹ To determine our ability to resolve such steps, we experimentally simulated them. Steps generated by stage motion lead to a common signal in both detector records—like stage drift—and their detection is therefore suppressed. To generate steps we translated one laser beam by 0.4 nm while leaving the other fixed, creating apparent bead motion.

As Brownian motion dominates the position signal of optical trapping experiments on short time scales (<35 ms), we used relatively long-duration steps (1 s) to ensure that there would be sufficient time to average positional uncertainty to <0.1 nm, a previously inaccessible resolution owing to instrumental drift. Our raw data record did not show 0.4-nm steps [Fig. 3(a)], as occurred for previous measurements that also had poor low-frequency (<10 Hz) performance.^{6,7} However, subtracting out the stage noise causes the differential trace to show clear steps, resolved over tens of seconds [Fig. 3(a)]. Importantly,

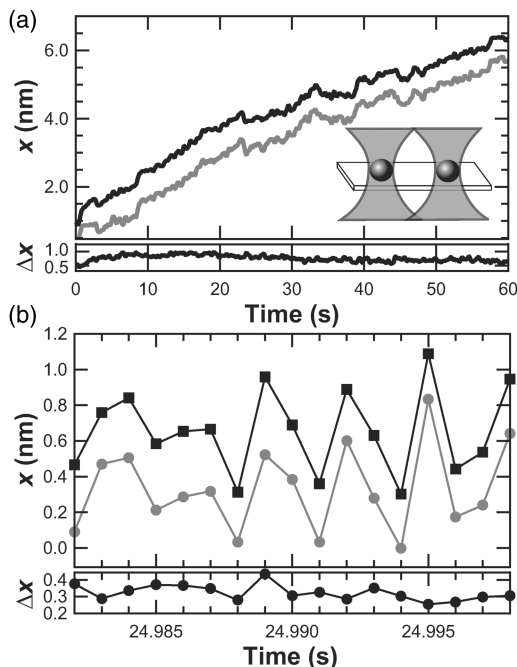


Fig. 2. (a) Position records of two beads measured separately by two detector laser beams at 10 Hz. Subtracting out the stage noise kept the difference (bottom trace) stable to 0.5 nm over 60 s. (b) In a separate test, two records of bead motion recorded at 1 kHz, and the difference.

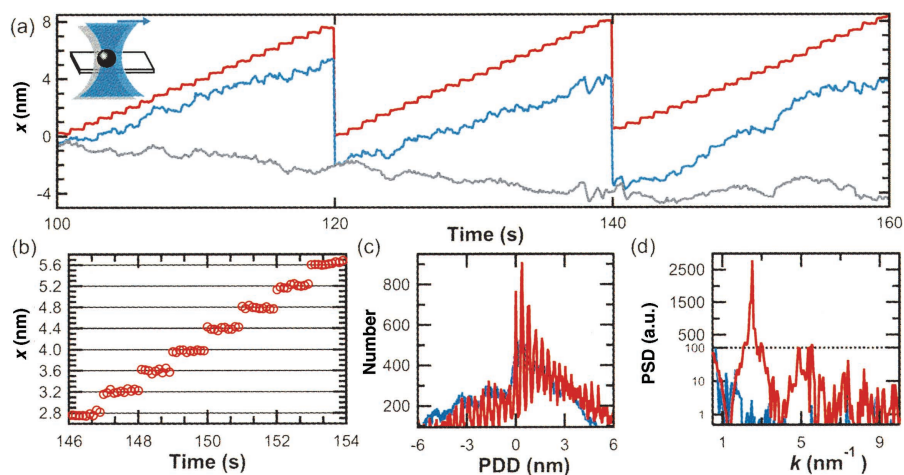


Fig. 3. (a) Two laser beams (blue and gray) measure the position of the same bead at 10 Hz. The blue laser beam is moved in 0.4-nm steps with a PZT mirror. Subtracting the common noise reveals 0.4-nm steps (red). Traces were moved vertically for clarity of presentation. (b) Individual 0.4-nm steps resolved at 10 Hz. (c) Trace of PDDs for the differential signal (red) and the normal signal (blue). (d) Fourier transform of the PDD histograms in (c). PSD, power spectral density.

these steps were stable to better than 0.1 nm over a number of steps for many portions of the trace, allowing for direct determination of the step size [Fig. 3(b)].

Even though these steps were clearly resolvable, we wanted an unbiased measurement of step size and an estimate of the signal-to-noise ratio. The pairwise distance difference (PDD) calculates the distance between pairs of points in the record.^{1,10,13} Ideally, every such difference would be a multiple of 0.4 nm. A histogram of PDD data would then show peaks at 0.4-nm intervals. A histogram of our raw data shows no peaks, whereas the differential measurement shows clearly resolved peaks [Fig. 3(c)]. A Fourier transform of the graphed data determines the spatial frequency components that are present in the data [Fig. 3(d)]. The dominant peak is at $k = 2.52 \pm 0.20 \text{ nm}^{-1}$ (peak \pm FWHM), corresponding to a step size of $0.40 \pm 0.04 \text{ nm}$. Compared with the differential signal, the uncorrected signal showed no detectable peak in the Fourier transform at the proper spatial frequency ($k = 2.52 \text{ nm}^{-1}$). An approximate signal-to-noise ratio for determining step size is 25, based on the next largest signal in the power spectrum [dotted line, Fig. 3(d)]. Although repeated steps smaller than 0.4 nm are resolvable, they could not be generated because of limitations in our PZT mirrors.

We anticipate that this differential measurement technique will significantly improve the resolution of a variety of single-molecule biophysics experiments. Many current single-molecule motility assays are surface based.^{1,4} Hence they are incompatible with a fully suspended, dual-beam optical-trap setup in which similar long-term stability was achieved.³ In particular, our measurements of 0.4 nm with a signal-to-noise ratio of >25 should allow for 0.338-nm (1-base pair) motion along DNA to be determined. Our technique can be adapted to aid the recent breakthrough in which single fluorophores are tracked with nanometer-scale precision.⁴

This study was supported by a Burroughs Wellcome Fund Career Award in the Biomedical Sciences to T. T. Perkins, by the National Science Foundation, by a Jane and Charlie Butcher grant, and by the National Institute of Standards and Technology (NIST). We thank Terry Brown and Carl Sauer for custom electronics, Hans Green and Blaine Horner for custom machining, and Kunio Toshimitsu for technical discussions. The authors are staff members of NIST's Quantum Physics Division. T. T. Perkins's e-mail address is tperkins@jila.colorado.edu.

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