II. DESIGN

Fig. 1 is the complete optical tweezers schematic, with an inset photograph of the microscope and its significant modifications. All components are mounted to a vibration isolation table (RS 2000, Newport Corp., Irvine, CA). For safety and increased laser stability, a plexiglass housing draped in laser safety fabric covers the entire laser pathway. The microscope oculars are kept covered during operation.

A. Microscope and Video-Enhancement System

The microscope (Axioplan2, Zeiss Inc., Thornwood, NY, Fig. 1 H, inset) is principally outfitted for video-enhanced DIC and epi-fluorescence microscopy, using a 1.3 NA objective (Plan-NEOFLUAR 100X, Zeiss Inc.). Two principal image processing techniques, contrast enhancement and background subtraction, allow small structures such as microtubules to be visualized with DIC [7]. A custom quantitative image analysis routine tracks the position of an object between captured images using a cross correlation algorithm (IDL v. 5.2, Research Systems, Inc., Boulder, CO) [8].

B. Laser and its integration into the Microscope

An Nd:YVO4 laser beam (T20-BL10-106Q, Spectra-Physics Lasers, Inc., Mountain View, CA, λ = 1064 nm) in continuous TEM-00 mode is expanded and collimated (Fig. 1, A & B, 1/e^2 diameter = 4.2 mm) to overfill slightly the back aperture of the microscope’s objective. The laser enters the microscope through a port machined above the objective turret, where a custom-machined mount holds a dichroic mirror (Fig. 1 inset). The beam exits the microscope through a hole machined in the microscope’s base. The standard DIC optics of the microscope include a linear polarizer and a green bandpass filter; both were moved to a filter wheel near the illumination source to prevent interference with the laser.

C. Beam Polarity and Attenuation

To create two non-interfering, independent traps, a polarizing beam-splitting cube (PBSC), preceded by a λ/2 wave plate, divides the laser into orthogonally polarized beams (Fig. 1, C). A liquid crystal attenuation system (LCR-IR05, Newport Corp.) controls the laser power for each beam (Fig. 1, D).

D. Alignment to Wollaston Prisms

To preserve the polarity of the laser beams, before entry into the microscope a λ/2 wave plate rotates the two beams to meet the orthogonal ordinary and extraordinary axes of the Wollaston prisms (required for DIC) (Fig. 1, G).

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Aligning the beams’ polarity to the Wollaston prisms requires several steps. A single polarity beam (S- or P-) is sent through the microscope without the Wollaston prisms in place, and strikes a linear polarizer after exiting the microscope. The polarizer is rotated to maximum absorbency. The λ/2 wave plate is introduced, oriented to introduce no beam rotation, and one Wollaston prism is put in place. In this configuration, any laser power measured beyond the linear polarizer represents a splitting of the beam’s polarity by the Wollaston prism. The λ/2 wave plate and linear polarizer are then rotated concurrently until the measured laser power is extinguished.

E. Beam Steering with Acousto-Optic Deflectors (AODs)

A two axis acousto-optic deflection system (DTD-406BA6, IntraAction Corp., Bellwood, IL) steers each beam through 1:1 telescope lenses into the microscope (Fig. 1, E & F). The telescope lenses create conjugate image planes at the AODs and the back aperture of the microscope objective lens, and also finely adjust the beams’ divergence, so that optical traps form exactly at the microscope’s image plane. The x and y axis deflectors are aligned to the Bragg condition at the AOD center frequency of 40 MHz. At optimal alignment, ~80% diffraction efficiency is achieved.

Custom computer programs (LabVIEW 5.1) control trap position by changing the acoustic wave frequency within each AOD. The control programs can also time-share each beam between multiple positions, allowing for the formation of an arbitrary number of optical traps per beam. The AOD rise-time for the beam (the time for laser power to go from 10% to 90% at a newly set angle) is 4.2 µs, giving a maximum switching frequency of 238 kHz between positions. Switching at this frequency between 2+ positions approximates multiple continually illuminated traps.

