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Combined optical trapping and single-molecule fluorescence

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Abstract

Background: Two of the mainstay techniques in single-molecule research are optical trapping and single-molecule fluorescence. Previous attempts to combine these techniques in a single experiment - and on a single macromolecule of interest - have met with little success, because the light intensity within an optical trap is more than ten orders of magnitude greater than the light emitted by a single fluorophore. Instead, the two techniques have been employed sequentially, or spatially separated by distances of several micrometers within the sample, imposing experimental restrictions that limit the utility of the combined method. Here, we report the development of an instrument capable of true, simultaneous, spatially coincident optical trapping and single-molecule fluorescence.

Results: We demonstrate the capability of the apparatus by studying force-induced strand separation of a rhodamine-labeled, 15 base-pair segment of double-stranded DNA, with force applied perpendicular to the axis of the DNA molecule. As expected, we observed abrupt mechanical transitions corresponding to the unzipping of DNA at a critical force. Transitions occurred concomitant with changes in the fluorescence of dyes attached at the duplex ends, which became unquenched upon strand separation.

Conclusions: Through careful optical design, the use of high-performance spectral notch filters, a judicious choice of fluorophores, and the rapid acquisition of data gained by computer-automating the experiment, it is possible to perform combined optical trapping and single-molecule fluorescence. This opens the door to many types of experiment that employ optical traps to supply controlled external loads while fluorescent molecules report concurrent information about macromolecular structure.

Background

Among the most useful single-molecule approaches are optical traps ('optical tweezers') and fluorescence. The combination of these two biophysical techniques in a single assay offers a powerful tool for studying molecular systems, by allowing direct correlations to be made between nanoscale structural changes, reported by single-molecule fluorescence, and biomechanical transitions, probed by piconewton forces generated with optical traps. Here, we demonstrate the feasibility of this combination by simultaneously observing single-molecule fluorescence changes and force-induced strand separations in dye-labeled double-stranded DNA duplexes.

Prior attempts to marry optical trapping with single-molecule fluorescence failed to achieve spatially coincident fluorescence and trapping within the sample. In all cases reported, the optical trap was physically separated from the region of fluorescence excitation by a distance of up to several micrometers [1,2], or the techniques were applied sequentially [3]. The comparatively large separation avoids problems caused by the high flux of infrared light within the optical trap, which can reduce fluorophore lifetimes (through unwanted two-photon excitation of dyes or other destructive photochemical mechanisms) and contributes to high levels of background light, which can readily obscure the weak fluorescence signal. A large physical separation imparts its own drawbacks, however, imposing severe restrictions on the experimental geometries that can be achieved. Moreover, it requires that optical forces be applied remotely, generally through a suspended filament geometry - that is, through a tether, rather than directly to the macromolecule of interest. The elastic compliance of a tether increases with its length, and thermal measurement noise (Brownian noise) increases in proportion to this compliance. Longer tethers therefore produce greater levels of measurement noise. Our design strategy circumvents many earlier problems and achieves spatially coincident optical trapping and single-molecule fluorescence, making this a true combination of the two technologies.

Results and discussion

The optical layout of our instrument [4] (see also the additional diagram available with this article online) was designed from the outset to permit simultaneous single-molecule fluorescence and optical trapping. The choices of dyes and excitation wavelengths were optimized to minimize two-photon excitation and achieve acceptably long fluorescence lifetimes in the trap. The laser wavelengths used for trapping, nanoscale position detection, and fluorescence were all broadly separated. Attention was paid to the rejection of stray light and the selection of all optics, including the use of high-efficiency holographic notch filters (Kaiser Optical, Ann Arbor, USA) to reject laser excitation and detection wavelengths before the photodetectors. Finally, the apparatus is computer-automated, greatly reducing the set-up time once a candidate fluorophore is identified and permitting rapid measurements prior to photobleaching. The instrument incorporates a microscope plus three lasers: one for trapping (1064 nm), a second for position detection (827 nm), and a third for fluorescence excitation (514 nm; see the additional diagram available with this article online). Laser excitation light is coupled through the microscope objective into the specimen as an evanescent wave (single-sided total internal reflection fluorescence, TIRF).

