Combining Optical Trapping and Single-Molecule Fluorescence Spectroscopy: Enhanced Photobleaching of Fluorophores

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To obtain high-resolution information on position or conformation of a molecule and at the same time apply forces to it, one can combine optical trapping with single-molecule fluorescence microscopy. The technical challenge in such an experiment is to discriminate a minute fluorescence signal from the much larger background signals caused by the trap and the fluorescence excitation laser light. We show here that this is feasible even when the fluorophore is directly attached to the trapped particle, by using optimized optical filters. We found, however, that the photostability of the fluorophores we tested suffered from the presence of the additional laser light used for trapping. We found that bleaching rates increased linearly with both the intensity of the trapping laser and the intensity of the fluorescence excitation light. Photobleaching rates were unaffected by the presence or absence of oxygen, but were significantly diminished in the presence of antioxidants. Our results indicate that the enhanced photobleaching is caused by the absorption of a visible photon followed by the excited-state absorption of a near-infrared photon. The higher excited singlet states generated in this way readily form nonfluorescent dye cations. We found that different dyes suffer to a different extent from the excited-state absorption, with Cy3 being worst and tetramethylrhodamine least affected.

Introduction

Over the past decade single-molecule techniques, such as single-molecule fluorescence spectroscopy[1,2] and optical trapping or optical tweezers,[3−5] have become important and successful tools in biology, chemistry, and physics. Optical tweezers can be used to three-dimensionally trap micrometer-sized particles near the focus of a laser beam, using laser powers of up to 1 W.[3−5] In general, near-infrared lasers that are not resonant with electronic transitions of the molecules present in the sample are used in order to reduce photodamage caused by the high light intensities (up to 100 MW/cm^2).[6] In single-molecule fluorescence, individual fluorophores are excited with laser light and the resulting fluorescence is measured with a fluorescence microscope. In general, excitation intensities on the order of hundreds of W/cm^2 are applied, generating fluorescence intensities on the order fW (assuming a typical emission rate of 10000 photons per second). An important limitation of the organic dyes used in single-molecule fluorescence experiments is photodamage, which limits the total number of emitted photons typically to 10^5-10^6.[7] A well-known cause of photodamage is oxidation of the dye by singlet oxygen. Singlet oxygen can be formed by sensitization of ground-state triplet oxygen present in the sample by dye in the triplet state.[8] Under typical illumination conditions the triplet state of most dyes is substantially populated. This source of photodamage can be reduced substantially by decreasing the oxygen concentration using deoxygenation or oxygen scavengers such as the glucose oxidase/catalase system.[9] In fluorescence experiments using two-photon excitation, excited states are generated with high-intensity light (most often femto- or picosecond laser pulses with peak intensities on the order of 100 GW/cm^2)[10] of half the photon energy needed for excitation of the dye molecules. It has been shown that the photobleaching rate under these excitation conditions is proportional to the third power of the excitation intensity, indicating that it is a process caused by three photons.[11] This bleaching behavior was explained by a mechanism not involving triplet states, but higher excited singlet states. These states are populated by three-photon excitation of the fluorophores and couple, in polar solvents such as water, efficiently to ionized states involving a solvated electron and a nonfluorescent dye cation.[12] Schwille and co-workers[13] have shown that the efficiency of this photobleaching channel can be reduced substantially by addition of antioxidants such as ascorbic acid to the sample at concentrations of 1 mM and higher. The idea is that these reducing agents recover the fluorescence signal by donating an electron to a bleached dye cation. A similar recovery effect of electron donors has recently been observed on the on-and-off blinking of the fluorescence from quantum dots.[14] This blinking phenomenon is due to ejection of an electron out of the core of the dot, leading to a dark state and subsequent recombination leading to recovery of the fluorescence signal. It was shown that electron donors such as dithiothreitol and β-mercaptoethanol substantially reduce the off times.[14]

