Anti-Brownian electrophoretic traps have been used to trap and study the free-solution dynamics of large protein complexes and long chains of DNA. Small molecules in solution have thus far proved too mobile to trap by any means. Here we explore the ultimate limits on trapping single molecules. We developed a feedback-based anti-Brownian electrophoretic trap in which classical thermal noise is compensated to the maximal extent allowed by quantum measurement noise. We trapped single fluorophores with a molecular weight of <1 kDa and a hydrodynamic radius of 6.7 Å for longer than one second, in aqueous buffer at room temperature. This achievement represents an 800-fold decrease in the mass of objects trapped in solution, and opens the possibility to trap and manipulate any soluble molecule that can be fluorescently labeled. To illustrate the use of this trap, we studied the binding of unlabeled RecA to fluorescently labeled single-stranded DNA. Binding of RecA induced changes in the DNA diffusion coefficient, electrophoretic mobility, and brightness, all of which were measured simultaneously and on a molecule-by-molecule basis. This device greatly extends the size range of molecules that can be studied by room temperature feedback trapping, and opens the door to further studies of the binding of unmodified proteins to DNA in free solution.

A longstanding challenge in single-molecule spectroscopy has been to observe a small molecule in solution for an extended time, without surface tethering or other mechanical immobilization. Stable observation becomes more difficult as the particle decreases in size, because smaller objects diffuse more quickly, in accordance with the Stokes–Einstein relation. Gold nanoparticles as small as 18 nm in diameter, corresponding to a mass of 35 MDa, have been trapped using laser tweezers (1). Below this size laser tweezers fail because the trapping force is proportional to the square of the volume of the trapped object. Real-time feedback provides an alternate strategy, and has been used to trap single atoms in vacuum (2). The anti-Brownian electrophoretic (ABEL) trap uses feedback to suppress Brownian motion in solution and can confine particles as small as the 800 kDa complex of the chaperonin GroEL (3–5). A 104 kDa protein, allohexocyanin, was recently studied in an ABEL trap in which the viscosity was increased with 50% glycerol to slow the Brownian motion (6). Past attempts to trap small-molecule fluorophores in aqueous solution resulted in transient confinement, but not stable trapping (3). Small-molecule fluorophores are the tiniest objects that one can conceive of trapping in aqueous solution. If a particular fluorophore can be trapped, then so too can any molecule to which it is attached.

Laser tweezers and the ABEL trap both confine small objects in solution, but the two technologies enable different kinds of measurements. The optical forces of laser tweezers enable precise (subnanometer) localization of the trapped object, and permit application of precisely calibrated point forces for the purpose of force spectroscopy (7). The ABEL feedback strategy enables trapping of smaller objects, including individual molecules, but tracking imprecision, algorithmic errors, and feedback latency permit residual Brownian motion, typically with an amplitude of several hundred nanometers. The feedback forces in the ABEL trap are applied as body forces to all molecules in the solution, so this trap is not appropriate for force spectroscopy. The ABEL trap is primarily suited to nonperturbative observation of single-molecule dynamics (8).

One goal of single-molecule trapping is to study binding interactions in solution that may be perturbed by surface tethering. An obstacle to reaching this goal is that if both species are labeled, at least one must be at a concentration incommensurate with single-molecule fluorescence for dissociation constants greater than approximately 0.1 nM. An alternative strategy is to detect binding via the influence of an unlabeled molecule on the photophysical or transport properties of a labeled and trapped binding partner. Binding of RecA to DNA has been extensively studied in bulk (9), structurally (10), and at the single-molecule level (11–13). Here we report observations of changes in transport coefficients of freely diffusing short DNA oligos upon binding of RecA.

The design of our ABEL trap is illustrated in Fig. 1. The position of an object is tracked in real-time via fluorescence, and electrophoretic feedback is applied via two orthogonal pairs of platinum electrodes to cancel Brownian motion in the plane. Out-of-plane motion is physically restricted by the fused silica walls of the nanofabricated sample cell. When necessary, the walls are chemically passivated to minimize surface interactions with the analyte. Fully three-dimensional electrophoretic trapping, which avoids problems of surface interactions, has been demonstrated on 0.6 μm polystyrene beads, but not yet for single molecules (14).