In our experimental geometry (Figure 1a), a 1,010 base-pair DNA duplex with an overhanging segment was attached at one end (via a biotin-avidin linkage) to a polystyrene bead 500 nm in diameter and annealed at its distal end to a 15 base-pair oligonucleotide, anchored directly to the coverglass surface (via a digoxygenin-antidigoxygenin antibody linkage). Tetramethylrhodamine (TAMRA) dyes conjugated to nucleotides were placed on complementary bases, one on the 3′ end of the shorter 15-mer and the other on the 5′ end of the long strand; in these positions, the dye proximity causes fluorescence to be quenched. A recent study has shown that rhodamine self-quenching arises from the formation of noncovalent dimers based on stacked xanthine rings, leading to an approximately 30-fold decrease in fluorescence signal [5]. The distance range probed by dye self-quenching is therefore exceedingly short (about 1-2 nm), and smaller than most distances probed by fluorescence resonance energy transfer (FRET). The DNA-bead complex was trapped and the microscope stage was moved at constant velocity, pulling the bead from the center of the trap and thereby increasing the load until rupture ('unzipping') of the 15 base-pair duplex occurred. Experiments were conducted in an assay medium consisting of 100 mM Na-phosphate buffer pH 7.5, 0.1% Tween detergent and 0.1% beta mercaptoethanol, with 3.0 mg/ml bovine serum albumin added as a blocking protein; buffers were degassed lightly under house vacuum before use in flow chambers.

Bead position, accurate to the nanometer level, was recorded with a quadrant photodiode [4] while fluorescence was monitored by counts on an avalanche photodiode (APD; EG&G Optoelectronics, Gaithersburg, USA), collected through a confocal pinhole. The area of regard of the APD was limited to a region of approximately 950 nm diameter in the specimen plane. Fluorescence and position traces were both collected at 200 Hz and filtered at 100 Hz; fluorescence count rates were further smoothed with a three-point boxcar filter. Records of bead position were converted to force based on the measured stiffness of the trap.

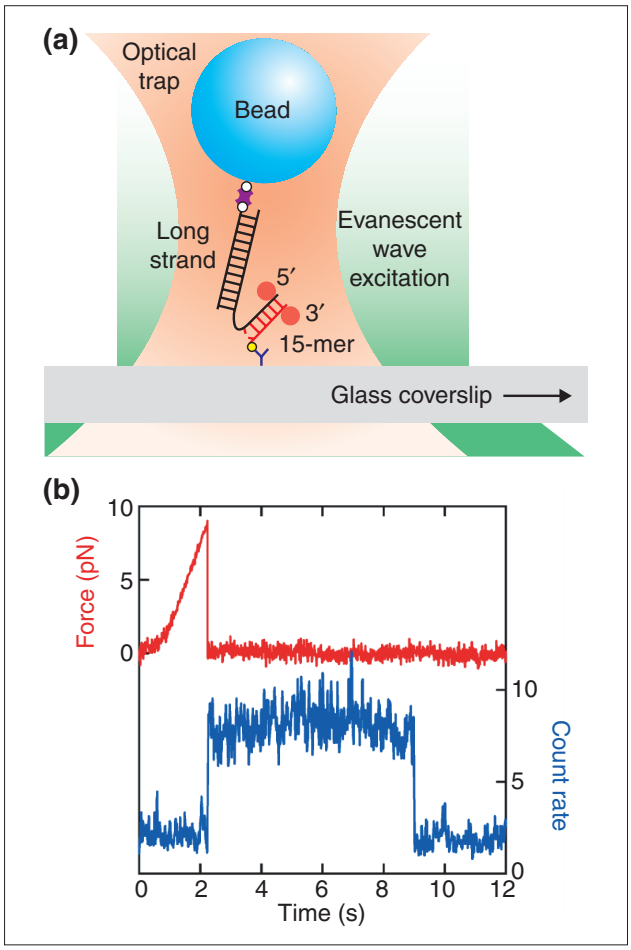

Figure 1
A combined optical trapping and fluorescence experiment to unzip DNA. **(a)** A cartoon of the simplified experimental geometry (not to scale). A bead was tethered by a digoxygenin-based linkage (blue and yellow) to the coverglass surface through a DNA molecule, consisting of a long segment (black) joined to a shorter 15 base-pair strand that forms a duplex region (red). The bead (blue) was captured by the optical trap and force was applied to unzip the short duplex. Tetramethylrhodamine (TAMRA) dyes attached at the ends of the DNA strands provide a fluorescence signal (red dots). **(b)** Simultaneous records of force (red trace) and fluorescence, measured as the photon count rate (blue trace). Rupture occurred at $t \approx 2$ sec at an unzipping force of 9 pN. The dye unquenched at the point of rupture, and later bleached at $t \approx 9$ sec. See text for further details.