In recent years there have been several attempts to apply single-molecule fluorescence spectroscopy and optical tweezers simultaneously in order to obtain detailed information on conformation and location using fluorescence, while at the same time manipulating the molecules with the optical tweezers. In one study two optical traps were used to extend a DNA molecule while the location of a single RNA-polymerase molecule moving along the DNA searching for promoter sites was measured with fluorescence.[15] In another experiment the same researchers held an actin filament between two traps and measured single

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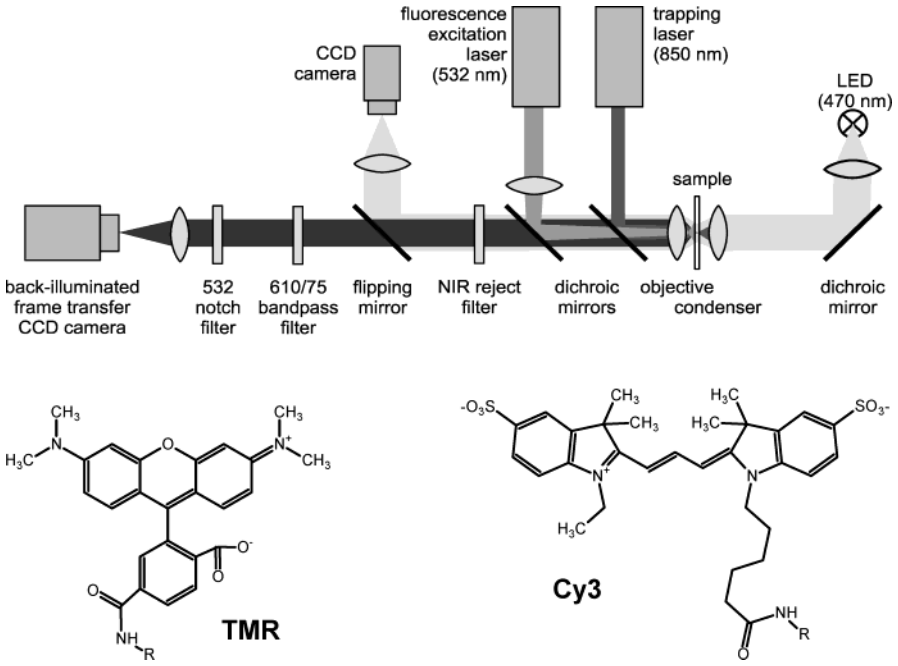

Figure 1. Diagram of the instrument used for combined fluorescence and trapping measurements and structures of two of the dyes used, carboxytetramethylrhodamine (TMR) and Cy3. For details, see text.

fluorescent ATP turnovers by a myosin motor bound to the actin.[16] In both studies the traps and the area from which fluorescence was detected were separated by several micrometers, which avoided potential problems caused by the high intensity of the trapping lasers, such as larger background signals and enhanced photobleaching. In a more recent study, the distance between trap and fluorescent molecules was smaller, only several hundred nanometers,[17] on the order of the trap diameter. A short length of double-stranded DNA was unzipped using an optical trap while at the same time the separation of the two single strands of DNA was measured with fluorescence. In this study it was demonstrated that detection of fluorescence from a single fluorophore close to an optical trap is feasible when the proper optical filters and excitation methods are used.

There are indications that enhanced photobleaching in the presence of the intense trapping beam could be a problem in such experiments.[17] Here, we investigate photobleaching of fluorophores in combined fluorescence and optical tweezers experiments when the dye molecules are located directly in the center of the trap. In these experiments two laser beams are present, a relatively weak one for fluorescence excitation, resonant with the optical transition, and another nonresonant one for optical trapping, with intensity 5−6 orders of magnitude higher. It should be noted that the wavelength of the trapping beam is similar to what is generally used in two-photon excitation of the fluorophore, but the intensity is 3−4 orders of magnitude lower. In the present study we investigate whether enhanced photobleaching indeed occurs in combined trapping and fluorescence experiments, we quantify the effect, and we examine under what experimental conditions it occurs and whether measures can be taken to reduce it. We also explore the mechanism of photobleaching under these conditions, to find out whether bleaching occurs through one of the known mechanisms (see above) or another, unknown one.

