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Evidence for Resonance Optical Trapping of Individual Fluorophore-Labeled Antibodies Using Single Molecule Fluorescence Spectroscopy

Haitao Li,[†] Dejian Zhou,[†] Helena Browne,[‡] and David Klenerman*,[†]

Contribution from the Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge, CB2 1EW, UK, and Division of Virology, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QP, UK

Received October 13, 2005; E-mail: dk10012@cam.ac.uk

Abstract: We report single molecule fluorescence studies of the diffusion of individual multiple fluorophore-labeled antibodies in solution, which show that a trapping potential of about 3.6 k_BT can be obtained at laser powers below 1 mW with resonant excitation. Individual antibodies can be trapped for up to 140 ms, and bound antibodies can also be used to trap a single virion for up to 1 s. Selective resonance trapping to sort and manipulate fluorophore-labeled biomolecules and complexes may be possible.

Introduction

The interaction of laser radiation with matter has been used trap atoms and particles, and this effect is now widely used in physics and biology.[1−5] A single focused laser beam can be used as an optical tweezer to trap and manipulate a dielectric particle toward the center axis of the focused laser without mechanical contact, producing a trapping potential of about 8−10 k_BT.[6−9] This has been exploited to manipulate viruses and bacteria,[6,7,10] measure the forces exerted by molecular motors,[11] manipulate cell organelles,[12,13] probe the cell surface by scanning small microspheres,[14] and recently separate cells in a microfluidic cell sorter.[15] All these applications are based on using a wavelength not absorbed by the particle so that there is no damage to biological samples. Typically the laser powers used are of the order of 10−1000 mW. Since the nonresonant trapping force is proportional to the particle volume, it decreases significantly for a particle of the size of tens of nanometers and hence very high laser powers are needed to increase the trapping force. Hence the smallest reported trapped objects in solution, to date, are gold particles, down to 18 nm in diameter.[16] An alternative is to use a laser close to an optical resonance of the particle. Trapping and cooling has been demonstrated in the gas phase for sodium atoms by using a single Gaussian laser beam close to resonance.[17] It has also been suggested by Ashkin[18] that tuning closer to an optical absorption of the particle may lead to resonance enhancement in solution. Resonance trapping requires lower powers and depends on the properties of the electronic states being excited and not the particle volume. It also offers selectivity in trapping. Resonant trapping has been predicted theoretically, with enhancements of between 50 and 10 000, depending on the electronic states of the particle being excited.[19,20]

The recent development on single molecule fluorescence detection provides sufficient sensitivity to detect individual fluorescent molecules under conditions of resonance excitation. However, since optimized filters and dichroics are needed in these experiments to obtain sufficient sensitivity, tuning experiments are very difficult to perform. Previous work showed some evidence for biased diffusion of single fluorophore labeled biomolecules of about 10 nm in diameter, when the fluorophore is excited at resonance, indicating that the force produced by the light was too small for trapping.[21,22] However at 200 mW laser power it was possible to trap long lambda phage DNA, 48 kilobases in length, with about 2400 YOYO dyes attached and manipulate the DNA.[21] Recently an alternative method, based on a novel anti-Brownian electrophoretic trap has been

[†] Department of Chemistry.
[‡] Department of Pathology.

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developed to trap and manipulate beads of 20 nm in diameter in two dimensions.[23] This can be potentially combined with single molecule detection to trap molecules; however this trapping would not be based on the direct interaction of resonance photons with the fluorophores attached to the biomolecule.

Based on the previous work described above, we reasoned that if we had multiple fluorophores attached to a nanometer size particle excited at resonance, sufficient force could be generated to trap the particle. In this paper, we describe experiments on individual multiple fluorophore-labeled LP2-IgG antibodies, $\sim 4.0 \times 8.5 \times 14.5$ nm^3 in size, studied in free solution using single molecule fluorescence. We then extended these experiments to demonstrate trapping of individual Herpes Simplex Virions (HSV), 200 nm in diameter,[24] using surface-bound fluorophore-labeled antibodies.

Experimental Section

Materials: The production of purified HSV virions and LP2-IgG antibodies has been described previously.[25] An Alexa-647 protein labeling kit was purchased from Invitrogen (Paisley, UK). LP2-IgG was labeled following the procedure described with the labeling kit, and the level of labeling was determined by measuring the absorbance at 280 and 650 nm. Two batches of the LP2-IgG antibody were labeled, with the average labeling of 14 and 2.5 fluorophores per antibody, respectively. All other chemicals and reagents used were purchased from Sigmal-Adrich (Dorset, UK). Phosphate buffered saline (PBS, 10 mM phosphate, 150 mM NaCl, 2 mM NaN_3, pH 7.4) was prepared with MilliQ water (resistance > 18 MΩ cm^{-1}). The virus sample was sonicated for 3 min before dilution into PBS for measurement in order to avoid any virus aggregation. The virus concentration in terms of virus particles/mL was determined by electron microscopy using an internal standard of latex spheres.[26] This analysis also confirmed that the sample only contained individual virions.