Our optical detection scheme uses a scanned laser and a single-photon counting detector, with spatial information derived from the location of the laser at the instant each photon is detected (3, 15). The position of the laser is specified by a field-programmable gate array (FPGA), similar to the strategy outlined in a recent theoretical article (16). The key innovation that improves our trap’s performance is a statistically rigorous real-time tracking and feedback system, implemented on the FPGA. This system operates at the quantum limit imposed by the finite information carried by each fluorescence photon. The LabVIEW FPGA code is publicly available (17).

Each detected photon specifies the particle’s location with a precision limited by the standard deviation of the Gaussian laser spot, measured to be 360 nm. Averaging over many photons improves localization precision, but neglects the motion of the particle between photon detections. A more sophisticated strategy is to construct a “running average” in which recently detected photons are weighted more heavily than those detected earlier. A Kalman filter (18, 19), schematized in the upper left of Fig. 1, appropriately weights the information from each photon (see Methods for details) and is simple enough to implement in the FPGA and to run at high speed. Thus the tracking system operates close to the physical limits imposed by diffusion, diffraction, and the finite rate of photon detection events.

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Results and Discussion

In the absence of feedback, fluorophores of Alexa 647 diffused across the laser scan pattern with an average residence time of 2 ms (Fig. 2A, Top). When feedback was applied, fluorophores that diffused into the trap were quickly pushed to the trap center and held for an average of 800 ms prior to photobleaching, photoblinking, or escape (Fig. 2A, Middle), corresponding to the collection of an average of 37,000 photons per event. Some single fluorophores were trapped for as long as 10 s, yielding as many as 450,000 photons. The time-averaged illumination was uniform throughout the region explored by the molecule, so residual molecular motion did not lead to brightness fluctuations. The fluorescence intensity was constant during each event and from one event to the next, and every event ended with a quantal step to background fluorescence, establishing that the trapped species contained only one fluorophore. Molecule-by-molecule analysis of diffusion coefficients (see below) yielded a narrow distribution peaked around 325 μm²/s, which matched the value obtained in bulk (20) and confirmed that every event corresponded to a free fluorophore. Occasional short-lived positive intensity spikes during trapping events signified the approach of a second fluorophore near the trap; the Brownian motion of the fluorophores was uncorrelated, so after a few milliseconds one diffused away. Segments of dsDNA (30 bp), doubly labeled with Alexa 647 showed two-step photobleaching steps, each equal in intensity to that of a trapped single fluorophore (Fig. 2A, Bottom), further confirming that the objects trapped in the free dye sample were single fluorophores. Fig. 2B shows in red a time-averaged CCD image of a series of trapped single fluorophores (Movie S1). Displayed in blue is the time-averaged laser scan pattern.

To determine the loss mechanisms from the trap, we studied the trapping time of single fluorophores as a function of laser power (Fig. 2C). A trapping event was considered to end when the fluorescence dropped to background for longer than 300 μs. The mean trapping time was nonmonotonic in laser power, indicating that trapping time was limited by photon statistics and diffusional escape at low power, and by photobleaching or photoblinking at high power. The trap was typically operated under conditions to maximize mean trapping time, in which case rates of diffusional escape and photobleaching or bleaching were approximately equal.

Photobleaching rates in the ABEL trap were higher than in typical surface-tethered experiments because high count rates were required to achieve stable feedback. Furthermore, we interpreted every blinking event as the end of a single-molecule trajectory. This procedure contributed to the shorter reported observation time in the ABEL trap compared to surface-tethered experiments because high count rates were approximately equal.
experiments where one typically averages over blinks. The distribution of trapping times had a long tail, possibly due to variations in rates of photobleaching and photoblinking caused by variations in the concentration of oxygen and triplet quenchers.

