

Two-photon fluorescence diagnostics of femtosecond laser tweezers

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We show how two-photon fluorescence signal can be used as an effective detection scheme for trapping particles of any size in comparison to methods using back-scattered light. Development of such a diagnostic scheme allows us a direct observation of trapping a single nanoparticle, which shows new directions to spectroscopy at the single-molecule level in solution.

Keywords: Back scattered light, femtosecond laser pulses, optical tweezers, sub-diffraction scale trapping, two-photon fluorescence.

Introduction

LASER-aided single-beam gradient optical traps or laser tweezers^{1,2} have revolutionized a vast field of research employing holding and manipulating particles³, say, for example, in force calibration of molecular motors inside a live cell⁴. The gradient force offered by a laser beam tightly focused by a high numerical aperture microscope objective leads to stable trap of particles with refractive indices higher than the surroundings.

Although events of trapping are directly observed via video imaging, it is obsolete for capturing the tweezing events of tiny particles that cannot be optically resolved using wide-field illumination. Use of other far-field microscopic techniques, such as differential interference contrast or dark-field fluorescence microscopy does not improve the situation as the resolution for all such techniques is governed by the diffraction limit. In practice, therefore, the smaller sub-diffraction limited-size particle is tethered to a larger particle of a few micrometres. Trapping forces for such a system are then calibrated based on the force exerted on the larger particle. This is a common practice for observing and studying forces in the piconewton order exerted by single biomolecules⁵, the sizes of which are in the sub-diffraction regime. The motivation of the present work lies in an attempt to observe directly the trapping of sub-diffraction regime particles that cannot be monitored by conventional bright field illumination.

A commonly employed approach to directly observe trapping of particles is to record the scattered light from the trapped object⁶. However, such scattered light is dependent on the size of the trapped particle and is accompanied with a large background; as the particle size reduces, signal-to-noise ratios can become overwhelmingly small. In fact, any detection scheme that directly depends on the first-order of the illumination field will be fraught with signal-to-noise problems. An innovative solution to the problem could be the use of such a detection scheme that has a nonlinear dependence on the illuminating field. The simplest way to induce nonlinearity with minimum perturbation under existing experimental conditions is to simply replace a continuous wave laser with a femtosecond laser. Quite a few instances of using femtosecond laser trapping now exist in the literature^{7–9}. In this work, we make a comparison between the back-scattering and two-photon fluorescence, and demonstrate that such a nonlinear signal can be an effective and fruitful diagnostic tool for laser tweezers, in particular for the trapping of tiny Rayleigh particles.

Experimental set-up

The schematic of the experimental set-up is shown in Figure 1. Our optical tweezer consists of a completely home-made bench-top inverted microscope with quite a number of useful features put together^{10,11}. The ~150 fs (obtained by auto-correlation technique) pulsed excitation from a mode-locked titanium-sapphire laser (MIRA 900F pumped by Verdi 5, Coherent Inc.) was used to simultaneously trap and induce two-photon absorption from polystyrene beads coated with fluorescent dyes (Molecular Probes Inc.) randomly floating in water. Before sending the laser beam (shown as thinner solid arrow in red, Figure 1) to the microscope objective (UPLSAPO 1.4 NA 100XO, Olympus Inc.), it was expanded using a beam expander (to fill the back-aperture of the trapping objective) and passed through a telescopic arrangement along with two steering mirrors. This allows us to manipulate the trapped object (not presented in this article). The back reflection is shown as short-dashed arrow in red, and the fluorescence is shown as long-dashed arrow in green (Figure 1). A lamp was used for bright-field illumination