Methods and Experimental Setup. The apparatus used to achieve single molecule fluorescence detection has also been described in a recent publication.[27] Briefly, a laser beam (633 nm model 25LHP151 He−Ne laser, Melles Griot) was directed through a dichroic mirror and oil immersion objective (Apochromat 60× , NA 1.40, Nikon) and was focused 5 μm into a 0.5 mL sample solution supported in a Lab-TeK chambered coverglass (Scientific Laboratory Suppliers Ltd, UK). The size of the beam diameter on the back aperture of the objective was measured to be 3 mm. Fluorescence was collected by the same objective and imaged onto a 50 μm pinhole (Melles Griot) to reject out of focus fluorescence and other background. Red fluorescence was filtered by long-pass and band-pass filters (565ALP and 695AF55, Omega Optical Filters) before being focused onto an APD (SPCM AQR-141, EG&G, Canada). The dark count rate for the APD was below 100 counts per second. Output from the APD was coupled to a PC implemented multichannel scalar card (MCS-Plus, EG&G, Canada). The power of the laser entering the microscope was adjusted using Neutral Density Filters (Thorlab, UK) from 10 μW to 2 mW to perform the trapping experiments (70% of this incident power reaches the sample). Time bins of 0.1−10 ms were used for the experiments. Shorter time bins, 0.1 ms, were used for accurate measurement of the untrapped burst times at low laser powers and longer bins to measure the trapped burst times.

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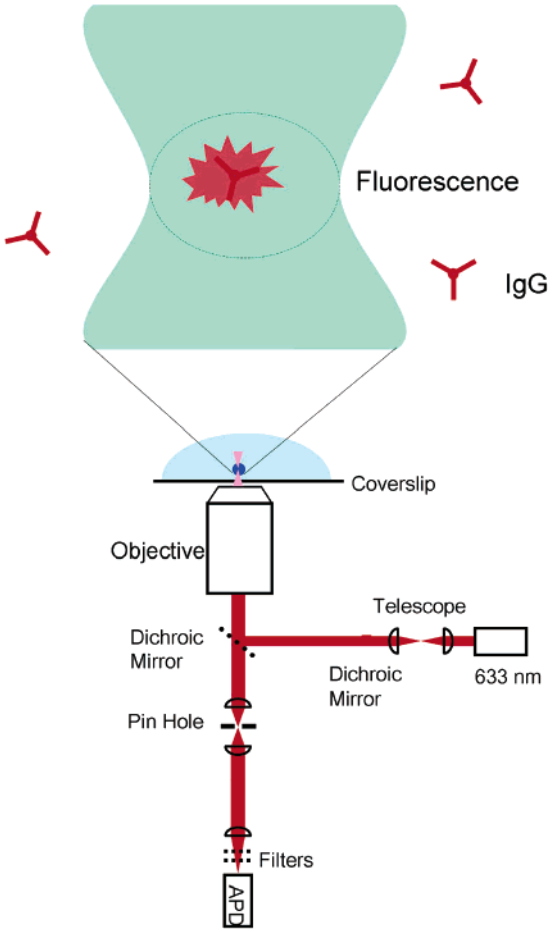

Figure 1. Schematic of apparatus used for resonance trapping (APD, avalanche photodiode).

To determine the mean diffusion time of antibody crossing the probe volume, a 5 nM Alexa Fluor 647 labeled IgG sample was prepared in PBS buffer on a BSA-treated cover glass surface. The beam waist of the laser had been previously determined,[27] and fluorescence correlation spectroscopy was performed to measure the mean diffusion time.[27] These experiments were carried on at room temperature (~20 °C) with the excitation laser power adjusted to 10 μW. A bin time of 20 μs was used, and the total time for one experiment was 3 min.

Trapping of LP2-IgG was performed at a 50 pM concentration. The trapping of virions was performed using 10 pM HSV-1 with 3 nM LP2-IgG. Since the background in both experiments increases with laser power, we used a threshold value 10 times higher than the mean background for all experiments. This criterion was chosen since it provided good discrimination from background without significant reduction in the number of detected events. A trapped antibody was only counted if the fluorescence burst was longer than 2 ms, 4 times longer than free diffusion. Only those fluorescence bursts lasting longer than 10 ms were counted as trapped viruses, 3.3 times longer than the free diffusion time. This slight difference in criterion is due to the discrete time bins available on the multichannel scalar board. For both experiments the mean value of the trapping time was obtained from a 90 min experiment.