The photon-by-photon recording of each trapping event enabled quantitative determination of the spatial trajectory, transport coefficients and photophysical properties of each molecule, with a precision far beyond that of any other single-molecule technique. We developed a maximum-likelihood assumed density filter (ADF) to perform these calculations (see Methods). We applied the ADF to trajectories of single trapped molecules of Alexa 647 to determine the strength and relaxation time of the trap (Fig. 3A and B). The fluorophore was tightly constrained to the center of the trap, with a rms deviation of 416 nm, well within the approximately 5 μm diameter of the laser scan.

To test our ability to measure diffusion coefficients of small objects, we studied a mixture of free Alexa 647 dye and 30 nt ssDNA, singly labeled with Alexa 647. Many single molecules were sequentially trapped until photobleaching. Based on their intensity or trapping time alone, the two species in the sample were indistinguishable, but they were clearly resolved by their diffusion coefficients (Fig. 3 C and D). From experiments in which only one species was trapped, we associate the lower diffusion coefficient with the DNA species and the greater one with free Alexa 647.

These highly precise measurements of single-molecule diffusion coefficients enable quantitative comparison with bulk measurements and theory. The diffusion coefficient obtained in the ABEL trap for Alexa 647, $D_{AF} = 325 \pm 2 \text{ μm}^2/\text{s}$ (SEM), is consistent with the value of 330 μm²/s measured by two-focus fluorescence correlation spectroscopy (FCS) (20). The diffusion coefficient of the 30-mer ssDNA, $D_{30\text{-mer}} = 178 \pm 2 \text{ μm}^2/\text{s}$, matches that predicted by the Zimm model for a polymer in good solvent, 166 μm²/s, calculated with persistence length and rise per base taken from laser tweezers experiments (22) and no adjustable parameters (see Methods and ref. 23).

The diffusion coefficients can be converted to hydrodynamic radii via the Stokes–Einstein relation. The ABEL trap data yielded a population-average radius for Alexa 647 of $6.76 \pm 0.03$ Å (SEM) and for 30-mer ssDNA of $12.3 \pm 0.2$ Å. The diffusion coefficient of each single molecule was determined more precisely as trapping time increased (Fig. 3D); we calculate a precision of roughly 20 μm²/(s/Hz) in determining $D$. Thus a single molecule of Alexa 647, trapped for 1 s, yielded an estimate of its hydrodynamic radius with a precision of 0.5 Å (SD). The ability to measure hydrodynamic radii of single molecules in free solution so precisely may be useful for observing subtle conformational shifts.

Encouraged by our success in trapping and characterizing single fluorophores and small DNA molecules, we next studied the interaction of ssDNA with Escherichia coli RecA, a protein known to form helical filaments on ssDNA in the presence of ATP (9). The ssDNA sample was 60 nucleotides long, singly labeled at its 5′ terminus with Alexa 647. In the absence of RecA, the ssDNA is expected to form a random coil with a contour length of 33.6 nm, a persistence length of 7.5 Å, and a radius of gyration of 27 Å (22). This length of DNA is sufficient to nucleate a RecA filament containing up to 20 monomers of RecA (24), but vastly shorter than the approximately 900 nm persistence length of the RecA filament (13). Binding of RecA is expected to convert the ssDNA from a random coil to a semi-rigid rod with a radius of roughly 4 nm and a rise of 5.1 Å per nucleotide (10), corresponding to a total length of 30.6 nm and a hydrodynamic radius of 10.5 nm (see Methods).

Before studying the interaction of RecA with ssDNA in the ABEL trap, we first used conventional FCS to confirm binding of RecA to ssDNA and to study the ensemble-averaged effects of binding upon the diffusion coefficient and fluorescence brightness of the ssDNA. Addition of unlabeled RecA (1 μM) and ATP (1 mM) to a sample of ssDNA induced a 60% drop in the ensemble-averaged diffusion coefficient of the DNA (Fig. 4A), and a 40% increase in the average molecular brightness. The decrease in diffusion coefficient was consistent with a change in geometry from a compact random coil to an extended rod. The increase in brightness upon binding of RecA to fluorescently labeled ssDNA likely reflects changes in the chemical environment of the fluorophore, and is consistent with a previous report in which a different fluorophore was used (25). RecA in the absence of DNA had no detectable fluorescence. These FCS measurements provided no information on the underlying distributions of single-molecule brightness and diffusion coefficient, and provided no information about the ensemble-averaged or single-molecule values of the electokinetic mobility.

