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Forbidden Light Detection from Single Molecules

Thomas Ruckstuhl,*,† Jörg Enderlein,‡ Stefan Jung,‡ and Stefan Seeger†

Physikalisch-Chemisches Institut, Universität Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland, and Institute of Analytical Chemistry, Chemo- and Biosensors, University of Regensburg, P.O. Box 10 10 42, D-93053 Regensburg, Germany

We present a new concept for ultrasensitive detection of surface-generated fluorescence which is made possible by a new optical module. The detection method leads to an enhancement in fluorescence collection efficiency to more than 65% of the total of emitted light, whereas high-aperture microscope objectives are able to collect 44% at best. Moreover, by employing this new optical module, the detection volume can be restricted to $\sim 10^{-17}$ L. This allows for an exceptional discrimination of bulk-generated against surface-generated fluorescence, which may be of great value when surface-binding processes are monitored. We demonstrate the performance of the new detection system by detecting single fluorescent molecules and by determining antigen concentrations down to 5 fmol.

In recent years, the fluorescence detection of single molecules (SMD) has become a standard laboratory technique with great potential in chemical and biochemical analysis, see, e.g. refs 1−3 and references therein. SMD is especially advantageous in applications where lowest molecule concentrations have to be analyzed. An already well established detection technique with single-molecule sensitivity is fluorescence correlation spectroscopy, where analyte molecules diffusing freely in solution are detected.[4,5] However, many bioanalytical applications employ heterogeneous assays based on specific receptor−ligand bindings at surfaces (e.g., enzyme-linked immunosorbent assays). For such applications, a sensitive fluorescence-based detection of molecules bound at a water−glass interface is of major importance.

When single molecules are detected by laser-induced fluorescence, the main task is to discriminate any background signal against the molecule's fluorescence. The background signal is mainly due to Rayleigh and Raman scattering of the exciting laser light, which increases with the fourth power of the optical frequency. The suppression of background signal is of particular importance when detecting on glass surfaces, because Raman scattering at the water−glass interface is considerably stronger than that of water. The most common way of suppressing scattering background is to employ spectral filters, taking advantage of the frequency shift of the fluorescence emission with respect to the laser excitation (Stokes shift). However, Raman scattering is also wavelength shifted, and it is therefore not completely rejected by emission filters. An additional way to increase the signal-to-background ratio is to reduce the volume of the detection region. In that case, one uses the fact that the background intensity is proportional to that volume, whereas the fluorescence signal of a single molecule is basically independent of it. The standard approach for minimizing the detection volume is to use a confocal epifluorescence microscope.[6,7] In common epifluorescence microscopes, high-quality microscope objectives with as high numerical apertures as possible are used for collecting the fluorescence. However, increasing the numerical aperture leads to more and more complicated multiple lens systems with increasing optical losses due to multiple interfaces. In the case of surface-bound molecules, the efficient collection of the fluorescence becomes even more intricate, because of the peculiarities of fluorescence emission on a glass surface. The angular distribution of the fluorescence emission of molecules bound to a water−glass interface differs significantly from that of molecules within a homogeneous medium,[8−12] which has been recently also demonstrated on the single-molecule level in refs 13 and 14. Because we are mainly interested in bio- and chemoanalytical applications where the dipole moments of different molecules are rather randomly oriented, only the case of isotropic dipole orientation will be studied here. Figure 1 shows the angular emission distribution of a fluorescent dye with arbitrary orientation directly immobilized on a glass surface. In this case, 72% of the fluorescence is emitted into the half-space of the glass (refractive index 1.5) and only 28% into the solution half-space (refractive index 1.33). The distribution shows a significant

* Corresponding author. E-mail: thomasr@pci.unizh.ch.
† Universität Zürich.
‡ University of Regensburg.