Results and Discussion

A schematic of our experiments is shown in Figure 1, where we used an inverted microscope with a focused He−Ne laser (633 nm) using a ×60 1.4 NA oil immersion objective. This instrument has been well characterized for single molecule measurements.[27] In these experiments we can analyze individual

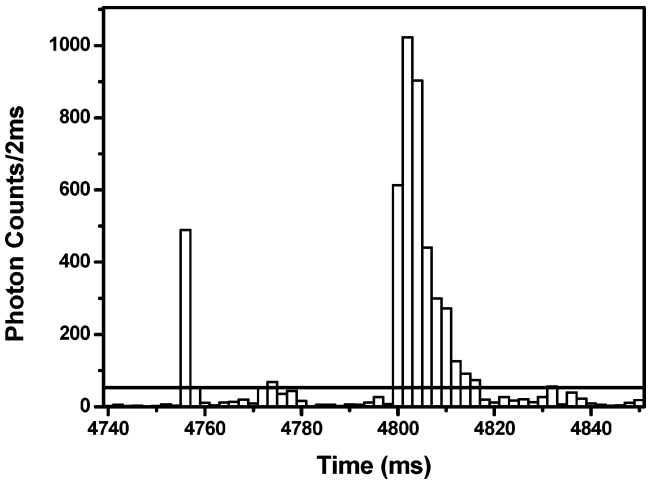

Figure 2. Trapping of individual LP2-IgG labeled with on average 14 Alexa-647 fluorophores using a 633 nm He−Ne laser at 200 μW power. The threshold level used to distinguish events from background is marked as a parallel line. There is an example of a trapped trajectory after 4800 ms. The fluorescence burst time is the time before the fluorescence decreases below the threshold level of 10 times the background. The burst time is 18 ms in this example. There is a burst of an untrapped antibody at 4755 ms.

molecules as they diffuse across the laser probe volume, by detecting the emission of fluorescence using an avalanche photodiode. Using fluorescence autocorrelation the beam waist diameter for the 633 nm laser beam was previously measured as 650 nm, close to diffraction limited spots.[27] We first performed experiments on the individual LP2-IgG antibodies labeled with on average 14 Alexa-647 fluorophores which we excited with 633 nm laser light (the absorption peak of Alexa-647 is at 650 nm, and maximum emission is at 668 nm). We also performed controls using the same antibody but labeled with on average 2.5 Alexa-647 flurophores.

Figure 2 shows some typical fluorescence intensity burst trajectories. One antibody spent 18 ms in the laser beam until the fluorescence was reduced, presumably due to photobleaching, down to the background level while another spent only 2 ms. The burst times showed a continuous distribution, as shown in Figure 3, and this distribution depended on the laser power used and number of fluorophores.

It is clear that the burst time histogram for the antibodies showed a power dependence. To analyze the antibody data in more detail we used the following method. We defined the burst time as the duration of the fluorescence burst when it is above threshold. We used the arbitrary criterion that apparent trapping corresponds to burst time at least 4 times longer than the mean diffusion time. We use the word apparent since this criterion may also include bursts that are long due to multiple crossing of the laser excited volume. The mean apparent trapping time is the average of all burst times that are defined to be trapped by this criterion. The apparent trapping efficiency is the fraction of all bursts that are defined to be trapped by this criterion.

Even for free diffusion there will be some events that are identified as trapped using this criterion, due to multiple recrossings of the laser probe volume. To correct for this we measured the burst histogram for the antibody with 2.5 fluorophores at as low laser power as possible, 10 μW, where we assumed that trapping was negligible, and determined the apparent trapping efficiency to be 2.0% using the above criterion. This value was subtracted from all measured apparent trapping efficiencies at higher laser powers to determine the corrected trapping efficiency. This corresponds to the fraction of molecules that are detected to enter the laser focus and are trapped. We also used these data at low power to determine the corrected trapping efficiency when different trapping criteria were used. We note that if there is any trapping at 10 μW laser power then the corrected trapping efficiency will be underestimated.

For the antibody labeled with on average 2.5 fluorophores, within experimental error the apparent trapping efficiency was the same as the percentage of multiple recrossings at both 80 μW and 750 μW. This indicates negligible trapping. There are no extended bursts. In contrast, for the same antibody with an average of 14 fluorophores the corrected trapping efficiency was 3% at 80 μW and increased to 8% at 750 μW. The mean apparent trapping time was 10 ms at 80 μW and increased to 18.6 ms at 750 μW (see Table 1 for dependence of corrected trapping efficiency and mean apparent trapping time on criterion used to identify trapped bursts). There are only a few long bursts at 80 μW, but at 750 μW we observed many long bursts up to 140 ms, as shown in the insets in Figure 3. Note that the mean burst time for the antibody, measured at low laser power, is 0.5 ms. This is in good agreement with that predicted by simple Brownian diffusion theory for the meantime taken for the antibody to diffuse across the laser beam focus. The extended bursts up to 140 ms that we have measured are about 300 times longer than the mean diffusion time.

The antibodies with 14 and 2.5 fluorophores would be expected to have the same distribution of burst times, in the absence of any resonance trapping, since the fluorophores make a negligible increase to the size of the antibody and so would diffuse at the same rate. Furthermore, any nonresonant effect would be the same on both antibodies. At each laser power, the data for the antibody with 2.5 fluorophores and 14 fluorophores were also taken with the same thresholds, 10 times higher than the background, so this could not contribute to any difference. However, we have clearly observed different distributions of burst times, significantly longer bursts, increased trapping efficiency and increased mean apparent trapping time when 14 fluorophores were on the antibody, and negligible trapping with 2.5 fluorophores present.

