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High-Performance Image-Based Measurements of Biological Forces and Interactions in a Dual Optical Trap

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Abstract

Optical traps enable nanoscale manipulation of individual biomolecules while measuring molecular forces and lengths. This ability relies on the sensitive detection of optically trapped particles, typically accomplished using laser-based interferometric methods. Recently, image-based particle tracking techniques have garnered increased interest as a potential alternative to laser-based detection, however successful integration of image-based methods into optical trapping instruments for biophysical applications and force measurements has remained elusive. Here we develop a camera-based detection platform that enables accurate and precise measurements of biological forces and interactions in a dual optical trap. In demonstration, we stretch and unzip DNA molecules while measuring the relative distances of trapped particles from their trapping centers with sub-nanometer accuracy and precision. We then use the DNA unzipping technique to localize bound proteins with sub-base-pair precision, revealing how thermal DNA “breathing” fluctuations allow an unzipping fork to detect and respond to the presence of a protein bound downstream. This work advances the capabilities of image tracking in optical traps, providing a state-of-the-art detection method that is accessible, highly flexible, and broadly compatible with diverse experimental substrates and other nanometric techniques.

Graphical Abstract

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Supporting Information Available: “Supporting Information” containing Supplementary Figures 1–11, Supplementary Tables 1–2, and Supplementary Note 1. “Supporting Video” containing Supplementary Video 1. This material is available free of charge *via* the Internet at <http://pubs.acs.org>.

Keywords

dual optical trap; image tracking; camera; FPGA; DNA unzipping; restriction enzyme footprint; DNA breathing

Optical traps (OTs) have made indelible contributions to our understanding of nanoscale biophysical processes through their powerful abilities to measure forces and distances while actively manipulating individual biomolecules.¹ Their determination of molecular forces with sub-piconewton accuracy on sub-millisecond timescales relies on a fundamental measurement: the displacement of a trapped microsphere (bead), a reporter particle attached to a biomolecule of interest, from the trap's center. Traditionally, this quantity is obtained through the technique of back-focal-plane interferometry (BFPI),² which uses a photodiode to measure the deflection of the trapping laser that occurs when a bead is displaced from the trap's center. The exceptional bandwidth, precision, and accuracy provided by BFPI has enabling such feats as the observations of single base-pair steps by translocating motor proteins,^{3,4} and the detailed characterization of histone-DNA interactions with near base pair resolution.⁵

Camera-based particle tracking is an appealing alternative to BFPI due to its ability to make direct measurements of positions and distances in the sample plane, its flexibility, and its relative ease of implementation on a commercial microscope. Over the past two decades, the use and efficacy of image tracking in scientific instruments has flourished in direct response to a remarkable revolution in digital camera technology that has yielded dramatic improvements in data bandwidth, sensor noise, and real-time tracking techniques.⁶⁻⁹ Indeed, image tracking is already widely used in the field of magnetic tweezers where the absolute positions of reporter particles are tracked with sub-nanometer precision at multi-kilohertz speeds,^{7,9,10} and has also been used recently to localize optically-trapped particles in solution with similar precision and speed.¹¹⁻¹³ However, despite these achievements, image tracking has not yet demonstrated the ability to make accordingly accurate real-time measurements of biological forces in an OT.

This discrepancy has a subtle but important source: optical traps present unique challenges for real-time image-based force measurements in that it is the bead's displacement from trap center, rather than its absolute position, that is of interest. While BFPI obtains this quantity inherently and accurately in a single measurement, cameras show only the bead's absolute position and offer no coincident measure of trap position. In practice, even the most precise pre-determined estimates of trap position in the sample plane are rendered inaccurate by laser pointing fluctuations and drift,¹⁴ which alter trap position by tens to hundreds of nanometers over experimental timescales. Consequently, an image-based OT able to perform advanced single-molecule manipulation experiments and obtain biological measurements of the same exceptional quality as its precision photodiode-based counterparts has not yet been realized.

Here we present an adept dual optical trap and a set of camera-based detection techniques that overcome these challenges. We determine biological forces by measuring the relative displacement of the beads within our optical traps with both sub-nanometer precision and sub-nanometer accuracy, across a trap movement range of nearly 10 μm , and at up to 10 kHz in real time using a low-latency field-programmable gate array (FPGA) tracking implementation. We demonstrate our instrument's capabilities by stretching and unzipping DNA molecules, and then use the versatile DNA unzipping technique to localize bound proteins with sub-base-pair precision. Revealed by the instrument's exceptional data quality, we additionally find that thermal DNA fluctuations enable an approaching unzipping fork to detect and respond to a bound protein that is downstream of the fork's mean position, and model the sequence-dependence of this behavior theoretically.

Results and Discussion

Instrument Design.

Maximizing the performance of camera-based optical trap detection requires both a stable optical trap and a capable image tracking system. Our instrument (Fig. 1a, Supplementary Fig. 1, further details in the Methods section and Supplementary Table 1) utilizes a dual optical trap, created by time-sharing a single laser trap between two locations in the sample plane. Because they share identical optical paths, traps generated by timesharing are remarkably self-consistent in their relative positions.¹⁵ We use an acousto-optic deflector (AOD) to timeshare our traps at 50 kHz, providing low-noise and high-stiffness traps¹⁶ and allowing independent control of the trap powers and positions along a single axis.