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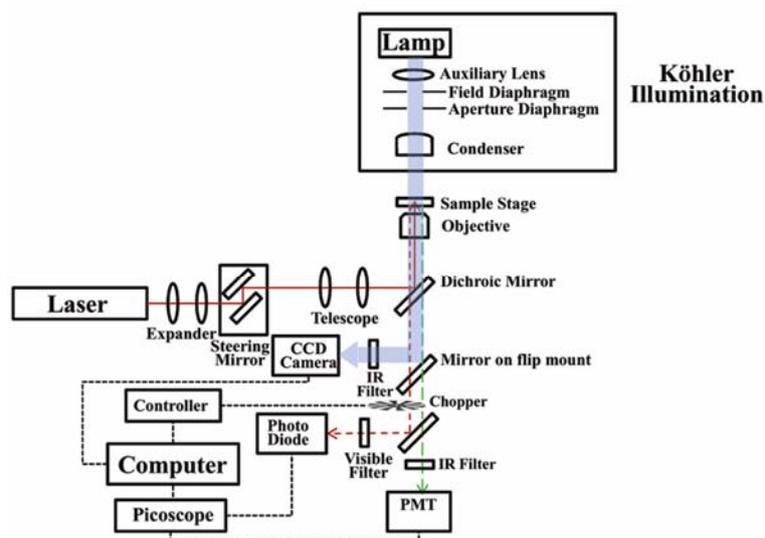


Figure 1. Experimental set-up.

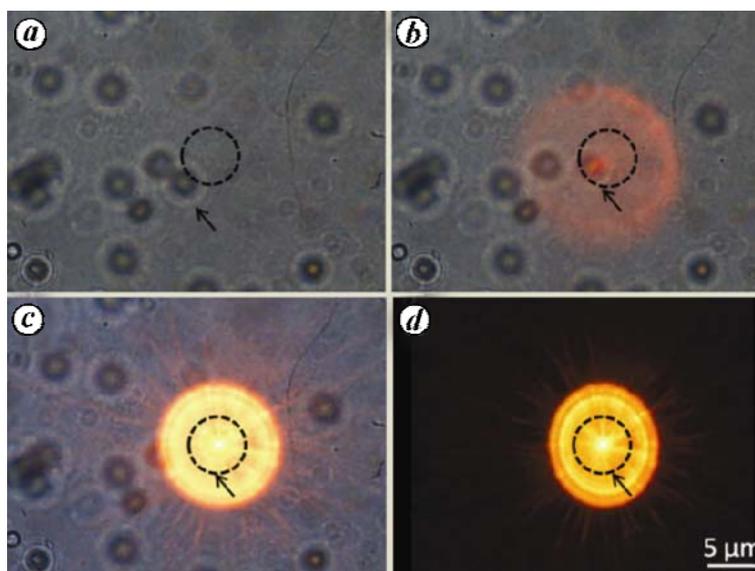


Figure 2. Observing the trapping of 4.1 μm -sized beads.

(shown as wider solid arrow in light blue, Figure 1) in a Köhler illumination scheme for uniform sample illumination. A mirror, mounted on a flip mount, sends the white light (along with the fluorescence) to a CCD camera (350 K pixels, e-Mark Inc.). An infrared (IR) filter was used in front of the camera just to avoid any back-reflection. When this mirror is flipped (turning-off the lamp), a dichroic mirror separates the back-scattered light from the fluorescence; the back-scattered light was collected by a silicon-amplified photo-diode (PDA100A-EC, Thorlabs Inc.) with large detection area (10 mm \times 10 mm) and the fluorescence was collected by a photo-multiplier tube (PMT). The large area photo-detection ensures collection of more number of photons. An IR filter was kept

in front of the PMT and a visible filter in front of the photo-diode to ensure sensitive optical detection at the desired wavelengths. The electrical cable connections are shown as dotted lines in black (Figure 1). Both the photo-diode and PMT were connected to an automotive oscilloscope or 'pico-scope' (Pico Technology Ltd). The signal was collected using a rotating-disk optical chopper (with 30 slot wheel) run by a tunable frequency driver (MC1000A, Thorlabs Inc.) and operating at 800 Hz. The pico-scope was triggered by the chopper; the peak-to-peak data collection enhances the signal-to-noise and allows detection of very small signal at much low average laser power (below 10 mW). The pico-scope and the camera were connected to a single personal computer where all

the video images and picoscope traces (using LABView program) were acquired. Thus in a single robust set-up, we were able to access various detection schemes of trapping with very high precision and sensitivity.

Results and discussion

Figure 2 shows the snapshots of trapping of a 4.1 μm diameter polystyrene bead. The figure depicts the gradual movement of a bead (indicated by a black arrow) towards the trap centre (shown as a black dashed circle). The last panel (Figure 2*d*) shows that the trapping is observable even when the bright-field illumination is absent, as also reported by others⁷.