The laser power dependence of the mean apparent trapping time of the antibodies with 14 fluorophores is shown in Figure 4A. Instead of a linear dependence with laser power as expected for nonresonant trapping, we observed a nonlinear dependence with a maximum at 750 μW, due to excitation saturation of the fluorophore, and then a decrease due to inactive fluorophores generated by additional photoinduced pathways. We observed that the mean brightness of an Alexa-647 fluorophore decreased significantly with high laser power and that the mean apparent trapping time also decreased at high laser powers, supporting the idea of additional photoinduced pathways at high powers (see Figure 4B). Experiments using glucose oxidase[28] to reduce photobleaching produced no detectable effect on the power dependence of the mean brightness (data not shown). This suggests that photobleaching is not the cause of the decrease in brightness so that other photoinduced pathways must be

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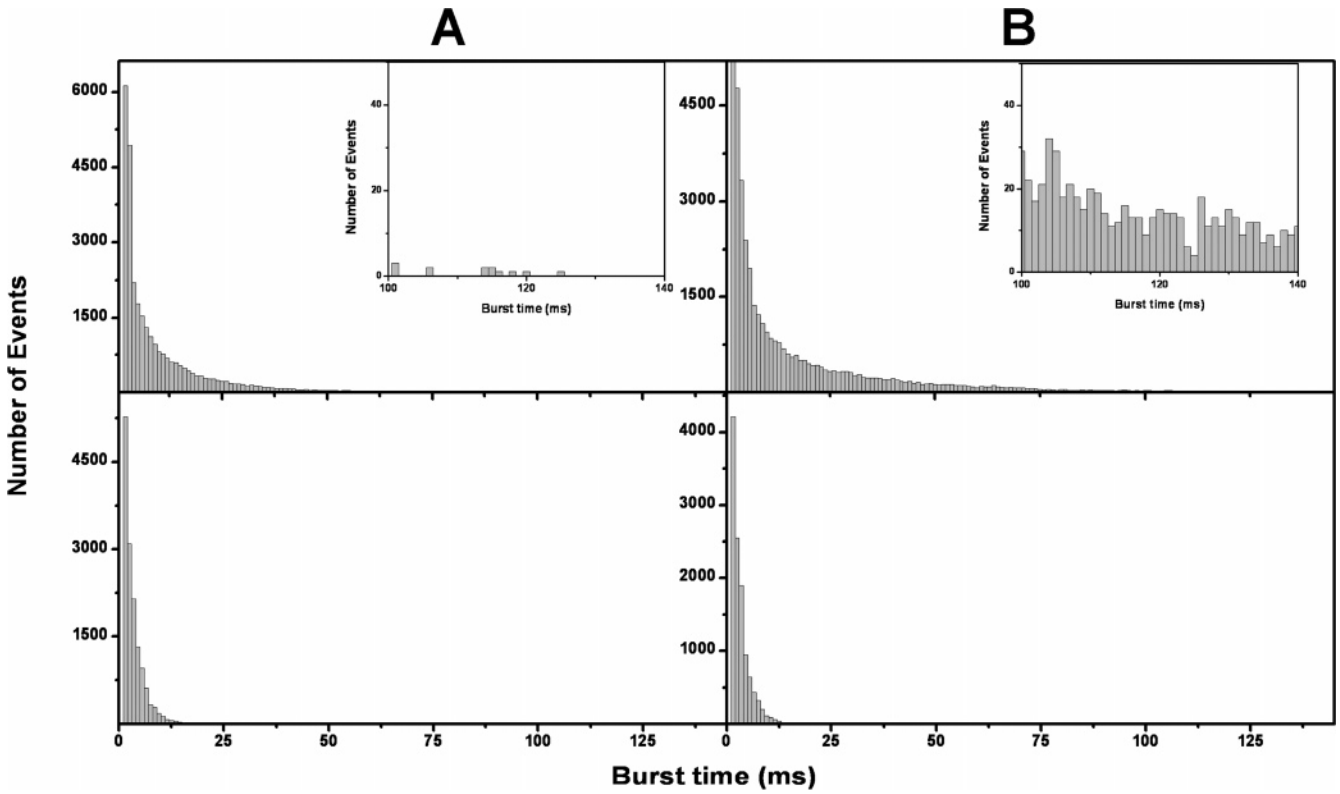

Figure 3. Burst time histograms at 80 μW, panels A, and 750 μW, panels B. The top panels are for the antibody with 14 fluorophores. The mean apparent trapping time was 10 ms at 80 μW and increased to 18.6 ms at 750 μW. Approximately the same number of events were observed in both measurements, 40 481 and 42 319 events, respectively. The insets show longer burst times. The bottom panels are for the antibody with 2.5 fluorophores where there was negligible trapping at both laser powers. All data were taken with 2 ms time bins.