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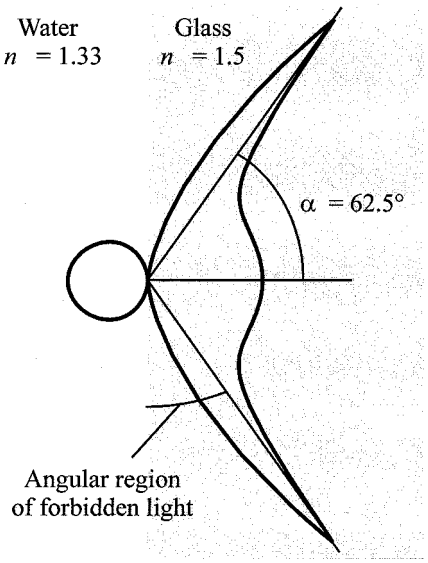

Figure 1. Angular distribution of the fluorescence intensity of a molecule positioned at a water−glass interface averaged over all possible emission dipole orientations. The discontinuity in the refractive index produces a significant emission maximum into the direction of the critical angle of total internal reflection.

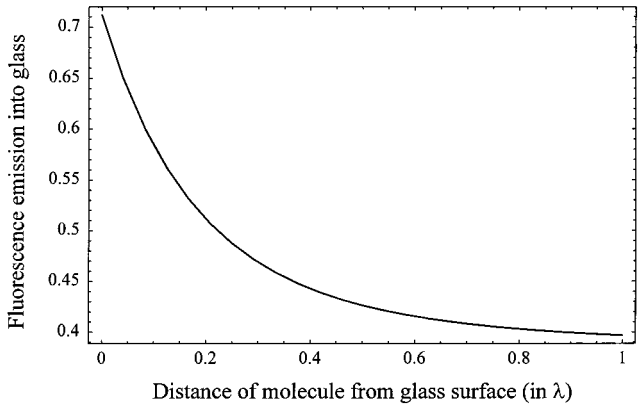

Figure 2. Dependence of the integrated intensity of the forbidden light on the distance of the fluorescing molecule from the water−glass interface.

emission maximum around the direction of the critical angle of total internal reflection, which is for a glass−water interface given by $\alpha_c = \arcsin(1.33/1.5) \approx 62.5°$. A significant part of the fluorescence radiation is emitted into the glass side above the critical angle. This radiation is called forbidden light[15] and amounts to 34% of the total fluorescence emission. The forbidden light is of particular interest for detecting surface-generated fluorescence due to the fact that its intensity decreases exponentially with increasing distance of the emitter from the surface. In Figure 2, the integrated intensity of all the forbidden light is plotted against the distance of the emitter from the glass surface. By detecting the forbidden light only, the penetration depth of the detection volume into the water solution is about $\lambda/6$, where λ is the wavelength of fluorescence emission. Consequently, when dyes emitting at visible wavelengths are used, the forbidden light signal stems only from molecules closer than ∼100 nm to the glass surface. It should be mentioned that the electromagnetic coupling

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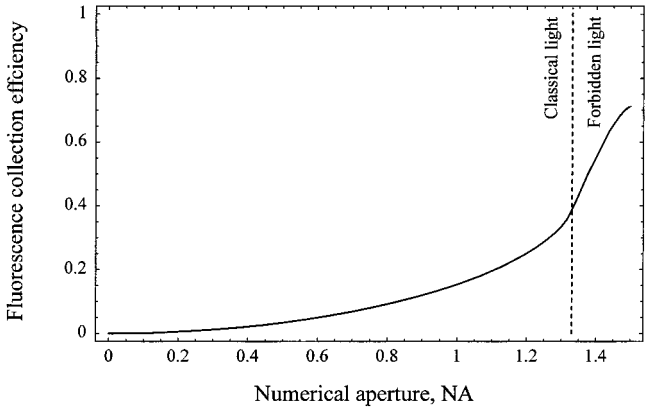

Figure 3. Fluorescence collection efficiency of conventional microscope objectives in dependence on their numerical aperture. Any internal light losses within the objective are neglected.

of the radiating fluorophore to the glass decays much stronger than exponentially with distance r from the glass surface (due to the $1/r^6$ decay of the dipole's near-field intensity),[16] so that the detection volume restriction next to the surface is even stronger than in case of an evanescent wave excitation as used in refs 17 and 18.