Prior to rupture, force increased monotonically, and the low light levels indicated the quenched dye state (Figure 1b). At rupture, the force dropped from around 9 pN to 0 pN and the light level increased, indicating that the two fluorophores separated, unquenching the dye bound to the 15-mer remaining on the surface. After several seconds, this dye photobleached in a single step and the light signal returned to background levels.

We also conducted similar experiments with single dyes bound to either the long strand or to the 15-mer (data not shown). As expected, dyes bound to the long strand fluoresced normally and light levels decreased abruptly upon rupture as the bead carrying the DNA strand was pulled beyond the excitation zone. Records of light levels with dyes bound to the 15-mer show no such decrease, but instead bleached at some variable time later. The latter experiment supplies a control showing that rupture corresponds to strand dissociation, as expected, and not to the breakage of the digoxygenin-based link holding the DNA to the surface. Using the methods described here, it is possible to build up histograms of the forces required to unzip (or to shear) duplex DNA molecules of various lengths under a variety of conditions and rates of loading (M.J.L., P.M.F., A.M. Engh, K.C. Neuman, and S.M.B., manuscript in preparation).

Conclusions

When suitably combined, optical trapping and single-molecule fluorescence supply information beyond what can be learned with either technique alone, permitting structure and mechanics to be probed within a single macromolecule. Traces corresponding to force-induced mechanical transitions may be challenging to interpret, because of difficulties in determining the precise location at which an applied force produces a conformational change. Simultaneous fluorescence measurements can resolve this ambiguity by pinpointing the location(s) of structural transitions. This allows precise assignment of specific mechanical changes to specific structural transitions. Such assignments are especially important in the study of complex systems: for example, the folding/unfolding studies of nucleic acids and polypeptides, or conformational rearrangements associated with the translocation of processive enzymes.

Moreover, simultaneous optical trapping and single-molecule fluorescence measurements supply information about the sequence of molecular events. Relative timing information can be used, for example, to establish the relationship between substrate binding and stepping motion in motor proteins, or to probe molecular mechanisms underlying conformational changes. We anticipate that this technique will have broad applicability to the study of fundamental biological questions, by providing a direct means to study coupling between biochemical and biomechanical reaction cycles.