Figure 3*a* and *b* shows the back-scattered (black curve) and fluorescence (red curve) signals for trapping of the beads having diameters 4.1 and 1 μm respectively. A number of significant points can be noted from the curves; first, the fluorescence signal exactly follows the back-scattered one, as expected. Under the experimental conditions the bigger particle leads to more stable trap than the smaller, which is due to the more random Brownian motion of the latter. However, the back-scatter signal is noisy (for the smaller bead) and always has a residual background signal arising from reflection from various interfaces, e.g. objective/air, immersion-oil/sample, etc., which is always present even when the particle is not trapped. In contrast, the two-photon fluorescence has better signal-to-noise ratio and has an almost zero-signal baseline. The presence of large background for the back-scattered signal reduces the signal-to-noise ratio for low signal in the case of smaller particles. Most importantly, for the smaller bead, the back-scattered signal shows additional spikes between two trapping events. This arises due to the scattering from out-of-focus beads (or any contamination) floating in the double-cone-shaped optical beam path across the focus, which tends to contribute more for even smaller particles. This is altogether absent in fluorescence signal because two-photon fluorescence is spatially confined only in the tiny focal volume¹². Also, the fluorescence signal can sense the arrival of another incoming bead to an already trapped bead (shown as blue arrow in Figure 3*b*), which the back-scattered signal cannot. It is important to note here that, though such arrival of an incoming bead may result in the replacement of the trapped bead; it may as well not result in such a replacement and the originally trapped particle remains in place. Nevertheless, this indicates the sensitivity of such a detection scheme and any fluorescence signal detected over time corresponds to a stable trap.

Now, under the assumption that the net fluorescence from the 4.1 and 1 μm beads is due to the contribution of all the fluorophores on the surface of the beads, we expect a ratio of fluorescence signal of the two beads as $\sim 17 : 1$. However, this is not the case for the experimental trapping events, as shown in Figure 3*c*. This result can be

arrived at as follows: the time-averaged background signal for both back-scattering and two-photon fluorescence is 50 counts. When the background signal is subtracted from the total signal (3697.202 counts for backscattering signal and 185.5572, 228.0703 and 211.3613 counts for two-photon fluorescence signal), then we get the absolute signal for backscattering and two-photon fluorescence. Now dividing the absolute values in each case we get the ratios to be 26.905, 20.48 and 22.60,