To collect light above the critical angle of total internal reflection, one needs collection optics with numerical apertures (NA) larger 1.33, the refractive index of the solvent. Using the knowledge of the angular distribution of the fluorescence emission, as shown in Figure 1, the collection efficiency of surface-generated fluorescence by conventional microscope objectives can be calculated in dependency of their numerical aperture. A thorough theoretical analysis of that collection efficiency was presented in ref 19, and all subsequent theoretical results are based on the theoretical approach as presented there. The computational result is shown in Figure 3. In this figure, optical losses within the objective due to its multiple refracting surfaces are completely neglected. For real objectives, the optical transmissivity is equal or lower than 80%. The highest achievable NA using standard cover slips with a refractive index of 1.5 equals 1.4. Consequently, the best possible collection efficiency with a 1.4 NA microscopy objective is ∼44%. The forbidden light can only be collected to ∼43%. A special solution to that problem represents the so-called solid immersion lens as described in refs 20 and 21. However, the solid immersion lens only becomes effective when made of glass with high refractive index (>1.67) and is therefore rather useless when light is collected through a cover slip made of glass with a refractive index near 1.5.

As can be seen from the angular distribution in Figure 1, to achieve high fluorescence collection efficiencies, it would be much more advantageous to have an optical element that collects light preferentially near and above the critical angle instead of having the usual microscope objective that collects light preferentially

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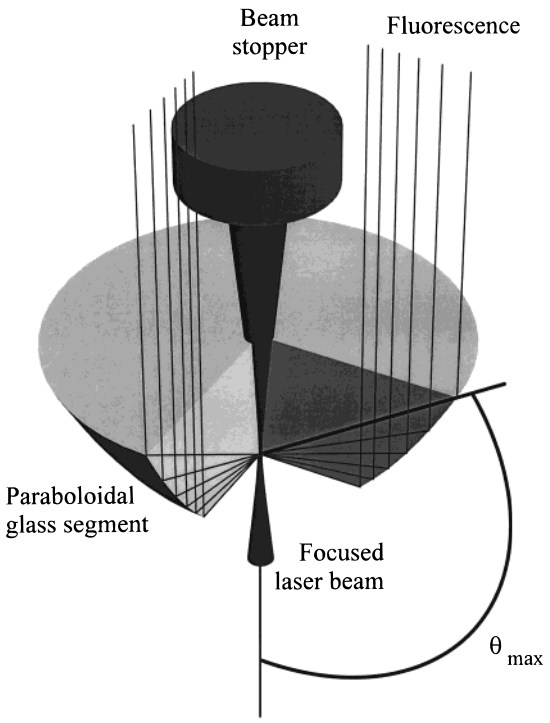

Figure 4. Schematic of the paraboloid glass reflector. The paraboloid glass segment has two parallel plane faces. The geometrical focus of the paraboloid is positioned directly on the glass surface. It represents the point of collection of surface-generated fluorescence. Fluorescence emitted from that point is reflected (by total internal reflection) and exits the segment as bundle of parallel light rays. The axial extension of the glass segment determines the angle θ_{max} up to which fluorescence emission is collected. Excitation light is focused onto the collection point and blocked by a beam stopper positioned behind the segment.

around the optical axis. For this purpose, we designed the paraboloid collector to replace the microscope objective. This collector comprises a glass paraboloid of revolution and is schematically shown in Figure 4. The paraboloid glass segment has two parallel plane faces. The focal point of the parabolic surface lies in the center of the smaller planar face, the front face. This is the position of the fluorescence detection region. The parabolic surface of the element acts as a loss-free mirror via total internal reflection. After reflection, the fluorescence light exits the glass segment as a ring of parallel light rays. With this glass reflector, both forbidden and allowed light (emitted into the region below the critical angle) can be captured. Increasing the thickness of the glass segment (by shifting the exit face) increases the amount of collected light. Collection efficiencies of more than 65% can be achieved, whereby the forbidden light is collected completely; see Figure 5.