The AOD is driven by an RF synthesizer, which we control using an FPGA. This "trap control FPGA" acts as a centralized instrument hub, acquiring and processing instrument data, and then determining and generating the driving signal for the RF synthesizer to control the optical traps (Fig. 1b). Trap powers are measured for each trap individually by a high-speed photodiode and stabilized *via* feedback (Supplementary Fig. 2). Bead position detection (detailed in the following section) is performed by a high-speed camera connected directly to a second "image tracking FPGA" dedicated to tracking bead positions. Thus the system provides an embedded platform for data acquisition, feedback, and instrument control, executed with hardware-timed determinism on the FPGA's 40 MHz clock. Custom LabVIEW 2015 software directs the top-level instrument operation.

Achieving both high-speed and precise image tracking requires a low-noise camera sensor and a bright light source to provide adequate illumination at sub-millisecond camera exposures.¹⁷ We acquire a cropped region of interest (Fig. 1a) at up to 10 kHz using a CMOS camera illuminated by a high-powered LED. Additionally, we incorporate a 4x optical magnification on top of our 60x water-immersion objective, resulting in images scaled at 57.3 nm per pixel. A large image magnification improves tracking accuracy by reducing the relative contributions, if present, of tracking algorithm bias and camera fixed pattern noise.⁷ Once acquired, image data from the camera are transferred over a Camera Link interface directly to the image tracking FPGA for processing.

Cross-Correlation Image Tracking on an FPGA.

Real-time image tracking at high camera framerates generates large amounts of data, which strains the throughput capabilities of traditional software tracking and requires a specialized solution for accelerated processing. This is typically realized by performing all or part of an image tracking algorithm on a graphics processing unit (GPU).^{7,9-12} Less commonly used, an FPGA provides a re-programmable hardware implementation of a tracking algorithm, yielding superior determinism and the low latencies required for demanding real-time control applications.¹⁸ On the other hand, their limited physical resources and lack of native floating point support constrain their aptitude for complex tracking algorithms. Here we have implemented the precise and accurate mirror cross-correlation (MCC) image tracking algorithm¹⁹ (Fig. 2a) on an FPGA platform to realize ultra-low-latency particle tracking in real time.

Image pixels are streamed directly from the camera to the image tracking FPGA, where we first correct for the camera's fixed pattern noise and then determine the positions of the bead centers. We track beads in one dimension (along the tethering axis), which is sufficient to reach the thermal limit of detection in dual optical traps.²⁰ The bead positions are then sent to the trap control FPGA over directly-wired digital lines. Overall, the system permits tracking and transfer of the dual bead positions at up to 625 kHz (though our camera configuration limits us to 10 kHz acquisition rates), with a latency between the finished camera exposure and the completed transfer of both tracked bead positions of about 50 μ s. The tracking exhibits both high precision and high accuracy; the variance in the tracked position of a bead fixed firmly to a coverslip stays below 0.2 nm² through 10 kHz acquisition rates, and a histogram of sub-pixel bead positions shows minimal bias (Supplementary Fig. 3). Sub-nanometer changes in the separation between two strongly trapped 800-nm beads (Fig. 2c) are clearly visible.

Accurate Force Measurements in Mobile Traps.

For small bead displacements from the trap centers, force in a dual optical trap can be expressed as $F = k_{\text{eff}} \cdot \chi_{\text{bead}}$ where k_{eff} is the effective trap stiffness of the two traps and χ_{bead} is the average displacement of the two trapped beads relative to their respective trap centers (Fig. 3a, Supplementary Note 1). While image tracking is suited to the task of determining absolute bead positions in the sample plane, the large uncertainty in the concurrent trap positions ultimately limits the accuracy of individual trap force measurements in real time. However, while the position of an optical trap in the sample

plane fluctuates and drifts substantially, the separation between two traps generated from a single laser source may be much more stable²⁰ (Supplementary Fig. 4). Our technique for measuring force (Fig. 3a) leverages the enhanced stability of differential detection in a dual optical trap by forgoing the use of absolute positions altogether and relying on an expression of χ_{bead} that uses only separations.

We begin by calibrating the separation of the traps. Holding an untethered (free) bead in each of the two traps, we track the separation between them (χ_{free}) as we maintain one trap stationary and step the second “active” trap across its remaining mobile range. The free bead separation, reflecting the absence of an externally applied force, represents the center-to-center distance between the two traps. The measured trap separation at each AOD frequency of the active trap (which drives its position in the sample plane), is sent to the trap control FPGA as a look-up-table (LUT) prior to an experiment. As Figure 3a illustrates, the use of a LUT in this process is critical to measurement accuracy due to the nonlinearity of the trap response to its control signal.

When experiments with a biological substrate are performed, we again fix the stationary trap, then manipulate the sample *via* movement of the active trap while measuring the separation between the two beads (χ_{tether}). The AOD frequency of the active trap serves to index the LUT of trap separation (χ_{free}). Half of the difference between χ_{free} and χ_{tether} represents the average displacement of the beads in their respective traps for that image frame, χ_{bead} . The accuracy of this technique relies on the stability of the trap separation calibration LUT afforded by the time-shared dual optical traps, which confers the repeatability required for a robust measurement of force. We characterize this by obtaining the Allan Deviation (ADEV)¹⁴ of χ_{bead} for two free beads as the active trap is scanned across its full movement range, and comparing it to that of stationary traps over a comparable time period (Fig. 3b). Inaccuracies in the LUT calibration of χ_{free} will manifest as non-physical fluctuations in the measured value of χ_{bead} as the active trap is scanned, and a consequent increase in the ADEV of the moving traps relative to the stationary traps. The results show that this technique enables us to obtain χ_{bead} with sub-nm accuracy across the full mobile trap separation range. Additionally, we assayed the drift of the calibration LUT over a time period of one hour and determined the drift rate to be on the order of 0.01 nm per minute in the region of the LUT in which our data were obtained (Supplementary Fig. 5).