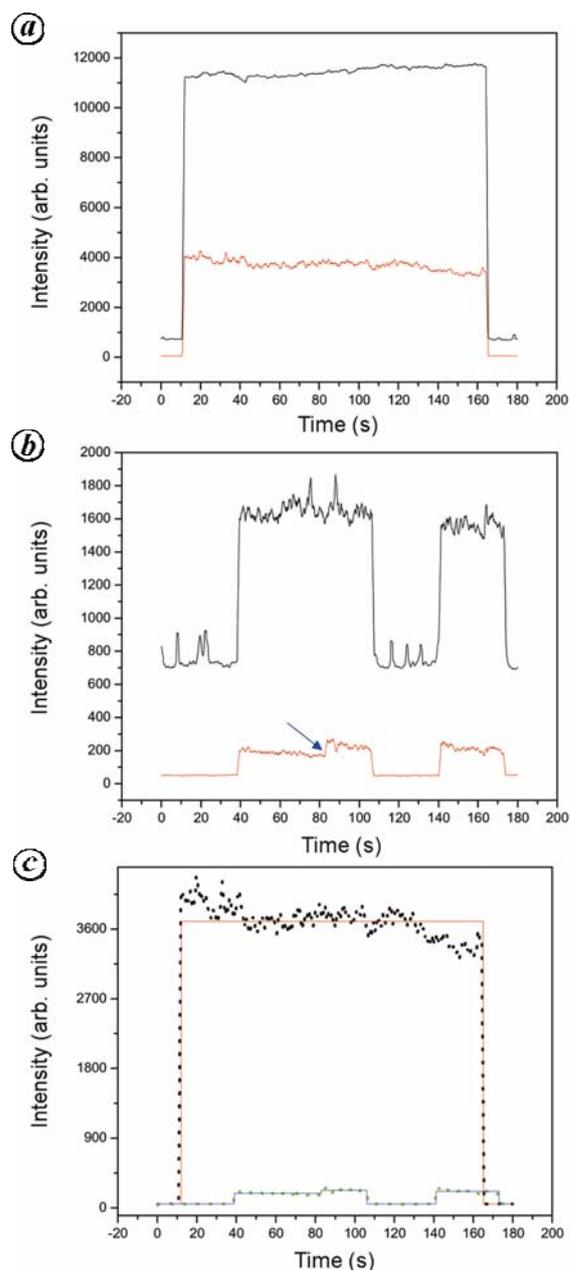


Figure 3. *a*, Trapping of 4.1 μm diameter bead showing back-scattered (black curve) and fluorescence (red curve) signals. *b*, Trapping of 1 μm diameter beads showing back-scattered (black curve) and fluorescence (red curve) signals. Blue arrow indicates arrival of another incoming bead to an already-trapped bead (see text for details). *c*, Relative two-photon fluorescence signal of trapped 4.1 and 1 μm beads (shown as black and green dots respectively) along with their time-averaged intensities (shown as red and blue lines respectively).

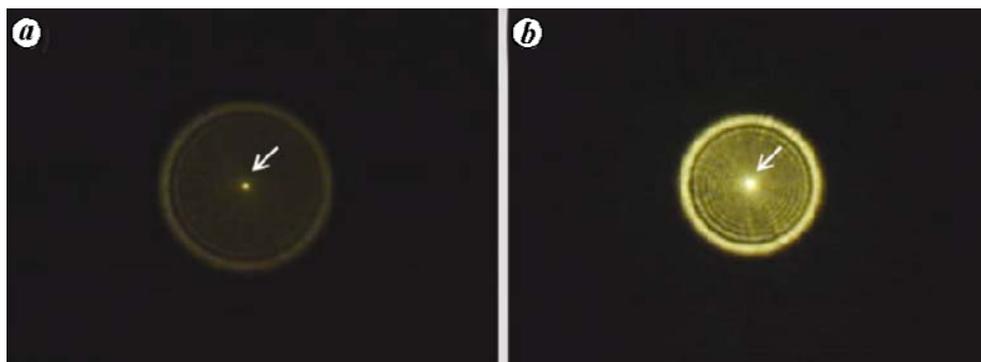


Figure 4. Snapshots of trapping and subsequent aggregation of 0.1 μm -sized beads at the focus (indicated by the arrow).

which are rounded off to 27, 20 and 23. This is due to the fact that the fluorophores not only coat the surface, but also penetrate the surface, and they do so at different depths for different beads. Furthermore, it is important to note that our assumption of all fluorophores contributing to the net fluorescence is not quite valid as the contribution only comes from the interaction at the focal volume. Overall, the two-photon fluorescence-based detection proves to be advantageous.

We also observed the trapping and aggregation of fluorescent beads with 100 nm diameter using video microscopy, as shown in Figure 4. Since the two-photon fluorescence is selective only to the trapped particles and is self-confocal, constraint on having small area detector is nonexistent. Thus, large-area position-sensitive detectors like the CCD array can also be used to visualize the sub-diffraction-sized particles. Such particles can also tend to aggregate in the trapping zone with time and this technique can also visualize the real-time dynamics of the aggregation of such particles. Further extension of this method is being pursued in the authors' laboratory to monitor the trapping of even smaller particles, not resolvable under bright-field illumination. Preliminary indication of our efforts in trapping 10–20 nm-sized q-dot sample (Q21031 MP, Invitrogen Inc.) has already been presented elsewhere¹³.

One can also think of using the two-photon fluorescence as a force calibration method in a laser trap. The obvious question is how to account for the recoil force due to absorption of the photons arriving in one direction with omni-directional emission¹⁴. However, since the probability of two-photon absorption is very low (e.g. only one in a million of the shining photons gets absorbed¹⁵), we can completely neglect such recoil effects. Thus, this is better than one-photon microscopic technique, which suffers from recoil effects as well as the presence of background fluorescence.

Conclusion

To summarize, we have used the two-photon fluorescence as a nonlinear detection scheme to directly detect and

visualize trapping events, even at sub-diffraction length scales. The advantages of two-photon fluorescence over backscattering for detection of trapping events have been demonstrated.

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