When approaching extremely low fluorophore surface concentrations, the most perfect light collection becomes useless if one is not able to reject scattered light efficiently. Minimizing the detection volume is of utmost importance in ultrasensitive fluorescence detection systems. For that purpose, the paralleled fluorescence light can be refocused by a lens through a small circular aperture. In this way, the volume of the detection region can be reduced to the same order of magnitude as in a confocal

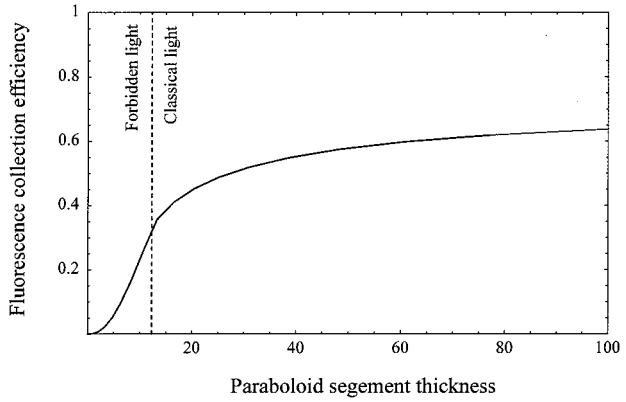

Figure 5. Fluorescence collection efficiency of the paraboloid collector for different values of its thickness.

epifluorescence microscope.[19,6,22] Additionally, the volume of the detection region can be further reduced by blocking off the allowed light and detecting the forbidden light only. This is easy to achieve since the allowed and forbidden light contributions are well separated within the light ring that exits the segment. The forbidden light exits closer to the symmetry axis, whereas the allowed radiation lays farther on the outside. Due to the strong dependency of the forbidden light intensity on the distance of the fluorophores from the water−glass interface, blocking off the allowed lights restricts the detection region to the direct vicinity of the surface. Assuming a diffraction-limited focusing of the exciting laser beam on the front surface, the detection volume for dye molecules at the glass−water interface can be made as small as 10^{-17} L.

EXPERIMENTAL SECTION

A schematic representation of our experimental setup is given in Figure 6. The excitation source was a multimode diode laser emitting at a wavelength of 635 nm (Laser Graphics, Dieburg, Germany) and was run with 1-mW output intensity. After passing an excitation filter (635DF15, Omega Optical, Brattleboro, VT), the laser beam was focused into a diffraction-limited spot onto a cover slip (20 × 20 × 0.15 mm; Menzel, Braunschweig, Germany) by a microscope objective (95×, NA 1.32, Leitz, Wetzlar, Germany). The cover slip was mounted onto the front surface of the paraboloid (BK7, BLS, Mannheim, Germany) with immersion oil (N518, Zeiss, Oberkochen, Germany). The geometry of the paraboloid is described by $z = -\alpha(x^2 + y^2)$, $0 > z_{front} \geq z \geq z_{rear}$, where z denotes the optical axis of the system, $(x^2 + y^2)^{1/2}$ the distance to the optical axis, z_{front} the position of the front face, and z_{rear} the positions of the rear face. In this coordinate system, the focal point of the paraboloid lies at $z = 0$, and the collector opens into the negative z-direction. The parameter values of the paraboloid are $\alpha = 0.035$, $z_{front} = 14$ mm, and $z_{rear} = 25$ mm. The z-position of the front face of the paraboloid is chosen in such a way that the focal point of the collector lies exactly at the surface of the cover slip.