Materials and Methods

Setup. A custom-built instrument, capable of high-sensitivity fluorescence imaging and optical trapping, was used for the experiments (Figure 1). Trapping light was provided by a continuous-wave Ti:Sapphire laser (Coherent Mira 900F with a triple-plate birefringent filter, pumped by a Coherent Verdi V10 frequency-doubled Nd:YVO4 laser (532 nm)), tunable from 730 to 1000 nm. The laser was used at 850 nm unless stated otherwise. The laser beam was expanded and coupled into an oil-immersion microscope objective (Nikon Sfluor 100×, 1.3 NA), using a 700 nm short-pass dichroic mirror (Chroma Technology, 700DCSX). Fluorescence excitation light was provided by the 532 nm laser. The beam was slightly decollimated in order to provide widefield epi-illumination and coupled into the objective with a 565 nm long-pass dichroic mirror (Chroma Technology, Q565LP). Fluorescence from the sample was collected with the same objective, filtered by the dichroic mirrors, a color-glass filter (Schott, BG39) or a short-pass filter (Chroma, E750SP), a 532 nm notch filter (Kaiser Optical Systems HPFN-532.0), and a band-pass filter (Chroma Technology, HQ610/75m), and detected with a back-illuminated, frame-transfer CCD camera (Roper Scientific, Micromax 512FTB). In most experiments, the fluorescence excitation light was circularly polarized with a Berek's variable wave plate (New Focus, 5540) while the trapping light was vertically polarized. In the combined trapping/fluorescence experiments in which the effect of polarization was investigated, the polarization of the fluorescence excitation light was altered using the variable wave plate and converted into horizontal and vertical (linear) polarizations. Silica beads used for trapping were visualized using trans-illumination with a blue LED (470 nm). The light from the LED was coupled into the sample with a condenser (Nikon, Achromat Aplanat) and detected with a CCD video camera (Watec 902B).

The (green) laser intensity was calibrated by measuring the Gaussian fluorescence profile from a sample of homogeneously spin-coated DiI (Fluka, dissolved in toluene). The intensity at the position of the laser trap (the maximum of the Gaussian) was determined from the width of the Gaussian. The half width at $1/e^2$ times the maximum (the waist) was 8.06 μm, leading to a peak intensity of 981 W cm^{-2}/mW (transmitted) power. As a measure for the intensity of the trapping laser we used the total transmitted power, P. The exact intensity, I, the trapped bead

is exposed to in the focus is not very well defined, because the bead performs Brownian motion in an approximately Gaussian intensity profile, but is on the order of 600 kW cm^{-2}/mW transmitted power, as calculated from $I = PNA^2/(0.61\lambda)^2$, with the numerical aperture NA = 1.3 and the wavelength λ = 850 nm.[10]

Fluorescence anisotropy was measured with the same setup. In these experiments, the fluorescence excitation light was alternated between horizontal and vertical polarizations using an electrooptical modulator (Conoptics 350-80), driven by a D/A computer board (National Instruments PCI-6733), in synchronization with the read-out of the camera. In these experiments the beads were nonspecifically attached to the glass surface by adding NaCl (~1 M).

Preparation of Fluorescent Beads. To test the feasibility of combining optical tweezers and single-molecule fluorescence, we coated silica beads (444 nm diameter, a kind gift of the Colloid Synthesis Facility, Utrecht University) with small quantities of dye. To attach fluorophores, beads were derivatized with reactive amine groups on the surface by incubation in a solution of 33% v/v aminopropyl-triethoxysilane (Aldrich) in water for 15 min. After washing the beads in water (by several centrifugation/resuspension cycles), they were incubated for 90 min in a sodium carbonate buffer (100 mM, pH 8.3) with a small quantity of aminoreactive fluorophores (Cy3 succinimidyl-ester (Amersham Biosciences), Alexa555-maleimide (Molecular Probes), or carboxytetramethylrhodamine succinimidyl ester (TMR, Molecular Probes)) dissolved in dimethylformamide. Finally, the beads were washed twice to remove unreacted dye. The structures of TMR and Cy3 are shown in Figure 1. For Alexa555 no structural information is available from the manufacturer.