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**Fig. 3.** Performance of the ABEL trap. (A) First 10 ms of the reconstructed trajectory of a single trapped molecule of Alexa 647 (red), plotted over a cartoon of the 27-point scan pattern (blue). Time-averaged probability densities are plotted along each axis (green). The mean precision with which each point in the trajectory was localized is 240 nm, which is less than the width of the laser spot (360 nm) because the estimate includes information from several photons. The molecule was confined with a rms deviation of about 416 nm, well within the approximately 5 μm diameter of the laser scan. (B) Time-averaged probability densities along each axis (green). The mean precision with which each point in the trajectory was localized is 240 nm, which is less than the width of the laser spot (360 nm) because the estimate includes information from several photons. The molecule was confined with a rms deviation of about 416 nm, well within the approximately 5 μm diameter of the laser scan. (C) Distribution of diffusion coefficients $D$ for Alexa 647 (red) and ssDNA (blue) measured by two-focus FCS. From experiments in which a single species was trapped, the diffusion coefficient for Alexa 647 is $325 \pm 2 \text{ μm}^2/\text{s}$ (SEM), consistent with the value of 330 μm²/s measured by two-focus fluorescence correlation spectroscopy (FCS) (20). The diffusion coefficient of the 30-mer ssDNA is $178 \pm 2 \text{ μm}^2/\text{s}$, matches that predicted by the Zimm model for a polymer in good solvent, 166 μm²/s, calculated with persistence length and rise per base taken from laser tweezers experiments (22) and no adjustable parameters (see Methods and ref. 23). (D) Distribution of diffusion coefficients $D$ for Alexa 647 (red) and ssDNA (blue) measured by time-averaged FCS. From experiments in which a single species was trapped, the diffusion coefficient for Alexa 647 is $325 \pm 2 \text{ μm}^2/\text{s}$ (SEM), consistent with the value of 330 μm²/s measured by two-focus fluorescence correlation spectroscopy (FCS) (20). The diffusion coefficient of the 30-mer ssDNA is $178 \pm 2 \text{ μm}^2/\text{s}$, matches that predicted by the Zimm model for a polymer in good solvent, 166 μm²/s, calculated with persistence length and rise per base taken from laser tweezers experiments (22) and no adjustable parameters (see Methods and ref. 23).
mean molecular brightness of the fluorophore is sensitive to the chemical environment at different aspects of the molecular structure: brightness, diffusion coefficient, and electrokinetic mobility (Fig. 4). These three parameters indicate different aspects of the molecular structure: brightness of the fluorophore is sensitive to the chemical environment at the 5' end of the ssDNA; diffusion coefficient is sensitive to hydrodynamic radius of the entire molecular complex; and electrokinetic mobility is sensitive to both charge and viscous drag. Thus each molecule was characterized with high precision in a multidimensional parameter space, allowing facile identification of heterogeneous subpopulations.