Table 1. Dependence of the Mean Apparent Trapping Time and Corrected Trapping Efficiency on the Burst Times Used to Define a Trapped Trajectory for IgG and HSV[a]

trapped burst time	≥1 ms	≥2 ms	≥3 ms	≥4 ms	≥5 ms	≥6 ms
mean apparent trapping time for IgG with 14 fluorophores	12.3 ms	18.6 ms	20.5 ms	24.5 ms	27 ms	29 ms
corrected trapping efficiency	10%	8%	7%	6.5%	5.7%	4.8%

trapped burst time	≥5 ms	≥10 ms	≥15 ms	≥20 ms	≥25 ms	≥30 ms
mean apparent trapping time for HSV	180 ms	248 ms	295 ms	352 ms	411 ms	491 ms
corrected trapping efficiency	15.7%	13.3%	11.2%	9.6%	7.8%	5.9%

[a] Bursts longer than this time were classified as trapped and used to calculate the trapping efficiency and mean apparent trapping time (see text for details). The IgG and HSV data were taken at 750 and 600 μW, respectively.

important, although the absence of an effect of glucose oxidase is not definitive. Photoisomerization is one such possible pathway as has been observed by Sauer and co-workers for Alexa-647 attached to DNA.[29]

We examined a large number of trapped burst trajectories (see Figure 5). We found there was no correlation between long duration bursts and intensity; this is probably due to variation in the number of fluorophores on different antibodies. Only about half of the long bursts had a tail due to photobleaching (Figure 5, top panels), and the rest had a sharper tail (Figure 5, bottom panels). This is because the molecules can leave the probe volume for two main reasons. First photobleaching of sufficient fluorophores will result in the force on the antibody reducing to a level where it can escape from the trap. Alternatively, there is a probability that the antibody can overcome the trapping potential before significant photobleaching occurs. Since we observed about equal amounts of both burst types, it suggests that photobleaching is not the dominant mechanism of removal from the trap.

One possibility is that a fraction of the antibodies are aggregates in solution, and it is these larger aggregates that we are trapping. However, using two color single molecule fluorescence, we have previously studied IgG, under identical solution conditions as those used here, and only observed a small amount of dimer and no higher oligomers.[25] Furthermore, the amount of the IgG dimers (2%) observed under these conditions is significantly lower than the fraction being trapped in these experiments. Another possibility is the trapping is due to the heating effect, which would also depend on the number of attached fluorophores. However the estimated temperature rise is only 0.01 K under the conditions of the experiment (see Supporting Information for details) and hence could not contribute to the observed trapping. Thus we conclude that we are indeed optically trapping individual IgG molecules.

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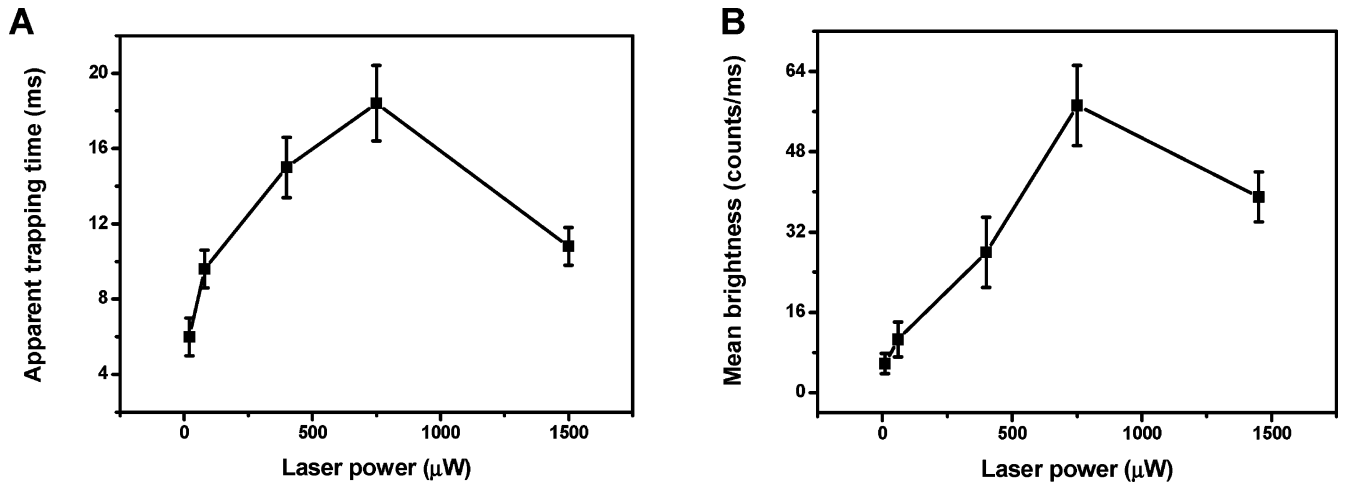

Figure 4. (A) Power dependence of mean apparent trapping time for the antibody with 14 fluorophores. (B) The dependence of the mean brightness of the single Alexa-647 fluorophore as a function of laser power.