In some of the experiments oxygen-free conditions were created in either one of two ways: (i) by vacuum degassing and flushing the sample with argon gas and (ii) by adding an oxygen-scavenging system (0.02 mg/mL glucose oxidase, 25 mM glucose, 0.035 mg/mL catalase, and 4 mM dithiothreitol) and subsequently sealing the sample chamber with vacuum grease. In another experiment ascorbic acid (100 mM, dissolved in water, pH set to 7) was added.

Results and Discussion

To study the possibility of measuring fluorescence from individual fluorophores in the center of the trap and the influence of the trap laser light, we measured the fluorescence from the dye Cy3, attached with short linkers (about a nanometer) to trapped silica beads of 444 nm diameter. It proved to be rather difficult to measure signals from single fluorophores, due to (i) the residual movement and rotation of the trapped beads, which led to substantial fluctuations of the fluorescence intensity, and (ii) the rapid bleaching of the signal. The former problem was solved by reducing the mobility of the beads by working in an agarose gel (1% w/v). In most biophysical assays used for combined trapping and fluorescence experiments this problem does not exist because the bead is attached to the cover glass or largely immobilized via the molecule to be manipulated. An example of the fluorescence signal from a single Cy3 molecule attached to a trapped bead (7.4 mW trapping laser power) in an agarose gel is shown in Figure 2a. The signal bleached in two steps, indicating that two fluorophores were bound to the bead. From the time trace it is clear that fluorescence arising from a single fluorophore in an optical trap can be detected above the background signal, although the Cy3 molecules appeared considerably more susceptible to photobleaching than without

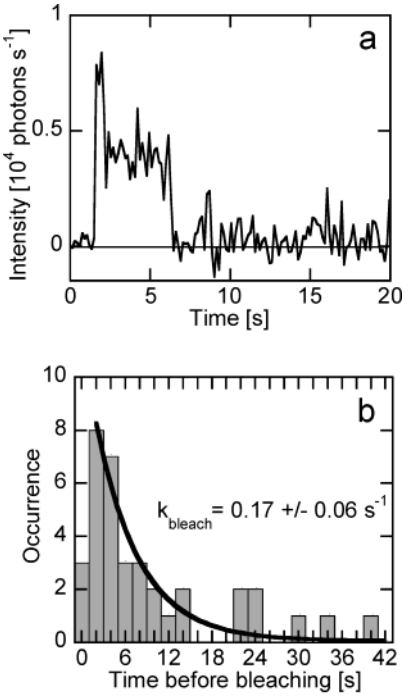

Figure 2. (a) Time trace of the fluorescence intensity of two Cy3 molecules attached to a trapped bead. (b) Histogram of the times before bleaching of 32 such Cy3 molecules with an exponential fit with decay constant 0.17 ± 0.06 s^{-1}. The power of the 850 nm trapping laser was 7.4 mW (in the sample); the intensity of the 532 nm fluorescence excitation laser was 350 W/cm^2.

trap. To quantify the photostability, we determined from traces as shown in Figure 2a the time before bleaching for 36 single fluorophores, shown as a histogram in Figure 2b. The histogram can be fitted by a single exponential with a bleaching rate of 0.17 ± 0.06 s^{-1} (the accuracy of the fitted value is rather low due to the low amount of data points). At higher power of the trapping laser (25 mW) the bleaching rate increased substantially to 0.32 ± 0.09 s^{-1} (data not shown).