Stretching and Unzipping DNA.

The ability to make sensitive real-time measurements of biological forces and distances is the cornerstone of single-molecule optical trapping studies. Thus to demonstrate our instrument’s ability to acquire high-quality data in single-molecule manipulation experiments, we begin with two classic and well-characterized optical trapping assays: stretching and unzipping DNA. Throughout both experiments, k_{eff} was held at ~0.3 pN/nm, thus small inaccuracies in the measure of bead displacement would have a large effect on the measured force.

The force-extension relationships from stretching 12 individual duplex DNA molecules over the course of an hour are shown in Figure 4a, along with the theoretical prediction²¹ (not a

fit). These data were aligned in extension *via* small (<10 nm) global offsets, accounted for by variations in bead diameters (Supplementary Fig. 6), but are unaligned in force. Next, we used our instrument to unzip DNA, which describes the mechanical disruption of the duplex DNA into its two constituent strands.²² In Figure 4b we show a zoomed in region of raw, unaligned data obtained from unzipping and re-zipping a single tether 3 times over a period of about 3 minutes. As the molecule is unzipped, the force is observed to vary according to the underlying base pair sequence, for which the theoretical prediction²² is shown in red. The data for both stretching and unzipping exhibit characteristics of an instrument with excellent accuracy in both force and extension measurements, high stability, and low drift over experimental timescales.

In Figure 4c we examine a portion of the unzipping data from Figure 4b which has been converted to display the number of base pairs unzipped over time. With this presentation, we show sufficient bandwidth and precision to resolve discrete hopping fluctuations between nearby unzipping states on millisecond timescales. This behavior is not unexpected; thermally-driven “breathing” -- rapid and spontaneous opening and closing of DNA base-pairing interactions -- is highly characteristic of DNA fork junctions and is thought to play important regulatory roles *in vivo*.^{23,24}

Fork Breathing Fluctuations Modulate Protein Unzipping.

Unzipping DNA reveals a detailed and distinct force signature shaped by the underlying energy landscape of its sequence, and thus unzipping also serves as a powerful tool for probing and localizing protein-DNA interactions.^{5,25–27} A bound protein presents a roadblock which increases resistance to unzipping, resulting in a detectable rise in force above the naked DNA baseline when the unzipping fork encounters the protein. The location of the force rise thereby communicates the location of the bound protein on its DNA substrate. To investigate the performance of our instrument in an advanced setting, we unzipped through proteins bound to sequence-specific sites on DNA molecules in order to localize their interactions. Interestingly, our experiments uncovered unexpected insights into the effects of fork breathing fluctuations on the protein unzipping process.

Figure 5 shows two sets of data obtained by unzipping through the bound restriction enzyme HincII on the same DNA sequence but from opposite directions. Thus the protein, its recognition sequence, and the sequence context of its binding site are the same for both data sets, but the DNA sequences unzipped upstream of the protein are distinct. Against the backdrop of the naked DNA unzipping trace, the bound protein produces an unmistakable vertical force rise, occurring when the progression of the unzipping fork is impeded by the tightly-bound protein (Figs. 5a, 5b). Though one might anticipate a sharp transition from the naked DNA unzipping behavior into the protein’s vertical force rise, the data revealed surprising deviations from the naked DNA unzipping trace beginning well before the expected vertical force rise (Figs. 5c, 5d). Similar behavior was also observed for adjacent HincII binding sites unzipped from the same direction on a single DNA template (Supplementary Fig. 7, Supplementary Video 1). The correct interpretation of these data was not immediately apparent, due to several physical phenomena that could plausibly produce such effects, including protein sliding, protein remodeling, weak protein-DNA interactions

outside the main binding region, and even instrument miscalibration. Ultimately, we determined this phenomenon to be consistent with just a single strong protein-DNA interaction, located at the position of the vertical force rise.

How might a protein affect the unzipping force many bases upstream of its binding location? A potential mechanism lies in the thermally-driven breathing dynamics of the unzipping fork, which cause the fork's location within the DNA sequence (the number of base pairs unzipped) to fluctuate rapidly about its mean position for a given value of DNA extension. Consequently, both the average fork position and the average unzipping force at any given time reflect the cohort of unzipping states the fork is able to explore, which varies with the underlying DNA sequence. GC-rich regions near the unzipping fork limit the extent of fork fluctuations due to their high base pairing energies, whereas AT-rich regions more readily open with thermal agitation, allowing the fork position to fluctuate through distant regions of the DNA sequence. In this way, sequence information both downstream and upstream of the mean fork location influences the force required to progress the mean fork location through the DNA molecule. Thus, when a protein binds to DNA and modifies the accessibility of the base pairs under its footprint, that information may be captured by the unzipping fork before its mean position reaches the protein.