In the absence of RecA, we observed a homogeneous population of trapped molecules with diffusion coefficient 113 ± 4 μm²/s (SEM), a mobility of −6.3 ± 0.1 x 10^{-6} μm²/(V s) and a mean molecular brightness of 32.0 ± 0.2 x 10³ photons/s, which we associate with bare ssDNA. Addition of RecA and ATP led to the appearance of a second subpopulation with diffusion coefficient 50 ± 2 μm²/s, mobility of −3.65 ± 0.07 x 10^{-6} μm²/(V s) and a mean molecular brightness of 44.7 ± 0.3 x 10³ photons/s. We compared the diffusion coefficients of the bare ssDNA and the nucleoprotein filament to theoretical predictions based on the expected geometries of these compounds. The measured diffusion coefficient of the ssDNA, 113 ± 4 μm²/s, is in good agreement with the Zimm prediction of 124 μm²/s for 60-mer ssDNA. The measured diffusion coefficient of the RecA-ssDNA nucleoprotein filaments, 50 ± 2 μm²/s, is lower than that of the bare ssDNA, consistent with an increase in hydrodynamic radius upon RecA binding. However, the measured diffusion coefficient is significantly larger than expected from a rigid-rod model of the filament, 21 μm²/s. Several modifications to the rigid-rod geometry may account for this discrepancy, including: bent or curved nucleoprotein structure [as seen in some electron microscope images (26)], incomplete coverage of the DNA by RecA, or multiple RecA domains separated by floppy dislocations. Distinguishing among these scenarios will require varying conditions such as the length of the DNA template and the RecA concentration. The discrepancy is unlikely to be due to transient dissociation of RecA monomers from the nucleoprotein filament, as transport coefficients were not affected by replacement of ATP with ATTyS, an analog known to reduce the rate of dissociation (27).

The decrease in absolute value mobility upon addition of RecA is consistent with a decrease in net charge or an increase in drag. The change in Stokes drag can be inferred from the change in diffusion coefficient, discussed above, but the relevance of Stokes to the electrophoretic mobility depends on the details of the ionic cloud around the complex. In the Hückel regime, in which the particle is much smaller than the Debye length of the buffer, the electrophoretic velocity is determined by a force balance between the Coulombic pull of the field and the Stokes drag on the moving particle. In the Smoluchowski regime, in which the particle is large compared to the Debye length, the electric and shear forces are both localized within the Debye layer and both grow proportionally to the size of the particle (28). Thus the electrophoretic mobility is independent of the size of the particle. In the case at hand, the size of the ssDNA-RecA complex is comparable to the Debye length of the buffer, estimated to be 1.5 nm. Thus, the molecule is between the Hückel and Smoluchowski regimes, where theoretical estimates of mobility are difficult. Free-solution electrophoretic mobilities of ssDNA (29) and DNA–protein complexes (30) have been measured and are similar to our single-molecule values, though the buffer composition in these experiments differed from ours, preventing quantitative comparison.
The unique ability to measure simultaneously the diffusion coefficient, electrokinetic mobility, and brightness of each trapped molecule, to high precision, allows species to be distinguished on the basis of size, charge, and photophysical properties, in free solution and in complex mixtures. Unlike fluctuation techniques such as FCS (32) and the photon-counting histogram (33), the ABEL trap characterizes individual molecules in isolation, allowing compilation of the full distribution of each measured parameter and enabling multiple parameters to be correlated at the single-molecule level. The long observation times per molecule in the ABEL trap provide significantly more precise information than is obtained from photon burst analysis (34, 35). Furthermore, the tracking data enables unambiguous separation of transport and photophysical dynamics, which are otherwise conflated in single-point confocal techniques.

Finally, the ABEL trap opens the possibility of observing molecular transitions as they occur; such transitions cannot be resolved in observations of passively diffusing molecules unless they occur on a timescale faster than the diffusion time (approximately 1–10 ms), but may be observed on timescales as long as seconds in the ABEL trap. In the present RecA data we observed occasional transitions indicative of RecA binding to an already trapped molecule of ssDNA; however, these transitions were too infrequent to merit detailed analysis.

In contrast to the ensemble-averaged FCS data, the ABEL trap data clearly discerned hidden heterogeneity in the sample of RecA-ssDNA. The multiparameter molecule-by-molecule data enabled a quantitative comparison to models of the RecA nucleoprotein filament. These capabilities are expected to be broadly useful in contexts beyond studying protein–DNA interactions.

Trapping small-molecule fluorophores in aqueous solution is the ultimate size limit of feedback trapping in solution. The median size of human proteins is 375 amino acids (36), corresponding to a diameter of approximately 4 nm (37). With previous trapping technology, only the largest of proteins and complexes, with diameter > 15 nm, could be trapped. The present ABEL trap extends this range to include all soluble proteins.