To track down the origin of the increased bleaching rate in the presence of the trapping laser, more statistics were needed. Since there is no necessity to do this experiment with single molecules, we used beads coated with 10−100 fluorophores (as judged from fluorescence intensity). At this surface density the fluorophores were far enough apart on the beads to not interact and to not transfer excitations. The advantages of this approach are that (i) the bleaching rate could be determined from the intensity time trace of a single trapped bead, which is also expected to decay exponentially, and (ii) the fluorescence intensities are about 10−100 times higher, so that the experiments could be done in water and no gels were needed to improve the signals. Figure 3a shows an example of such a time trace, fitted with a single exponential. Using this approach we determined that the bleaching increased substantially with trapping laser intensity (Figure 3b). The bleaching rate dependence on trapping power was linear, indicating that the enhanced bleaching was caused by a process involving one and not two 850 nm photons. Note that the straight line does not extrapolate to the origin, reflecting the fact that at zero trapping power, but with fluorescence excitation light present, bleaching still occurs. In similar measurements performed with 1000 nm trapping light (data not shown), lower bleaching rates were observed (70 ± 10% of those with 850 nm trapping light). This difference can be explained by the smaller area of the focal spot at 850 nm (about 70%) than at 1000 nm.

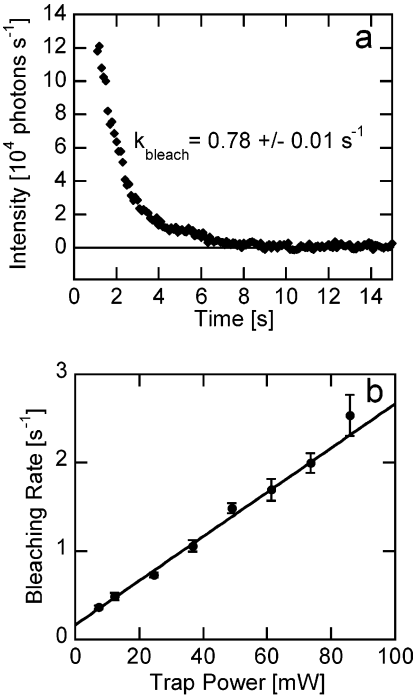

Figure 3. Effect of the power of the trap laser on photobleaching. (a) Time trace of the fluorescence intensity of many Cy3 molecules attached to a trapped bead (diamonds) with an exponential fit with decay constant 0.78 ± 0.01 s^{-1} (gray line). The power of the 850 nm trapping laser was 25 mW; the intensity of the 532 nm fluorescence excitation laser was 350 W/cm^2. (b) Trap laser power dependence of the bleaching rate. Shown are the average bleaching rates from about 10 beads per power (solid circles, the error bars represent the standard error of the mean) and a linear fit to the averaged data (solid line), with intercept 0.16 ± 0.02 s^{-1} and slope 25 ± 1 s^{-1} W^{-1}. The intensity of the 532 nm fluorescence excitation laser was 350 W/cm^2.