To determine if such a phenomenon could explain our protein unzipping data, we modeled the effects of a bound protein in our unzipping theory calculations. Theoretical unzipping curves are generated by calculating, for a series of DNA extension values, both the force and the fork location probability density at each potential fork position (number of base pairs unzipped) in a DNA molecule. The predicted values for force and base pairs unzipped at each value of DNA extension are then obtained from the expectation values of the distributions.²² We simulated a tightly-bound restriction enzyme as an infinite energy barrier to fork progression at a defined base pair location, forcing the fork location probability density to zero at and beyond this point.

In the lower panels of Figures 5c and 5d, we compare the calculated fork location probability distributions both with and without a simulated bound protein that is positioned at the base pair location suggested by the high force unzipping data. For each distribution, the value of DNA extension was selected such that the resulting mean fork position was located 10 bp upstream of the simulated protein interaction. The presence of the bound protein truncates the available fork fluctuation states as the fork approaches the binding site, forcing a redistribution of probability density into the unzipping states upstream of the protein and a concurrent increase in the unzipping force (Supplementary Video 1). In figure 5c, this does not occur until the fork is immediately adjacent to the protein binding site due to the limited extent of the fork fluctuations in this region. In contrast, large amplitude fork fluctuations across the protein binding site in figure 5d are strongly perturbed when a protein is bound, resulting in a detectable unzipping force rise a substantial distance upstream of the protein.

Thus we find that the unzipping signature of a DNA-bound protein is highly sensitive to the DNA sequence in the vicinity of the protein binding site, *via* the protein's ability to perturb the extent of the sequence-dependent breathing fluctuations of an approaching unzipping

fork. This understanding is critical to interpreting protein unzipping data below ~20 pN, in order to differentiate weaker protein-DNA interactions at a given sequence location from a strong barrier further downstream. Additionally, the sensitive sequence-dependence of the low-force unzipping signature can contain valuable information on the protein's location. To take advantage of this, we generated unzipping theory curves for a protein positioned at integer-bp sequence locations in the vicinity of its apparent binding site, and determined the χ^2 value of our unzipping data against each theoretical prediction (Figs. 5e, 5f, Supplementary Fig. 8). The sub-bp minimum of the χ^2 versus protein position plot was determined by parabolic fit, allowing us to localize a protein-DNA interaction with sub-bp precision, even when high force data were unavailable.

Breathing fluctuations are an intrinsic feature of single-to-double stranded DNA fork junctions both *in vitro* and *in vivo*.²⁸ Within the cell, these junctions occur at replication forks where, instead of mechanical unzipping, DNA is unwound by helicases. In this process, breathing fluctuations are believed to play an important regulatory roll by influencing helicase movement into the replication fork.²⁴ Our data suggest that, additionally, thermal fork fluctuations may allow an approaching *in vivo* unzipping fork to detect and respond to the presence of a bound protein that is well downstream of its mean location. The sequence-dependent slowing of a replication fork approaching a bound protein could potentially facilitate early information transfer before full fork arrest occurs, *e.g.* by enhancing recruitment and binding of other proteins to assist in the roadblock's removal. This presents a distinct mechanism for "action at a distance,"²⁹ by which a protein at one location on a DNA sequence communicates information to a protein some distance away.

Unzipping Footprints of DNA-Bound Proteins.

With this knowledge of protein unzipping behavior in hand, we are well-positioned to probe the interaction landscape of a bound protein. We unzipped three type II restriction enzymes, each with a unique binding site along a DNA sequence, from either direction (Fig. 6, Supplementary Fig 9 including naked DNA traces), then used the χ^2 analysis technique to determine the locations of the interactions. Notably, while the data for the enzymes HincII (Fig. 6a) and XbaI (Fig. 6b) were well-described by a single strong protein-DNA interaction on either end of the protein, the data for the enzyme BsiWI (Fig. 6c) were not. Though the unzipping signatures for BsiWI resemble those of the other two enzymes superficially, our theoretical model allows us to distinguish its unzipping behavior. In this case, the data are more consistent with multiple weaker interactions surrounding a central, stronger region of contact.

As expected, the footprints of all three enzymes are centered with respect to their recognition sites to within 1 bp, with sizes of 14.3 ± 1.0 bp (HincII), 12.3 ± 0.9 bp (XbaI), and 7.0 ± 1.2 bp (BsiWI). This is in good general agreement with previous DNAase I³⁰ and unzipping³¹ footprint measurements on restriction enzymes of the same class, which have obtained footprints ranging from 5 to 20 bp for 8 different species. Of the three enzymes we unzipped, only HincII has a published crystal structure.³² The protein shadows the bound DNA across a region of ~50 Å or 14.8 bp, within error of the 14.3 ± 1.0 bp we mapped by unzipping. The precision of the localization measurements is state-of-the-art; individual

interactions were localized with standard deviations from 0.37 to 1.01 bp. These data show that, rather than being hindered by image tracking, dual optical trapping measurements can be enhanced by image tracking's direct and robust measurement of extension in biological systems.

Conclusions

The photodiode-based technique of back focal plane interferometry (BFPI) has been widely acknowledged as the only detection method suitable for attaining the highest level of optical trapping performance for biophysical studies.^{33–35} This has persisted in the field, even after advances in camera technology have improved the precision and speed of image tracking, due to the inability of cameras to measure the relative displacement of a bead from its trap center with the same accuracy and speed as BFPI, particularly in the important case of mobile traps.