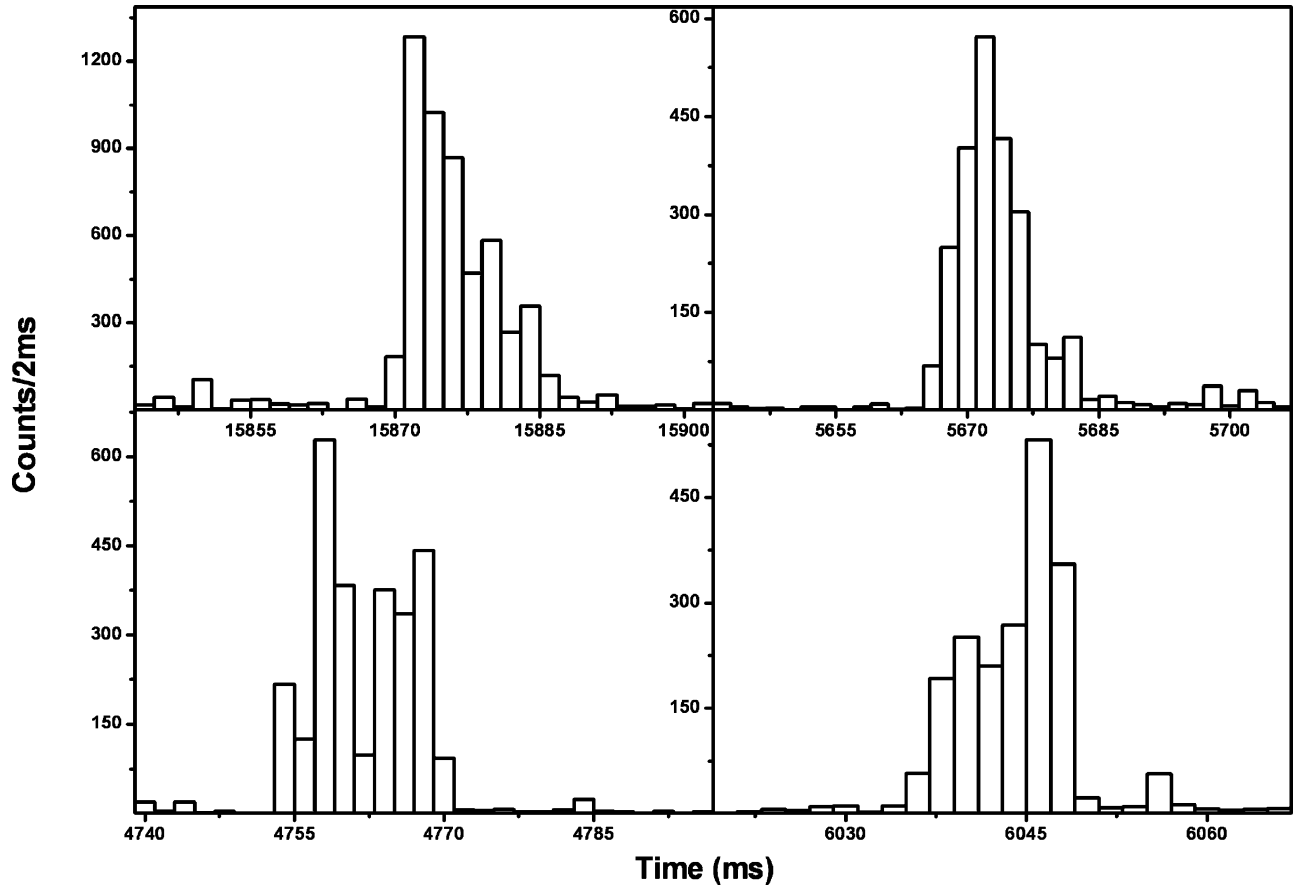

Figure 5. Examples of trapped burst trajectories for the antibody labeled with 14 Alexa-647 fluorophores.

In normal optical traps the particle is trapped for sufficiently long that analysis of the particle position or increasing viscous drag can be used to measure the trapping potential.[3] This is not possible in our experiments due to the short duration that the antibodies are trapped, so we used the mean apparent trapping time to estimate the trapping potential as has been used previously in a situation of a weak trap.[30] Following our previous work,[22] a particle randomly diffusing with thermal energy k_BT has a probability given by a Boltzmann factor, $\exp(U/k_BT)$, of diffusing out of a potential U caused by the focused laser beam. The time spent in the laser beam or trapping time, τ, is then given by

$$\tau = \tau_0 e^{U/k_BT}$$

If U is set to zero then the time spent in the laser beam is τ_0, the time taken for free untrapped diffusion across the laser focus. At 750 μW, the mean apparent trapping time for trapped antibodies with 14 fluorophores is 18.6 ms and the untrapped diffusion time, measured at low laser power, is 0.5 ms. This gives a ratio of τ/τ_o of 37 and hence an estimate of U of 3.6 k_BT or 0.25 k_BT per Alexa-647 fluorophore. Thus the trap

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produced here is weaker than the usual criteria of 8−10 k_BT but still significant, and further optimization of the fluorophore, the excitation wavelength, or number of fluorophores may increase the trapping potential. It is also noteworthy that the highest trapping efficiency we have observed, 8%, is comparable to the axial trapping efficiencies reported for microspheres.[31−32]

We then used the fluorophore-labeled antibodies to resonantly trap HSV, a large enveloped virus about 200 nm in diameter.[24] The virus envelope contains over a dozen different glycoproteins, and one of them, glycoprotein D, binds to a monoclonal antibody, LP2.[33] We labeled the purified LP2 IgG with Alexa-647 fluorophores, on average 14 per antibody. We selected a molar ratio of antibody to virus of 300 since we found that the number of surface-bound antibodies did not increase above 300. We observed three types of events. Background, low intensity burst due to antibodies alone diffusing through the probe volume, and two types of high intensity events, due to the antibody-bound virus diffusing across the probe volume. We defined a high intensity burst as a burst with a fluorescence count rate at least 10 times higher than the average count rate of an antibody alone (the main contributor to the background signal). The majority of the high intensity bursts showed a mean burst time of 3 ms, as expected for a particle of 200 nm diffusing across the probe volume. However, some intense bursts were significantly longer, up to 1 s (see Figure 6).