For determining surface concentrations of immobilized fluorescent molecules, the surface of the cover slips was scanned with the system described above. Surface scans were performed by

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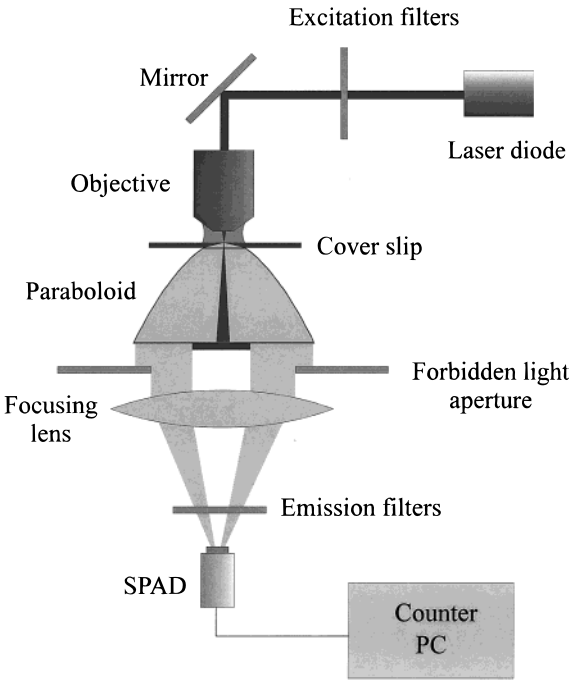

Figure 6. Schematic of the complete fluorescence detection setup.

moving the cover slip perpendicularly to the surface by means of a micropositioning system in a stepwise way: Fluorescence measurements at fixed positions of the laser focus were alternated with movements of the focus to a new spot on the surface. Between fluorescence measurements, the laser beam was blocked by a mechanical shutter, which opened only during the measurement at the fixed positions. The beam stopper behind the back face of the paraboloid collector blocked the excitation light. The fluorescence light was collected within a range of polar angles between 55° and nearly 90° (compare with Figure 1). The collected light was reflected at the paraboloid's glass−air interface into parallel light rays by total internal reflection. An additional beam stopper was used to completely reject the allowed light. A biconvex lens (01MFG028, diameter 100 mm; Melles Griot, Irvine, CA) focused the remaining light through two emission filters (670DF40 and 682DF22, Omega Optical) onto a single-photon avalanche diode (SPCM-aq; EG&G, Quebec, Canada). This diode acted not only as the photon detector but also as a spatial aperture (diameter of photoactive area 177 μm).

Fluorescence sample preparation was performed in the following way. In an initial step, glass cover slips were covered with several monolayers of aminotrimethylsilyl ether cellulose by a Langmuir−Blodett technique (using a Langmuir−Blodgett trough FW2; Lauda, Königshofen, Germany).[23] Next, the trimethylsilyl groups were cleaved off by a HCl vapor treatment. By placing the cover slips for 1 h into an aqueous solution of cyanuric chloride (0.6 mg/mL), cyanuric chloride was covalently bound to the cellulose by nucleophile substitution of chloride by the cellulose hydroxy and amino groups. After rinsing with phosphate-buffered saline (PBS, pH 7.4; Sigma Aldrich, Milwaukee, MI), goat anti-rabbit antibodies (Dianova, Hamburg, Germany) were covalently linked via their free amino group to the functionalized cellulose by placing the cover slips into a PBS−antibody solution of 6 μg of antibodies/mL. Subsequently, the cover slips were put into 2-mL solutions of different concentrations of Cy5-labeled rabbit anti-horse antibodies (Dianova) in PBS. The resulting immunoreaction was carried out for 1 h. Because the surface density of immobilized antibody receptors was always in excess of the total number of solved antigen molecules, a linear dependency of the number of surface-bound Cy5 molecules on antigen concentration in solution is expected.[24] Finally, the cover slips were rinsed with H_2O (Milli-Q plus, Millipore, Bedford, MA) and dried within a nitrogen atmosphere.

For fluorescence measurements, the cover slips were mounted onto the paraboloid collector system with immersion oil. A drop of H_2O (Milli-Q plus) was placed between the laser-focusing objective and the cover slip. For each sample, the fluorescence was measured at 1000 different spots 4 μm apart. Each spot was measured for 300 ms with 3-ms time binning.