By achieving an accurate and robust real time measure of force in an image-based optical trap, and by extending this ability to mobile optical traps without sacrificing accuracy or precision, we have developed an image-based optical trapping detection platform that yields state-of-the-art biological data. Thus equipped, we are now able to take full advantage of image tracking's greatest strength: unlike BFPI, image tracking is able to measure *extension* in a dual optical trap directly, making it potentially superior to other indirect detection techniques in this aspect (Supplementary Fig. 6). This is evidenced by the exceptional quality of the instrument's data, which has resulted directly in the elucidation of the mechanism by which a bound protein can affect the progression of an approaching unzipping fork.

With their compatibility with commercial microscopes and broad flexibility in implementation configurations, image-based techniques are familiar to biologists and biophysicists across a diverse array of applications. Along with these features, the added ability to realize the highest levels of performance in optical trapping detection renders image tracking an appealing option for making even the most demanding dual-trapping single-molecule measurements. This is particularly true in cases where BFPI is challenging to implement, *e.g.* with opaque substrates,³⁶ and with certain hybrid instruments³⁷ where traditional optical trapping detection cannot be used. Ultimately, the fundamental limits of this detection method are set by the stability of the dual trap separation relative to the bandwidth and signal-to-noise ratio of the camera. Thus as the speed and noise characteristics of imaging technologies continue to improve at a rapid rate, image tracking may be poised to become the preferred detection method in dual optical traps.

Methods

Instrument Construction.

A part list is located in Supplementary Table 1, and a full instrument layout is presented in Supplementary Figure 1. Dual optical traps are generated by timesharing a single 1064-nm laser at 50 kHz *via* an AOD. The traps are sourced from a 5W, 1064-nm fiber-coupled laser (IPG Photonics). A 60x, 1.27 NA, IR-corrected water immersion objective (Nikon) permits

stable trapping deep within the sample chamber, facilitating DNA dumbbell tether formation and experimental flexibility. An acousto-optic deflector (AOD) (Gooch and Housego) is used to generate timeshared dual traps from the single CW laser source. Lenses map the beam rotation at the AOD onto the back focal plane of the microscope objective, and expand the beam to overfill the objective's back aperture. The AOD is driven by an RF synthesizer (Gooch and Housego), from which frequencies between 35 and 45 MHz produce a trap displacement in the sample plane of up to about 10 μm . An FPGA (National Instruments 7852R) directs the output of the RF synthesizer. This "trap control FPGA" runs on a 40 MHz internal clock, enabling flexible, deterministic timing for trap modulation and signal acquisition with a resolution of 25 ns. We modulate the traps at 50 kHz, resulting in an "on" time for each trap of 10 μs .

The diffraction efficiency of an AOD is not constant with respect to the driving frequency. To produce traps of equal and uniform stiffness across their mobile range, we perform feedback on the trap powers (Supplementary Fig. 2). This requires a high-speed detector in order to measure each trap's power individually during its portion of the modulation cycle. Silicon detectors are not well-suited to this purpose due to parasitic filtering at near-infrared wavelengths,³⁸ therefore we use a high-speed InGaAs photodiode (Thorlabs DET20C). Using the timing capabilities of the FPGA, a measurement sample of the analog photodiode output is acquired during the center of each trap's on period, and then individual trap power feedback is performed on the FPGA to determine the trap output powers for the next modulation cycle.

To generate images for bead position detection, we use a high-speed CMOS camera (Mikrotron CAMMC1362) and illuminate the microscope sample from above with a high-powered LED (Thorlabs) emitting at 445 nm. The LED provides up to 5.4W of power, allowing high contrast and low-noise images even at 10,000 frames per second (fps) (Supplementary Fig. 3). An unusual aspect of our instrument compared to other image-based single-molecule instruments is that our illumination is focused onto the sample plane, from a diameter of about 3 cm down to about 0.5 cm. Many instruments employ collimated illumination in order to produce an extended pattern of diffraction fringes around the beads being tracked,^{9,10,12} which aids in tracking beads along the "z" axis perpendicular to the sample plane. Because bead motion along this perpendicular axis is not important to our measurements, focused illumination aids our tracking precision (by allowing us to maximize the contrast in our images to the camera's dynamic range with less input light), and our tracking speed (by reducing the useful tracking region to the bead's central bright spot and first surrounding dark ring). We performed tracking simulations to confirm that lower contrast diffraction rings do not contribute significantly to lateral tracking precision.

A low-powered LED operating at 630 nm also illuminates the sample from above for wide-field-of-view imaging at 30 fps by an additional CCD camera (Andor), which in our case is a high-sensitivity camera which can also be used to acquire fluorescent images. This second camera primarily aids in tether formation and sample chamber navigation, and any basic CCD camera will suffice in this role. The two imaging wavelengths are combined for illumination using a dichroic mirror (Thorlabs). A second dichroic mirror (Chroma, 5 mm thick) below the objective transmits the trapping laser, and reflects visible wavelengths

towards our imaging apparatus. The visible light is collected by a tube lens (Thorlabs), and then directed to their respective cameras by a third dichroic (Chroma). A 4x optical magnification attachment (Nikon) on the Mikrotrotron camera provides additional image enlargement. The conversion from camera pixels to nanometers in the sample plane is determined using a calibration grid printed on a microscope slide (Thorlabs), from which we obtained a value of 57.3 nm per pixel (0.01745 ± 0.00001 pixels per nm) using a cross-correlation-based analysis of the grid images.