**Methods**

The optics, device design and fabrication, electronics, and sample preparation are described in SI Text.

**Kalman Filter Feedback Algorithm.** The Kalman filter is an algorithm that interprets the past record of detected photons and applied voltages to construct a Gaussian likelihood distribution for the location of the particle in the present. The estimate for the particle’s position during the jth time bin, given all of the information recorded up to and including the jth time bin, is characterized by a mean $\hat{x}_j$ and variance $P_j$. The filter operates recursively: To calculate a new estimate, the previous estimate is revised to account for the most recent observations and the expected motion of the particle.

The number of photons, $n_j$, detected during the laser’s residence at the kth scan point are tallied and used to “update” the estimate according to

$$\hat{x}_{k|j} = \frac{n_j \hat{x}_{k-1|k-1} + w \hat{p}_{k-1|k-1}}{n_j + w},$$

where $w$ and $n$ are the mean and standard deviation, respectively, of the laser spatial intensity distribution during bin $k$. In this way, the information from photon arrivals is combined with the previous position estimate, with weighting coefficients determined by the uncertainty in each. The beam profile and spot positions were measured prior to trapping experiments by scanning a small bead immobilized on a coverslip through the scan pattern using a piezo scanning stage (Thorlabs SCXY2100).

When the laser’s residence at the kth spot ends, a new estimate for the location of the particle is constructed, with mean and variance “predicted” according to

$$\hat{x}_{k+1|k} = \frac{\hat{x}_{k|k} + \mu \Delta t \hat{v}_k}{\mu \Delta t},$$

$$\hat{p}_{k+1|k} = \frac{\hat{p}_{k|k} + 2D \Delta t}{\mu \Delta t},$$

where $\mu$ is the user-estimated electrokinetic mobility, $\hat{v}_k$ is the voltage applied during bin $k$, $D$ is the user-estimated diffusion constant, and $\Delta t$ is the duration of the bin. At the beginning of the experiment, the estimated location of the particle is initialized at the trap center and the variance in this estimate is set to an arbitrary large value. The influence of these initial conditions decays after a few tens of microseconds. The Kalman filter is propagated each time the laser is moved to a new position (every 3.1 μs).

Feedback voltages are calculated according to the equation

$$V_{k+3} = \frac{-\hat{x}_{k+3|k}}{\mu \Delta t},$$

where

$$\hat{x}_{k+3|k} = \frac{\hat{x}_{k+1|k} + \mu \Delta t (V_{k+1} + V_{k+2})}{\mu \Delta t}.$$
The sums converge, and we truncate when the fractional changes due to additional terms are $< 10^{-6}$.

The prediction equations are

$$\dot{s}_{k+1} = s_k + \mu \Delta V_k \quad \dot{\Sigma}_{k+1} = \Sigma_k + 2 D \Delta t + \nu^2 \Delta V_k V_k^T,$$

where $I$ is the two-by-two identity matrix, $\nu$ is the dimensionless ratio between the variance and the mean square of the effective mobility, and the other parameters are as defined previously. The effective mobility is treated as a Gaussian random variable to reflect the observation that molecules do not always respond identically to an applied voltage, perhaps due to spatial inhomogeneity of the field, or to unconstrained fluctuations in the vertical position of the particle in the trap and consequent changes in field strength and drag on the particle.

The overall log-likelihood of the entire data series is

$$\ln(\mathcal{L}) = \sum_k \ln(\mathcal{L}_k).$$

Maximum-likelihood parameter estimates are found by gradient ascent of this function. To convert the units of the calculated mobility parameter, the electric field strength was estimated as the applied voltage divided by the length of the trapping region (30 $\mu$m).

**Diffusion Coefficient Calculation.** Theoretical diffusion coefficients were calculated using the Zimm model presented in (23), specifically equation (4.84)

$$D_G = \frac{0.2030 \gamma k_B T}{\sqrt{6} \eta b R_g}.$$

Nonlinear least-square five-parameter fits were performed using MATLAB.

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