Ultrahigh-resolution optical trap with single-fluorophore sensitivity

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We present a single-molecule instrument that combines a time-shared ultrahigh-resolution dual optical trap interlaced with a confocal fluorescence microscope. In a demonstration experiment, we observed individual single fluorophore–labeled DNA oligonucleotides to bind and unbind complementary DNA suspended between two trapped beads. Simultaneous with the single-fluorophore detection, we clearly observed coincident angstrom-scale changes in tether extension. Fluorescence readout allowed us to determine the duplex melting rate as a function of force. The new instrument will enable the simultaneous measurement of angstrom-scale mechanical motion of individual DNA-binding proteins (for example, single-base-pair stepping of DNA translocases) along with the detection of properties of fluorescently labeled protein (for example, internal configuration).

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Single-molecule techniques have evolved into powerful tools to study many fundamental biological processes. They have been used to quantify the mechanical properties, conformational dynamics and interactions of biological macromolecules, providing previously unobtainable precision and clarity[1–4]. In particular, single-molecule fluorescence microscopy and optical 'tweezers' have provided crucial insights into the mechanism of a wide range of nucleic acid binding proteins and molecular motors involved in genome maintenance.

Fluorescence approaches are varied[5]. With a single fluorophore, fluorescence microscopy is used to detect the presence or absence of a single labeled protein[6], to count the number of subunits in a protein complex[7] or track the movements of proteins with nanometer precision[8]. With two fluorophores, fluorescence resonance energy transfer (FRET) between pairs of molecules provides a spectroscopic measurement of inter-pair distances[9,10], enabling the detection of conformational dynamics in a doubly labeled protein or translocation of a labeled motor protein along a labeled nucleic acid substrate[11]. In optical trap measurements, a single molecule of DNA or RNA can be tethered between two attachment points, and its extension can be monitored by the trap (or traps). The tether is designed so that changes in its extension relay information about the biological system under study, for example, the binding of a molecule or the motion of a molecular motor.

In many cases, a detailed understanding of the proteins involved in genome maintenance requires sensitivity to base-pair length scales. For example, molecular motors that translocate along DNA or RNA (for example, polymerases and helicases) likely move in discrete steps on the order of only a single base pair: only 3.4 Å in duplex DNA. The direct detection of such steps provides an important clue into the inner workings of these proteins. The recent technical development of ultrahigh-resolution optical tweezers[12–14] has made possible, to our knowledge for the first time, the direct observation of molecular motion on the scale of 1 base pair (bp) of DNA. This technique is only beginning to be applied to biological questions[12,15–18].

Despite such advances, single-molecule techniques currently have important limitations. For example, the operation of motor proteins consists of complex internal conformational transitions driving translocation along a substrate. Though optical traps can provide ultrahigh spatial resolution of motor translocation over long distances, they cannot reveal the internal state (conformation or number) of the protein. In contrast, single-molecule fluorescence techniques are well-suited to probe the conformational state of a protein of interest, but (in the case of FRET) have much more limited spatial range. More generally, current single-molecule techniques are often ill-equipped to capture the multifaceted and three-dimensional dynamics of protein complexes, as they typically project all motion onto a single measurement axis. In the case of FRET, for instance, molecular conformational changes are measured along the vector between donor and acceptor fluorophores; in optical trap measurements, movements are detected only along the direction of applied tension. These limitations motivate the development of hybrid techniques that allow for simultaneous measurement of multiple observables. Moreover, for these techniques to be used to probe these dynamics on relevant length scales and to be applicable to a large class of biological processes, particularly those associated with DNA metabolism, it is essential that they resolve motions on sub-nanometer length scales.

Hybrid instruments combining fluorescent capabilities and mechanical manipulation provide a promising direction to attain

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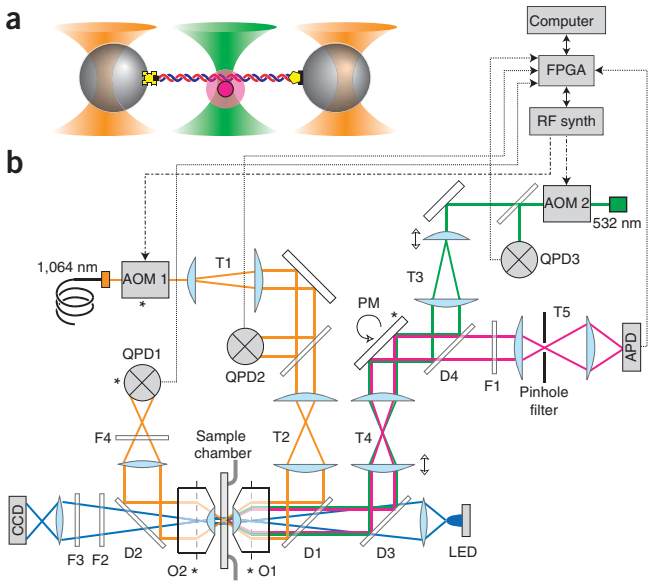