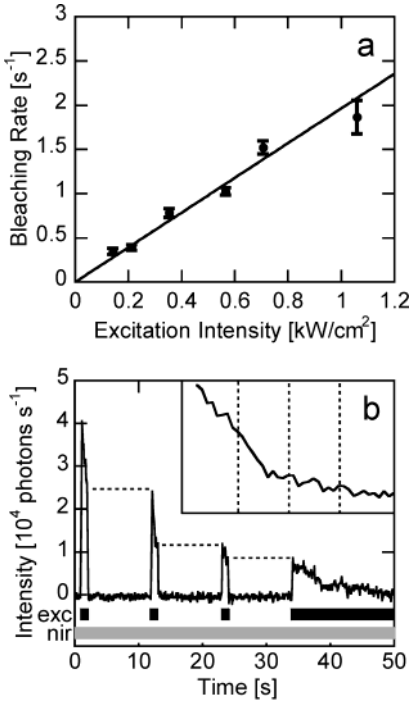

Figure 4. Effect of the intensity of the fluorescence excitation light on photobleaching. (a) Fluorescence excitation intensity dependence of the bleaching rate of hundreds of Cy3 molecules attached to a trapped bead. Shown are the fitted rates, the average bleaching rates from about 10 beads per power (solid circles, the error bars represent the standard error of the mean), and a linear fit to the averaged data (solid line), with slope 1.96 ± 0.05 cm^2 s^{-1} kW^{-1} and the origin as intercept. The power of the 850 nm trapping laser was 25 mW. (b) Time trace of the fluorescence intensity of many Cy3 molecules attached to a trapped bead with the fluorescence excitation laser chopped as indicated by the black bar in the bottom of the figure marked "exc". The three horizontal lines are drawn to indicate that no bleaching takes place when only the laser trap is present. The power of the trapping laser was kept constant at 25 mW, as indicated by the gray bar in the bottom of the figure marked "nir". In the inset the periods without fluorescence excitation light are cut out of the trace. The transitions between two periods with the green laser on are indicated by the vertical dashed lines.

We also measured the effect of the intensity of the fluorescence excitation light on photobleaching. Figure 4a shows that the bleaching rate again increased linearly with the excitation intensity. The data can be well fitted with a straight line through the origin, indicating that the absorption of a green photon is necessary for photobleaching to occur. To prove this point, we performed an experiment with fluorescence excitation light that was repetitively switched on and off. The resulting time trace (Figure 4b) clearly shows that the fluorescence intensity was equal before and after a dark period during which only the trapping laser was present. These results indicate that the enhanced bleaching of fluorescence in the optical trap is a two-photon process, involving the absorption of first a visible and then an NIR photon. In all our experiments we did not observe fluorescence arising from multiphoton excitation by the NIR trapping laser. Fluorescence was detected only when green excitation light was present (as can be seen in Figure 4b).

The two-photon bleaching process could involve triplet and ionized states of the dye. To find out whether triplet states are involved, we measured the bleaching rate in the presence of an oxygen-scavenging mixture based on glucose oxidase, glucose, catalase, and dithiothreitol. In Table 1 it is shown that the oxygen scavenger decreases the bleaching rate more than 2-fold. It should be noted that the oxygen scavenger mixture consists of dithiothreitol, an antioxidant. It could well be that the decrease of the bleaching rate is caused in this case by the antioxidant and not the oxygen-free conditions. To confirm this, we measured the bleaching rate under oxygen-free conditions achieved by degassing the sample and found that the absence of oxygen does not influence the bleaching rate. However, in the presence of the antioxidant ascorbic acid the rate decreased 4-fold (Table 1). From these results it can be concluded that triplet states are not intermediates in the enhanced bleaching process, since reduction of the oxygen concentration does not influence the bleaching rate. It has been shown that oxygen-free conditions can cause increased triplet-state lifetimes[18] and prevention of the production of singlet oxygen (a well-documented cause for photobleaching[8]). On the basis of the strong effect of antioxidants we suggest a model for the photobleaching process consisting of the sequential absorption of two photons, ending up in a higher excited (singlet) state, from which the dye can readily ionize to a nonfluorescent state, forming a solvated electron and a dye cation (see Figure 6 and

TABLE 1: Effect of Antioxidants and Oxygen Depletion on the Bleaching Rates[a]

condition	relative bleaching rate
untreated	1
degassed under argon	0.97 ± 0.03
oxygen scavenger	0.46 ± 0.03
ascorbic acid	0.25 ± 0.01

[a] The data were measured with 89 mW trapping power and 350 W/cm^2 fluorescence excitation intensity and are represented relative to the values for the untreated sample under the same optical conditions.