FPGA Image Tracking.

Image tracking is performed on a second “image tracking FPGA” (National Instruments 1473R). The high-speed camera is connected directly to the FPGA *via* CameraLink in the base configuration. Pixels from a cropped region of interest 200×7 pixels in size are sent in single tap, 10-bit mode on the camera’s 80 MHz clock. On the image tracking FPGA, three parallel, independent loops perform image acquisition and pre-processing, bead position tracking, and transfer of the tracked bead positions, respectively (Fig. 2a). In the acquisition loop, pixels are read out one at a time and processed as they arrive. Sequential processing steps are pipelined to allow for full parallelism; once a pixel leaves an individual processing step to the next downstream function, a new pixel is clocked in, so that all functions are acting on a different pixel simultaneously.

Pixel pre-processing begins by applying a correction for the camera sensor’s fixed pattern pixel noise by subtracting a constant offset for each pixel. The appropriate offset for each pixel is determined before an experiment by averaging 1,000 image frames and calculating each pixel’s difference from the mean frame intensity, and then sent and stored in the FPGA’s block memory. Next, the pixel stream is routed to one of two memory queues, one for each bead’s pre-defined sub-ROI within the full image ROI. As the pixels for a given frame are being sorted into memory, the FPGA determines the positions of the brightest pixel within each bead’s sub-ROI. This will provide a coarse estimate of the bead centers about which the tracking algorithm can later focus. On the last pixel of an image frame, these starting positions are passed to the FPGA tracking loop running on a 40 MHz clock.

The tracking loop is triggered to begin once it receives the results from the acquisition loop. Pixels are read out of their respective queues for each bead sequentially. The tracking loop first constructs a line profile centered around the starting position identified in the acquisition loop. The size of the line profile may be varied, but for this work we used a line profile 15 pixels in length, summing across three pixels in the orthogonal direction to reduce noise. The line profile is zeroed by subtracting the value of the first element of the array from all array elements. Informed by tracking simulations, the line profile size and baseline subtraction methods were chosen to minimize tracking bias and maximize precision. We then cross-correlate the zeroed line profile with its mirror image to generate a one-dimensional cross-correlation array, the peak of which can be mapped back to the original pixel space, giving the computed location of the bead. The sub-pixel maximum of the cross-correlation array is found by performing a parabolic least squares fit to the array maximum and its single nearest neighbor to either side. FPGAs are not generally well-suited to nonlinear curve fitting, however, the task is greatly simplified in this case by the fact that the

Vandermonde Matrix and its transpose are fixed and can be calculated in advance. For a least squares fit about the cross-correlation array peak value R_i and its nearest neighbors R_{i-1} and R_{i+1} , the maximum of the resulting curve fit reduces to the following “three point estimator”:

$$R_{\max} = \frac{3R_{i-1} - 4R_i + R_{i+1}}{2R_{i-1} - 4R_i + 2R_{i+1}} \quad [1]$$

Because multiply and divide operations performed on an FPGA require specialized hardware multipliers of which an FPGA typically has just a few dozen, the ability to simplify a curve fitting process involving matrix calculations and determinants to a single, simple arithmetic expression is crucially important for the viability of performing this image processing algorithm on an FPGA.

The pixel locations of each bead determined in the tracking loop are passed to the data transfer loop, which sends these values to the trap control FPGA. The two FPGAs are connected *via* a dedicated I/O extension board (National Instruments), which equips the image tracking FPGA with 8 configurable digital input/output (DIO) lines which we wired to 8 of the DIO lines on the trap control FPGA. The image tracking FPGA connects to the I/O extension board directly *via* a ribbon cable; signals are not routed through the computer’s PCIe bus. The tracked pixel locations of the two beads are stored in a 30-bit fixed point data type yielding a discretization of 2.38×10^{-7} pixels or 1.37×10^{-5} nm, which are here encoded in a length-30 Boolean array suitable for digital TTL transfer. We use one DIO line to signal the trap FPGA that data are ready for transfer, a second to clock the data transfer at 10 MHz, and the remaining 6 DIO lines to transfer the 30-bit bead positions over 5 clock cycles. The full transfer of one bead position data point takes 800 nanoseconds. The data are read by the trap control FPGA and translated back into a numeric form, at which point the current AOD frequency of the active trap is used to index the trap separation LUT for calculating χ_{bead} . The latency of bead tracking is thus a critical parameter for this technique, as the bead separation data lags the trap separation data by the tracking latency time which will result in force errors when the traps are in motion. Because our tracking is implemented on an FPGA, the total latency between the finished camera exposure and the completed transfer of the tracked bead positions is around 50 μs , yielding errors less than an Angstrom in χ_{bead} for active trap speeds up to 400 nm/s.

Free Bead Separation Calibration and χ_{bead} Determination.