A representative burst is shown in Figure 7A. This shows an initial high fluorescence count that reduces with time until the fluorescence decreases below the threshold and the virion is no longer detectable. This fluorescence decay is exponential, and a decay of similar shape was observed for the antibody alone, supporting the assumption that both decays are due to photobleaching of the fluorophores. The dependence of the mean apparent trapping time of the long high intensity burst with laser power is shown in Figure 7B. The mean apparent trapping time increases nonlinearly with power, reaching a maximum at 600 μW, and then decreases significantly at 1 mW; this is similar to the power dependence observed with the labeled antibody alone.[34] Under maximum trapping conditions, about 13% of the HSV was trapped.

The antibody binding makes a negligible increase in the virus particle size, only 5%, but for the trapped HSV the addition of bound fluorophores leads to a significant increase in the burst time, from 3 ms to a mean apparent trapping time of 248 ms at 600 μW, a factor of 80. Using the same method as that used for the antibody, based on the average time the virus spends in the trap, gives an estimated trapping potential of 4.4 k_BT. This trapping potential is only 20% larger than a single antibody. An experiment with 10 and 300 antibodies bound to the HSV

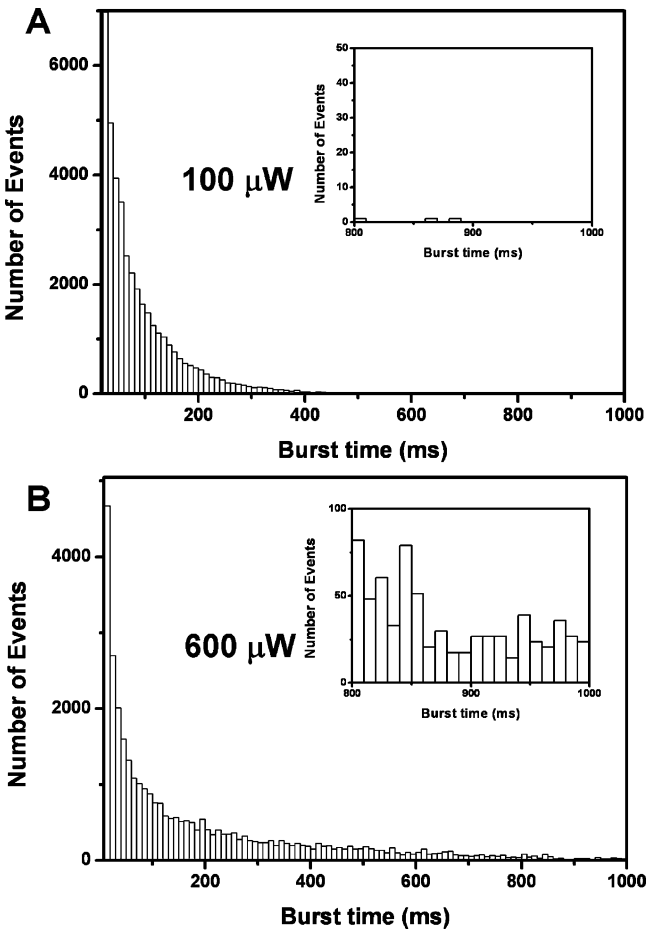

Figure 6. Burst time histograms for HSV bond with ~300 LP2-IgGs (each labeled with on average 14 Alexa-647 fluorophores per antibody) excited with a 633 nm laser beam at (A) 100 μW and (B) 600 μW. The mean apparent trapping time was 140 ms for panel A and 248 ms for panel B.

was also performed to check this result. The factor of 30 increase in the number of bound-antibodies only led to an increase in mean apparent trapping time of about a factor of 5 (data not shown). These results suggest that the force does not scale simply with the number of antibodies. This is possibly because the virion is a significant size compared to the width of the laser trap, so the forces exerted on the labeled antibodies on the virus surface may counter each other, leading to a significantly reduced overall trapping force. However more work is needed to explain this observation.