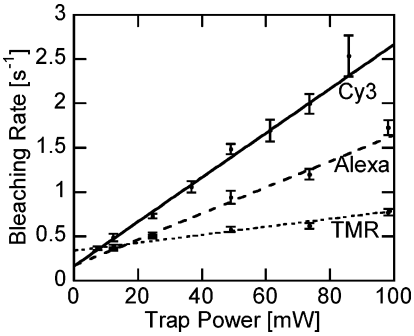

Figure 5. Dependence of the bleaching rate on trapping laser power with many dye molecules attached to a trapped bead, for three different dyes: Cy3, Alexa555, and TMR. Shown are the average bleaching rates from about 10 beads per power (solid circles, the error bars represent the standard error of the mean) and linear fits to the averaged data, with intercept 0.16 ± 0.02 s^{-1} and slope 25 ± 1 s^{-1} W^{-1} for Cy3, intercept 0.17 ± 0.02 s^{-1} and slope 15 ± 1 s^{-1} W^{-1} for Alexa555, and intercept 0.33 ± 0.03 s^{-1} and slope 4.5 ± 0.5 s^{-1} W^{-1} for TMR. The intensity of the 532 nm fluorescence excitation laser was kept constant at 350 W/cm^2.

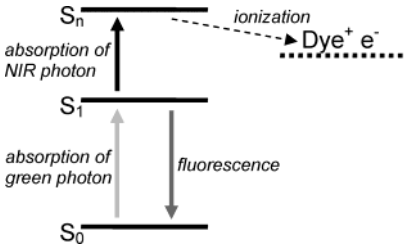

Figure 6. Proposed model for the photobleaching process. Electronic energy level scheme indicating the states and transitions involved. S_0, S_1, and S_n are the singlet ground state, the lowest singlet excited state, and higher excited singlet states, respectively. Dye^+ e^- is an ionized state consisting of a dye cation and a solvated electron.

conclusion below). In the presence of antioxidants this cation can be reduced back to its neutral, fluorescent form.[13]

To further characterize the bleaching process, we studied the dependence of bleaching rates on the relative polarization of the fluorescence excitation and the trapping beams. In these experiments we measured a bleaching rate that was 1.7 ± 0.3 times higher with parallel polarizations of the two beams than with perpendicular polarizations (data not shown). Given our model for the bleaching process as the consecutive absorption of first a visible and then an NIR photon, this experiment is in some ways equivalent to a fluorescence anisotropy[19] or a pump−probe transient-absorption anisotropy measurement.[20] The NIR photon plays the role of the fluorescence or the probe pulse in the respective cases. Any anisotropy measured by comparing parallel and perpendicular polarizations of visible and NIR beams reflects relative orientations of the transition dipoles of first and second absorption process. These transition dipoles can be intrinsically nonparallel, or they can become nonparallel due to rotation of the molecule during the two events (absorption−absorption or absorption−fluorescence).

Using the formula for anisotropy ($r = (k_{||} - k_{\perp})/(k_{||} + 2k_{\perp})$, with $k_{||}$ the bleaching rate with parallel polarization and $k_{\perp}$ the bleaching rate with perpendicular polarization) the anisotropy in our experiments was calculated to be 0.19 ± 0.07. For perfectly aligned transition dipoles the anisotropy would be 0.4.[19,20] The measured lower value indicates that the two absorption processes have transition dipole moments that are not exactly parallel and/or that some polarization is lost by rotational motion of the chromophores during the lifetime of the (first) excited state. To find out which of the two possiblities is the cause of the decreased anisotropy, we also determined the fluorescence anisotropy of the same bead/dye system, which was 0.15 ± 0.03. In Cy3 the transition dipole moments of absorption and emission are nearly parallel,[21] so it can be assumed that the fluorescence depolarization is due to rotation of the dye within the excited-state lifetime (due to their size, the beads hardly rotate in this time). The relatively low value of the fluorescence anisotropy indicates that the linkage of the dye to the bead is rather flexible, even more so than in previous studies using similar chemistry for dye attachment to proteins, in which values of 0.25−0.32 were observed.[21,22] The higher flexibility in our experiments could be caused by the longer linker we used than in those studies (the silane is flexible as well) and the absence of nonspecific interactions between protein and dye.[7] The fluorescence anisotropy is nearly equal to the bleaching anisotropy. This provides additional proof that the time scale of the two-photon bleaching process is set by the lifetime of the first excited singlet state. If this were not the case, the fluorophores would have more (or less) time to rotate, leading to a bleaching anisotropy substantially lower (or higher) than the fluorescence anisotropy. Within this model, the fact that the dye rotation already accounts for the observed bleaching anisotropy leads us to conclude that the dipole moments of the two transitions involved are approximately parallel.