To calibrate the free bead separation, we start with an untethered bead in each trap. The stationary trap remains fixed at 35 MHz and the active trap is stepped from 37 to 45 MHz across 330 intervals spaced evenly in frequency as the bead separation is measured. The spacing of the trap separation LUT, resulting in one data point approximately every 22 nm of trap movement, was determined empirically to minimize the variance of the resulting χ_{bead} measurement. At each LUT position, 50 unique points of trap separation values from the 1-kHz-filtered data acquisition buffer are sampled and averaged together. The resulting array of trap separations is transferred to the trap control FPGA and stored in block memory. The full calibration process takes about 30 seconds. During an experiment, upon arrival at the

trap control FPGA of the tracked bead positions from the image tracking FPGA, the bead-to-bead separation is calculated and the current position of the active trap is used to index the trap separation LUT with linear interpolation. During our unzipping experiments, a new LUT was obtained at the start of each data acquisition session immediately prior to data collection and at the same trap power with which the unzipping data would be obtained. LUTs were updated periodically during experimental data collection, approximately every 10 to 30 minutes. The calibration LUT and its stability are examined further in Supplementary Figure 5.

Stretching and Unzipping Experiments.

The generation of stretching and unzipping templates has been described previously.^{25,39,40} The DNA sequences for the HincII, XbaI, and BsiWI binding sites and the surrounding 200 bp (100 bp in the up and downstream directions) are provided in supplementary Table 2. The designation of a “forward” and “reverse” direction for unzipping templates is ultimately arbitrary, but is consistent with the 5' to 3' direction of the sequences listed in this table. For forming DNA dumbbell tethers, carboxylated beads (Polysciences) were coated with (either) streptavidin and anti-digoxigenin in house. The process of forming a DNA dumbbell tether is described in detail in Supplementary Figure 10.

For Figure 4, our template for both stretching and unzipping consisted of 6644 bp of double-stranded DNA “arms” and an 832-bp unzipping “trunk”. Experiments were performed in phosphate-buffered saline (10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4, 137 mM NaCl, 2.7 mM KCl). In Figure 4a, the beads were 500 nm in diameter and the trap stiffness was constant at ~0.28 pN/nm. The active trap was moved away from the stationary trap to stretch the DNA arms at a constant velocity of ~200 nm/s. Data traces were truncated at a force of 12 pN, prior to the onset of unzipping. In Figure 4b, the beads were 800-nm in diameter and the trap stiffness was constant at ~0.36 pN/nm. Unzipping was performed using a constant active trap velocity of 20 nm/s, and reziping was performed using a constant active trap velocity of -40 nm/s.

Protein unzipping experiments for data shown in Figures 5 and 6, Supplementary Figures 7–9, and all related calibration experiments were performed in a buffer containing 50 mM Tris-HCL pH 7.9, 100 mM NaCl, and 1 mM CaCl₂. Unzipping templates consisted of 6644 bp of double-stranded DNA arms and unzipping trunks of various lengths between 0.7 and 4.4 kbp. All experiments were performed under conditions of constant trap stiffness (ranging from 0.27 to 0.38 pN/nm) and constant active trap velocity (ranging from 40 to 100 nm/s). Proteins were purchased from New England Biolabs. One microliter of stock protein corresponding to 10 (HincII, BsiWI) or 20 (XbaI) vendor-calibrated units was added to a 15μL pre-incubation of DNA and beads just prior to introduction into the experimental flow cell for tether formation. Additionally, protein was included in the unzipping buffer in which unzipping experiments were performed, at concentrations ranging from ~100 Units/mL (1:100 dilution) to ~700 Units/mL (1:15 dilution)

Data Analysis.

While force in an optical trap is generally treated as being linearly proportional to bead displacement, the validity of this approximation decreases with increasing distance from the trap center. To improve data accuracy throughout the wide range of forces encountered during protein unzipping experiments, we developed a technique (Supplementary Fig. 11) to measure the non-uniformity of trap stiffness as a function of bead displacement. This calibration, performed once, is used to convert all measured bead displacements into forces in later experiments. The technique utilizes the knowledge that, in our constant trap velocity unzipping experiments, a DNA molecule will unzip at the same force regardless of the nature of the trapping potential.

Accordingly, we unzipped DNA molecules multiple times at laser trapping powers ranging from 120 to 300 mW in the sample plane, resulting in average bead displacements during unzipping of 117 to 33 nm. From our theoretical model of unzipping we obtained the predicted unzipping force, and plotted this force per watt of trapping power *versus* the measured bead displacement at each power. The resulting data was well-fit by the derivative of a Gaussian (DoG) function. This function and the obtained fit parameters were subsequently used to convert measured bead displacements into values of force. For protein unzipping data only, we allowed the amplitude of the DoG function to be a free parameter in order to correct for small observed variations in unzipping force (typically 1 to 3%) between traces, which could be due, *e.g.*, to variations in bead sizes or drift of the trapping power in the sample plane. The unzipping force calculated by our theoretical model was used to determine the appropriate DoG amplitude for the conversion of each trace.

Thusly determined values of force and extension during unzipping were converted to the number of bases unzipped in the sequence using a modified Marko-Siggia model²¹ for double-stranded DNA (dsDNA), and the freely-jointed chain model⁴¹ for single-stranded DNA (ssDNA). DNA elasticity parameters may be obtained by fitting double and single-stranded DNA stretching data with their respective models. For this work, we performed this calibration for ssDNA and used previous calibration results²¹ for dsDNA (persistence length of 42 nm, elastic modulus of 1200 pN, and contour length per base of 0.338 nm, also used for stretching theory curve in Figure 4a). To stretch single-stranded DNA, a hairpin was placed at the end of an unzipping segment, and the construct was unzipped to completion and then stretched. From the resulting values of extension, the contribution of the double-stranded portion of the unzipping construct was subtracted, leaving the force-extension relationship of only the single-stranded portion for fitting. Fitting resulted in an ssDNA persistence length of 0.71 nm (half the Kuhn length), elastic modulus of 420 pN, and counter length per base of 0.52 nm. Once represented as force *vs* the number of base pairs unzipped, individual protein unzipping traces were aligned horizontally to the DNA baseline theory using small global shift and stretch adjustments (typically < 10 nm and < 1%, respectively) determined by a simplex algorithm.