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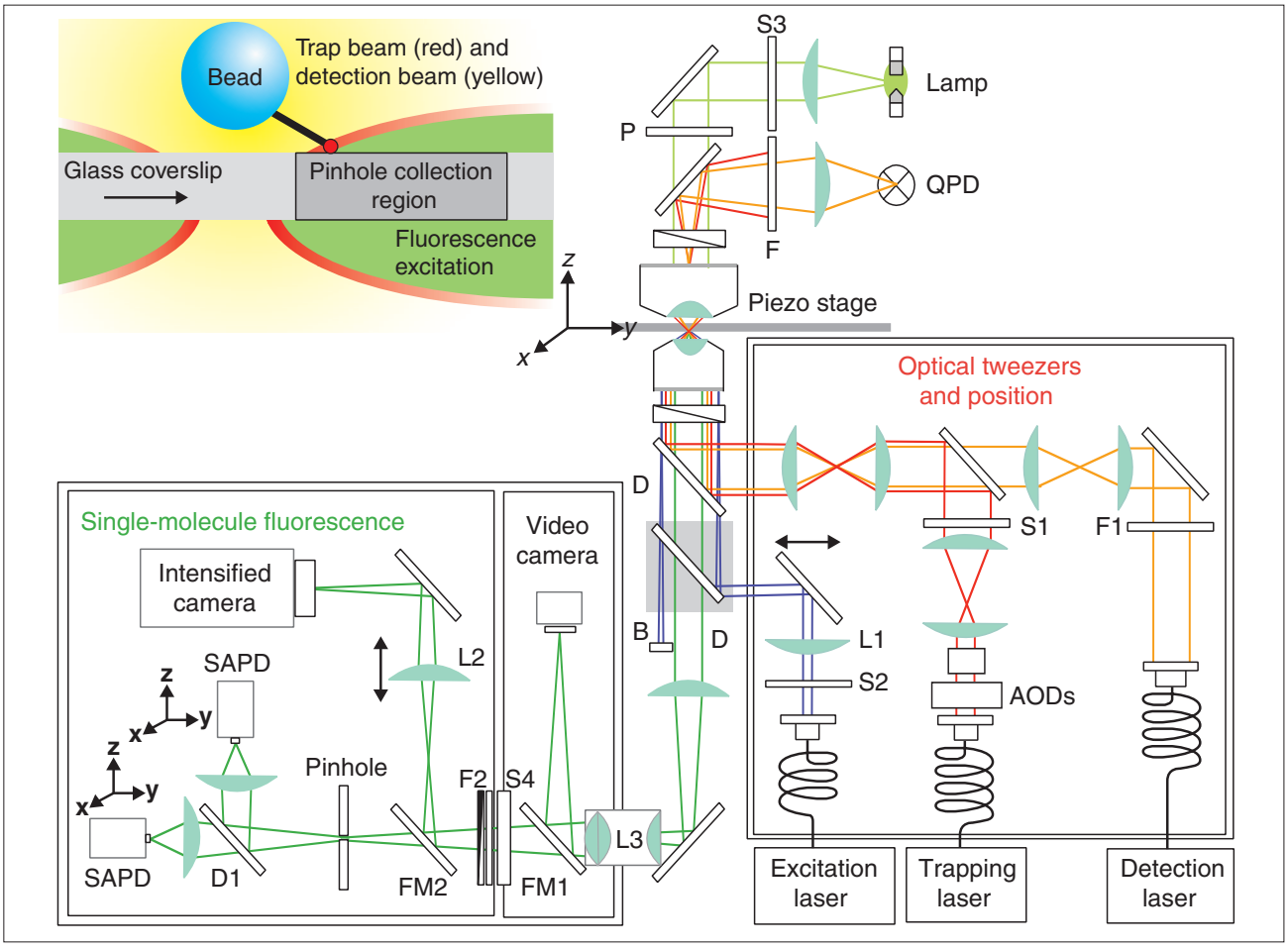

Figure S1
A simplified diagram of the optical layout of our instrument (see also [4]). The inset is a cartoon showing a detail of the specimen in the region illuminated by the three lasers (not to scale). Fluorescence excitation of the sample is supplied by evanescent wave illumination through the microscope objective over a region of several square micrometers (green). Overlapping within this region, the optical-trapping laser (pink) and position-detection laser (yellow) beams are coaxial and brought to diffraction-limited spots near the coverglass, as shown. An optically trapped bead tethered to the coverglass surface is shown for reference (blue). The area of regard of the fluorescence photodetectors through a confocal pinhole is indicated (dark gray). The main diagram shows the instrument itself, which is based on an inverted microscope with a nano-positionable three-dimensional piezo stage and equipped with a mercury arc lamp; the key components are shown in the center of the diagram. Also shown is the quadrant photodiode (QPD) subsystem used to detect changes in the position of the trapped bead. The input optics, including all three lasers, are shown to the right of the microscope inside the box labeled in red. The position-detection pathway is shown in orange, the trapping-laser pathway in red, the fluorescence-excitation pathway in blue and the fluorescence-emission pathway in dark green. The normal microscope transillumination pathway is shown in light green. The trapping laser beam can be moved electronically by means of acousto-optic deflectors (AODs) placed at optical planes conjugate to the back focal plane of the objective. The output optics, including a cooled, intensified charge-coupled device (CCD) camera, a conventional black-and-white CCD camera, and two silicon avalanche photodiodes (SAPDs), are shown to the left of the microscope, inside the box labeled in green. The identities of other optical elements are: B, beam; D, dichroic; F, filter; L, lens; P, polarizer; S, shutter; FM, flipper mirror.