F. Back Focal Plane Interferometry with Quadrant Photodiode Detectors (QPDs)

After exiting the microscope, the beams are separated by polarity and terminate onto separate QPDs (Spot 9DMI, UDT Instruments, San Diego, CA) located at a plane made conjugate with the back focal plane of the microscope’s condenser (Fig. 1, I) [6]. For maximum sensitivity, the magnification of the optics creates a spot size >80% of the QPD active area. Interchangeable neutral density filters placed in front of each QPD attenuate the beam’s power to within the sensitivity range of the QPD system. Differential amplifiers (431 Position Sensing Unit, UDT Instruments) amplify and sample the QPD signal at 7 kHz. A data acquisition card digitizes this signal at up to 100 kHz (PCI-MIO-16XE10, National Instruments), and software analyzes the signal and records it to disk (LabVIEW v.5.1).

G. Other Modifications

The calibration procedures described below use a piezoelectric nano-positioning stage (NPS-xy-100B, JDS Uniphase Corp., Berkshire, UK) to move the microscope slide, either in minute steps, or at constant known velocities. A custom-machined platform was built to mount the nano-positioning stage to the microscope (Fig. 1 inset).

III. CALIBRATION AND PERFORMANCE

A. Characterization of QPD Sensitivity (β)

QPD sensitivity, β (in V/nm), is determined experimentally by moving an immobilized glass bead through a low power trap. A solution of 0.97
Fig. 2. QPD detector response for an immobilized 0.97 µm glass sphere stepped through the trap. The fit to the center linear region gives the sensitivity.

µm diameter glass beads at 0.03% solids by mass (SS03N, Bangs Laboratories Inc., Fishers, IN) is prepared in BRB80 buffer containing a 1:80 dilution of saturated casein solution. (All future references to beads assume a 0.97 µm diameter and 0.03% solids by mass.) In a flow chamber constructed of a microscope slide and a #1 cover slip separated by double-stick tape, a bead is pushed against the cover slip with the optical trap until it adheres. The nanopositioning stage moves the slide and the now immobilized bead through the laser focus in 25 nm increments. (Nanopositioning stage applies a known velocity of fluid flow past the trapped microsphere. Fig. 3B shows raw data, and Fig. 3C shows final curves of force as a function of displacement taken at 28 °C. For all practical purposes the trap's force is a linear function of the position of the trapped bead.

As an alternative, the equipartition theorem can be used to provide a convenient calibration method. Combining the hookian nature of the trap with the equipartition theorem and assuming that \( \langle x^2 \rangle = 0 \) gives [11]

\[
\kappa = \frac{k_B T}{\text{Var}(x)}
\]  

Bead position data is sampled at 14 kHz for 45 seconds, butterworth filtered with a cut off set to 7000 kHz, broken into 90 pieces and the variance of each piece is calculated. The average of these 90 variance values is used with (1) to calculate \( \kappa \).

Power spectral analysis can be used as a third method to determine the trap stiffness. The power spectral density function, \( S(f) \), for a bead in an optical trap is expected to be Lorentzian:

\[
S_c(f) = \frac{B}{f_c^2 + f^2}
\]  

and \( f_c \) is the corner frequency of the Lorentzian power spectrum [12]. Fig. 3D shows a typical power spectrum. The corner frequency in turn depends upon the spring constant of the trap:

\[
\kappa = 2\pi f_c
\]

The same 90 filtered pieces of data used in the equipartition theorem method are windowed with a simple triangle function and 90 power spectra are calculated, averaged and re-normalized. The DC component is removed, the spectrum is truncated at 3.5 kHz and the Lorentzian described by (2) is fit to the truncated spectrum with \( f_c \) and \( B \) as fitting parameters (SPSS 10.0, SPSS Inc., Chicago, IL). (4) calculates trap stiffness, \( \kappa \), from corner frequency \( f_c \).