Figure 1 | Combined ultrahigh-resolution optical trap and single-molecule fluorescence microscope setup. (**a**) Schematic of experimental setup showing dual optical traps (orange cones) trapping two beads with DNA tethered between them, and confocal laser excitation and detection (green cone) for measuring fluorescence from a single fluorophore-labeled molecule (magenta disk) bound to the DNA. Bead-DNA attachments were made via biotin (DNA)–streptavidin (bead) and digoxigenin (DNA)–anti-digoxigenin (bead) linkages. (**b**) Instrument layout showing optical paths for 1,064-nm trapping laser (orange), 532-nm fluorescence excitation laser (green), collected fluorescence (magenta) and blue LED for brightfield imaging (blue). Trap and fluorescence lasers were interlaced by AOM1 and AOM2, respectively, driven by RF synthesizers (RF synth) directly controlled by an FPGA chip-based data acquisition and control personal computer (PC) card. Synchronous with laser modulation, the FPGA reads three QPDs that measure trapped bead positions (QPD1) and trap and fluorescence excitation laser intensities (QPD2 and QPD3, respectively, enabling laser intensity stabilization) along with a single-photon–counting APD measuring fluorescence. D1–D4, dichroic mirrors; F1–F4, filters; O1 and O2, objective lenses; PM, piezo mirror stage; T1–T5, telescopes. Conjugate image planes are indicated by asterisks.

this goal[19–24]. Though such instruments have long existed in the field, they have suffered from two major limitations: (i) they lack the sensitivity to detect individual fluorescent molecules, and/or (ii) they cannot be used to measure displacements with adequate (sub-nanometer) mechanical resolution and are thus limited in their applicability to biological problems. We combined ultrahigh-resolution dual-trap optical tweezers formed from a single laser beam with a single-molecule confocal fluorescence microscope to measure the fluorescence of labeled molecules immobilized on the tether between the two trapped beads with single-fluorophore sensitivity (**Fig. 1a**). The design provided angstrom-scale stability necessary for high resolution by eliminating the most common source of noise found in traditional optical tweezers, the drift between the microscope stage and the optical trap. We demonstrated our instrument's ability to detect individual fluorophores and resolve sub-nanometer motion simultaneously by measuring the hybridization of a probe strand of ssDNA oligonucleotides to a complementary sequence.

RESULTS

Instrument design

Combining optical tweezers with single-molecule fluorescence detection poses a severe and well-documented technical challenge: decreased fluorophore lifetime owing to enhanced photobleaching. This enhanced photobleaching is believed to be due to absorption of the near-infrared optical trap photons while in the excited state[25]. One solution to this problem is to separate the optical trap and fluorophore by a large distance[20,21]. This approach, however, is not practical for measuring angstrom-scale changes in tether extension because tethers must be as stiff as possible and hence must be short (typically ≤ 3 kbp or 1 μm in length). Instead we minimized photobleaching by combining the optical tweezers and confocal microscope via interlacing: the optical traps and confocal microscope were turned on and off in sequence so that they were never both on simultaneously[26].

Interlacing the optical tweezers while achieving ultrahigh resolution required modifications to previous ultrahigh-resolution optical tweezers designs[27]. To maintain sufficient trap stiffness while interlacing, the traps must be turned on and off at rates >10 kHz[26]. However, ultrahigh-resolution optical tweezers operate near noise thresholds and have extremely low tolerance for additional vibrational noise. Acousto-optic devices are all-electronic devices that can switch laser intensities at >100 kHz rates and are commonly used in lower-resolution optical traps[26,28]. We used the first acousto-optic modulator (AOM1) to directly control the intensity of the trap laser (that is, the trap stiffness) and whether it is on or off (**Fig. 1b** and **Supplementary Note 1**). Though this is not widely recognized, an AOM can deflect a laser beam similar to an acousto-optic deflector. We used a water-immersion microscope objective to focus the beam deep inside the sample chamber (~50 μm from the coverslip), and we used the AOM to rapidly switch the trap laser between two deflection angles to create traps at two positions, a technique known as 'timesharing'[29]. Working at this depth provided improved stability by decoupling the trap position and stiffness from the chamber position[27]. Scattered laser light from the trapped beads was collected by a second identical objective and imaged onto a quadrant photodiode detector (QPD), which measured the bead positions (Online Methods). The optical trap portion of the instrument could clearly resolve artificially generated single-base-pair steps (**Supplementary Fig. 1**).

For the confocal microscope, we interlaced a 532-nm fluorescence excitation laser via a second AOM (AOM2), and a piezo mirror stage deflected the excitation beam and provided lateral positioning of the confocal volume as well as raster-scan fluorescence imaging capability. Fluorescence from within the confocal volume was collected by the front objective, directed backward along the excitation laser path, focused through a 100-μm pinhole and imaged onto a single-photon-counting avalanche photodiode detector (APD).

We used a field-programmable gate array (FPGA)-based data acquisition and control system to interlace the optical traps and confocal microscope at 66 kHz. This provided high-speed synchronous control of beam modulation and data acquisition (**Fig. 2**). Each of the two traps was on in sequence for only one-third of the repetition cycle. Fluorescence was excited and collected only during the final one-third of the cycle when both traps were off. To control the optical traps (for example, timing,

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