Around the approximate apparent protein position as indicated by the location of the vertical force rise asymptote, we generated theory curves for proteins located at integer-spaced bp locations along the template. The theory assumes an infinite energy barrier at the specified point along the sequence, such that the unzipping fork cannot progress further. For each

generated theory curve, we computed the χ^2 value comparing our measured base pairs unzipped to the theoretical base pairs unzipped, using DNA extension as the common independent variable. To determine protein location with sub-bp precision, we plot the resulting χ^2 vs theoretical protein position and fit the minimum point plus its nearest neighbor to either side with a parabola.

The footprints for HincII and XbaI were determined using χ^2 fitting on the full unzipping signature, from the onset of the deviation from the naked DNA theory to a force of 26 pN. To analyze the BsiWI footprint, we performed χ^2 fitting on cropped regions of the protein unzipping signature, from the onset of the force deviation from the naked DNA to a force of 20 pN for the outer interaction, and from 23 to 26 pN for the inner interaction.

All analysis steps were performed with custom LabVIEW 2015 software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Instrument Overview.

(a) Dual optical traps are generated by timesharing a single laser *via* an AOD, which controls the positions and powers of both traps in the sample plane. Bead positions are tracked in real time at up to 10 kHz using a high speed camera. The drawn scale bar is 400 nm. (b) The control flow of the instrument. A “trap control” FPGA generates the driving signal to control the AOD (and thus the traps) in Fig. 1a, and acquires and processes instrument data including trap powers from a high speed photodiode and bead positions from a second “image tracking” FPGA. Acquired data sampled at up to 50 kHz are streamed to the the main host software for display and writing to disk.

Figure 2. FPGA Image Tracking and Results.

(a) FPGA image tracking using a cross-correlation algorithm is implemented in three independent loops, the first for image pre-processing, the second for bead tracking, and the third for data transfer. (b) An image of an 800-nm polystyrene bead cropped from 10-bit experimental image data, with its corresponding line profile shown below. The result from the cross-correlation of the line profile with its mirror image (“MCC Array”) is shown in the bottom panel. The scale bar is 400 nm. (c) With a bead in each trap and a trapping stiffness of ~ 0.5 pN/nm, we held one trap stationary and stepped the other trap in increments of 0.9 nm while measuring the bead separation. The raw data (2.5 kHz) are shown in gray, while the black trace is filtered to 250 Hz. The trap control driving signal is shown in light red.

Figure 3. Accurate Image-Based Force Measurements with Mobile Traps.

(a) Force in a dual optical trap can be expressed in terms of the relative trap separation coordinates x_{free} and x_{tether} . The free bead separation, x_{free} , versus the position of the active trap is first measured and stored as a look-up-table (LUT) on the trap control FPGA prior to an experiment. Half the difference between x_{free} indexed from this calibration LUT, and x_{tether} measured during an experiment, yields Δx_{bead} for each image frame. (b) The Allan Deviation (ADEV) of the Δx_{bead} signal for stationary traps was compared to the ADEV of the Δx_{bead} signal as the active trap was scanned across its mobile range, revealing the accuracy of Δx_{bead} measurements in mobile traps.

Figure 4. Stretching and Unzipping Naked DNA.

(a) The measured force-extension relationships for stretching twelve different DNA molecules are shown, along with the theoretical prediction²¹ (not a fit). (b) A single DNA molecule was unzipped until just before strand dissociation and then re-zipped. This process was performed three consecutive times over a period of about 3 minutes. The 10 kHz data is displayed without filtering or alignment. (c) A portion of the data in Figure 4b was converted to the number of base pairs unzipped *versus* time. Rapid transitions on millisecond timescales between discrete unzipped states are visible. A dwell time histogram is also shown.

Figure 5. A Bound Protein Modulates Unzipping at a Distance.

(a, b) The restriction enzyme HincII was bound to a DNA template and unzipped from either direction. A single naked DNA trace (grey) is included for comparison. (c,d) Featured deviations from the naked unzipping data (grey) and theory (black) are observed as the protein is unzipped. This was predicted well by our unzipping theory (red) by placing an infinite energy barrier at the base pair position indicated by the dashed red line. Below the data is the calculated likelihood for the fork to reside at a given sequence position with (red) and without (black) a protein bound at the dashed red line, for a mean fork position (black dashed line) 10 bp upstream of the protein location. A force rise relative to the naked DNA is observed when the protein perturbs the distribution of states the fork is able to explore through thermal fluctuations, which varies with the DNA sequence. (e, f) Theoretical protein unzipping signatures were generated for a protein bound at integer base positions along the DNA sequence, and the χ^2 value of our measured unzipping data against each separate

prediction was determined and fit with a parabola to extract the protein position with sub-bp precision (Supplementary Fig. 8).

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