Fig. 3E shows the trap stiffness as a function of laser power for all three calibration methods. Trap stiffness is proportional to laser power with a best fit to the data from all three methods of \( 6.7 \times 10^4 \) pN/nm/mW and standard error \( 1.5 \times 10^5 \). Generally the equipartition method agrees with the viscous drag method, within 5%. The power spectrum method agrees only within \( \sim 15% \). The traps are also uniform in the \( x \) and \( y \) directions, with a percent difference of 2.7% between \( \kappa_x \) and \( \kappa_y \) as determined by the equipartition method.
Fig. 3. Stiffness Calibration. (A) Geometry of a viscous drag calibration: Averaged unfiltered (grey trace) and low pass filtered (black trace, cut-off=25 Hz) QPD digitized at 1 kHz signal for five uniform velocity (V=0.23 µm/msec) stage movements. 50 to 100 milliseconds of data are averaged from the region of the filtered signal corresponding to the displaced bead (1) and from the region corresponding to the unforced bead (2). These are subtracted to yield the net displacement. (B) Force as a function of displacement from a viscous drag calibration for two laser powers: 15 mW (■) and 44 mW (●). (C) Example power spectrum (grey trace) with best fit Lorentzian (black trace). The corner frequency is 825 Hz, which gives stiffness $\kappa = 4.7 \times 10^{-2}$ pN/nm. (D) Trap stiffness as a function of laser power for all three calibration methods: viscous drag (■), equipartition theorem (●), and power spectral analysis (△). The best fit line of all of the points gives a slope = 7 $\times$ 10$^{-4}$ pN/nm/mW.

C. Characterization of AOD Performance

As the acoustic wave frequency within each AOD deviates from the center frequency, and thus the Bragg condition, the diffraction efficiency decreases, causing a decrease in laser power entering the microscope. The effective steering range of the two axis acousto-optic deflector system is the distance from center frequency that maintains 95% of the center frequency power. AOD steering range was characterized by measuring relative laser power across the field of view using the 4-quadrant sum signal from the QPD amplifiers.

A dramatic power fall-off occurs at ±4–6 MHz from the center frequency, thus the Bragg condition, the diffraction efficiency decreases, causing a decrease in laser power entering the microscope. The effective steering range of the two axis acousto-optic deflector system is the distance from center frequency that maintains 95% of the center frequency power. AOD steering range was characterized by measuring relative laser power across the field of view using the 4-quadrant sum signal from the QPD amplifiers.

IV. DISCUSSION

The optical tweezers design presented here has broad capabilities for studying biological systems. Importantly, this design integrates the most advanced features from previously described optical tweezer designs into a single instrument, and these features are incorporated into the upright microscope without compromising any imaging modalities. The alignment to the Wollaston prisms and the repositioning of other DIC optics make top-notch DIC possible while optically trapping objects. Epi-fluorescence can also be performed concurrently with DIC and optical trapping; indeed, the fluorescence filter cubes can be changed without disrupting the laser, allowing for tweezer...
The dual-trap setup allows the researcher to manipulate multiple objects simultaneously—with the AODs used for time-sharing, three or more traps are possible. The BFP interferometry system allows the optical traps to serve as high-resolution force transducers, and has two primary advantages over imaging techniques: (1) bead position within the trap can be measured with no knowledge of absolute trap position, allowing the user to make reliable bead position measurements at any trap location without repositioning the QPDs, and (2) changes in the image background (drift, stage movements, etc.) do not interfere with measurements. Agreement between the three methods of stiffness calibration allow a high level of confidence in the absolute measurement ability of the system.

Acousto-optic trap steering allows the user to control trap position easily. Most importantly, the AOD trap maintains comparable detection capabilities to a non-steerable trap, despite the minor power decreases observed within the AOD steering range.

Integrating the QPD signal with the AOD control program allows force-clamping or position-clamping of trapped objects. The QPD system detects a minute displacement of the object, and the AODs displace the trap center to maintain constant position or force. As an example, force-clamping and position-clamping ability has been crucial for studying motor proteins.

In summary, the present optical tweezers design uses advanced technologies (AODs, QPDs) to create a flexible, sensitive instrument that functions alongside any microscopy technique. The sub-nm resolution of the BFP interferometry detection system meets the demands of precise micromechanical experiments.

Our laboratory currently employs the optical tweezers to study (1) mitotic motor proteins, and (2) microtubule (MT) polymerization forces. In (1), the optical tweezers are used to bring a MT into contact with an isolated mitotic chromosome, using glass beads as handles on the MT. The motor proteins which localize to chromosomes during mitosis bind to and act upon the MT, and the QPDs track changes in the motion of the trapped beads. In (2), dynamic MTs linked to trapped beads are polymerized against barriers. By monitoring or controlling the trapping force, we study the effect of force on polymerization at the MT tip. Fig. 6 shows images taken during these experiments.

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