In the measurements described so far we used the dye Cy3, a dye that is widely used in single-molecule fluorescence experiments because of its high photostability upon direct excitation with green light.[7,12] When used in two-photon excitation microscopy (with NIR light) this dye bleaches rather fast.[23] In a last set of experiments we therefore compared the bleaching of Cy3 in combined trapping and fluorescence experiments with that of other frequently used dyes in the same wavelength range, TMR and Alexa 555. The measured bleaching rates, shown in Figure 5, clearly show that Cy3 is much more sensitive to the enhanced bleaching effect than the other two dyes. In fact, the dye that is least photostable in one-photon experiments, TMR (which therefore also has the highest bleaching rate extrapolated to zero trapping power in Figure 5), is most resilient to the presence of the high-intensity trapping beam. The difference between the three dyes can be caused by three photophysical properties. One would expect that (i) the shorter the singlet excited-state lifetime of the dye is, the lower the bleaching rate is, (ii) the lower the absorption cross sections of the excited-state absorption from the first singlet excited state to higher singlet states is, the lower the bleaching rate is, and (iii) the lower the coupling between the higher singlet excited states and ionized states is, the lower the bleaching rate is. Unfortunately, to our knowledge, neither experimental data nor quantum-chemical calculations on excited-state absorption spectra or on ionization potentials are available to compare the three dyes with respect to the latter two properties. However, there should be large differences between the dyes in these respects, since the lifetime (property (i)) of Cy3 (<0.3 ns, data from Amersham Biosciences) is substantially smaller than that of TMR (~2.5 ns[24]), while it nonetheless bleaches substantially faster in our experiments.

Conclusions

In experiments that combine single-molecule fluorescence spectroscopy with optical tweezers, attention has to be paid to limiting additional photobleaching of the fluorophores by the trapping laser. We have shown that an additional channel for photodestruction of the dyes is opened up in such experiments, causing, in general, a substantial increase of photobleaching.

The bleaching mechanism we propose, which is consistent with all our results, is shown in Figure 6. The visible laser used for fluorescence excitation generates dye molecules in their (lowest) singlet excited state (S_1), from which fluorescence can take place. The intensity of the near-infrared trapping light is so high that even during the short lifetime (~ns) of this state there is a considerable chance that a second photon is absorbed and higher excited states are populated (S_n). In general, these states couple efficiently to bleached, ionized states, in particular in polar solvents such as water,[12] and as a consequence, dye cations and solvated electrons are readily produced. We noticed that for Cy3 the bleaching is not much different when trapping light with a wavelength of 1000 nm is used instead of 850 nm.

The bleaching process we observed in the presence of a visible, resonant fluorescence excitation and a much stronger nonresonant, near-infrared trapping beam is related to enhanced photobleaching in two-photon excited fluorescence experiments, which show a bleaching rate proportional to the third power of the excitation intensity.[11,13] In those experiments in which only a nonresonant near-infrared laser beam is present at much higher power, bleaching takes place from higher excited states produced via a three-photon process, either by direct three-photon excitation or by two-photon excitation into the lowest singlet state immediately followed by an additional excitation by one photon into the higher excited states. In contrast, in our experiments the lowest singlet state is populated with one-photon excitation and higher excited singlet states, from which photobleaching can occur, are populated via excited-state absorption of one photon of different energy, from a different laser.

In summary, we have shown that measuring fluorescence from a single fluorophore in an optical trap is difficult, but possible. Several measures can be taken to reduce the additional photobleaching in the combined fluorescence and trapping experiments: (i) choice of the right fluorophore (TMR is better than Alexa 555, which is better than Cy3); (ii) use of low trapping and fluorescence excitation powers; (iii) use of antioxidants such as ascorbic acid; (iv) use of perpendicular polarization of the fluorescence excitation and trapping laser beams.

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