Optical trapping is based on the forces induced on a particle within a focused laser beam. The dissipative scattering force is due to the spontaneous scattering of photons and depends on the optical intensity and points in the direction of the incident light. The gradient force arises due to induced dipoles in the light electric field and points along the intensity gradient. To trap a particle, the gradient force must exceed the scattering force to generate a potential well that is deeper than the particle's kinetic energy. The effect of tuning close to resonance has been considered theoretically for a two-state system.[35] Close to resonance, the imaginary part of the polarizability is large and changes sign on passing through resonance. For trapping of a two-state system, it is necessary to tune the frequency below resonance because when the excitation is tuned above resonance

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(34) There is evidence that the average environment of the fluorophore changes on binding to the virus since the intensity of the fluorescence decreases by 50% on binding to HSV. The fluorescence signal increased linearly with the number of antibodies, indicating no interaction between fluorophores on different antibodies, and then reached a plateau at 300 antibodies per HSV virion. These observations suggest that fluorophores on antibodies bound to HSV may not necessarily undergo photophysical processes at the same rate as those on free antibodies and that this is one possible explanation for the steeper decline in apparent trapping time at higher laser powers observed for the virus. It is also a possible explanation of the difference in photobleaching times for the antibodies on the virus and the antibodies alone.
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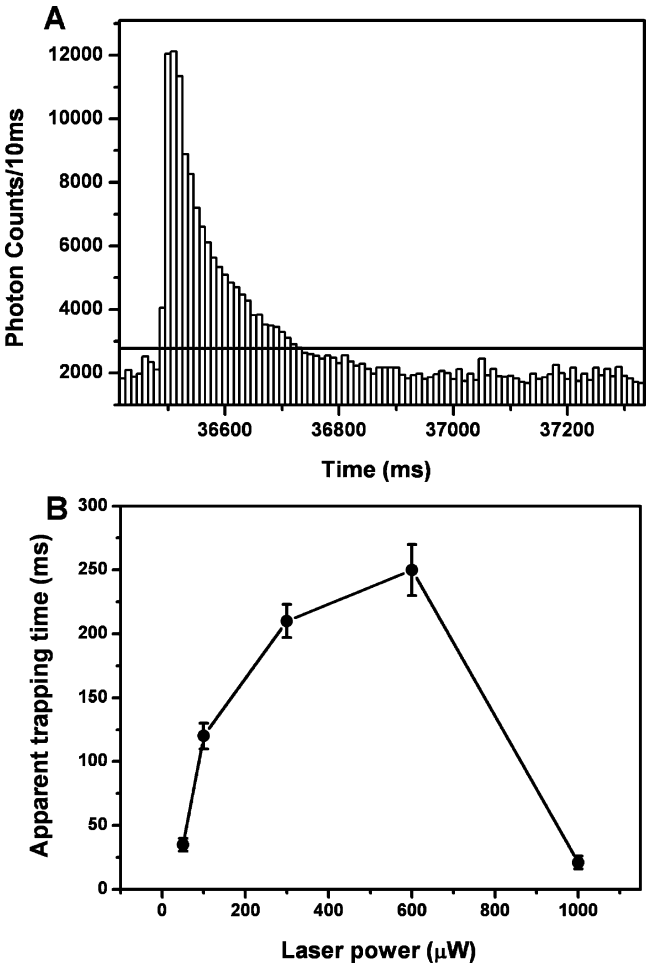

Figure 7. Trapping of HSV using Alexa-647-labeled LP2-IgG antibodies. (A) Example fluorescence burst of a trapped virus starting at 36.5 s and lasting for 240 ms before decreasing below the threshold level of 10 times the mean fluorescence count rate of an antibody alone. The laser power was 600 μW. (B) Power dependence of mean apparent trapping time of HSV at a ratio of antibody to virus of 300:1.

the force is positive and ejects the particle from the laser focus. However all systems which have shown biased diffusion or trapping have been much more complex and have been excited to the blue of the resonance.[21,22,36−37] This is also the case for this work where the excitation is blue-tuned from the maximum absorption peak of the Alexa-647 dye by 20 nm. It is therefore clear that these cannot be treated as a simple two-state model and that further work is required to understand the physical basis of the phenomenon. In particular it may be necessary to consider the interaction between multiple fluorophores on the same molecule.

Conclusions

Our work provides more evidence for resonance optical trapping of nanometer particles with an estimated trap potential of about 3.6 k_BT, and it should be stressed that this is not optimized. Although weaker than normal optical traps of 8−10 k_BT, the effect shown here is already sufficient for sorting applications in microfluidic devices where traps of only 4 ms duration are required.[15] In this case the use of channels smaller than the beam size could ensure that all molecules pass through the trap and can be sorted.[38] The work shown here indicates that resonance trapping of nanometer size objects may be possible opening up many intriguing possibilities. For example, since fluorescence is widely used to detect and mark specific biomolecules using fluorophore-labeled probes such as antibodies as used here, it appears that fluorescence detection could straightforwardly be combined with resonance optical trapping for selective sorting and subsequent analysis of a range of biological samples. For example, it may be possible to use fluorophore-labeled antibodies as handles to first identify and then selectively deflect down a channel specific larger objects, such as molecular complexes or viruses, using resonance excitation and hence sort complexes in a flow system. However further theoretical and experimental work is required to understand and increase the strength of the trap, and fluorophore photobleaching may be the ultimate limitation of this method.

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Supporting Information Available: Estimation of the temperature rise due to local heating. This material is available free of charge via the Internet at http://pubs.acs.org.

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