Measurements of the fluorescence of free dye in solution were performed both with the paraboloid detector and with a conventional confocal epifluorescence microscope. To ensure comparable experimental conditions, within the epifluorescence microscope (see ref 25 for a comprehensive characterization) an identical excitation laser, an identical set of interference filters, and the same photon detector were used. The core optical element of this system was a 100× microscope objective with 1.25 NA (Edmund Scientific, Barrington, NJ). The measurements were carried out on cleaned cover slips covered by a 5×10^{-9} mol/L solution of Cy5 in H_2O (Milli-Q plus) at room temperature. The cover slips were cleaned by incubating them with concentrated H_2SO_4 for 10 min, rinsing with H_2O (Milli-Q plus), and drying in nitrogen atmosphere.

RESULTS

To test the performance of the paraboloid collector, fluorescence detection experiments with different surface concentrations of Cy5-labeled antigens were carried out. Because the surface density of immobilized antibody receptors was always in excess of the total number of solved antigen molecules, a linear dependency is expected between the number of surface-bound Cy5 molecules and the antigen concentration in solution as used during the cover slip preparation.

Typical tracks (from 10 spots of 1000) for a blank and for four different antigen concentrations are shown in Figure 7. The track of Figure 7a was obtained by measuring a cover slip with immobilized antibodies that was not incubated with antigen-Cy5. It shows the pure background signal emanating from the detection region. The average background was equal to 67 counts/3 ms. The dwell of the mechanical shutter caused the count rate to increase rapidly at the beginning of each fluorescence measurement at a given spot. Panels b−e of Figure 7 present fluorescence measurement results for the following antigen concentrations as used during the cover slip preparation: 10^{-14} (b), 10^{-13} (c), 10^{-12} (d), and 5×10^{-12} mol/L (e). As can be seen, at higher concentrations, the signal shows characteristic exponential decays toward the background level. These exponential decays were caused by photobleaching of the fluorescent molecules within the

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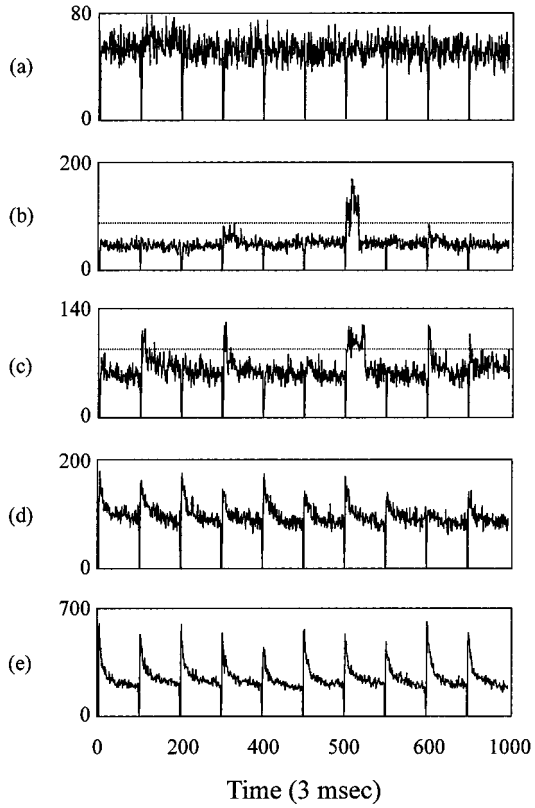

Figure 7. Typical tracks of fluorescence signals measured at different analyte concentrations. The ordinate always shows numbers of photon counts. The diagrams show signals at 10 subsequent surface spots of (a) pure glass and of cover slips prepared with rabbit anti-horse-Cy5 of (b) 10^{-14}, (c) 10^{-13}, (d) 10^{-12}, and (e) 5×10^{-12} mol/L. In tracks b and c, the threshold value (88 counts/3 ms) for the single-molecule counting is shown as a dotted line.

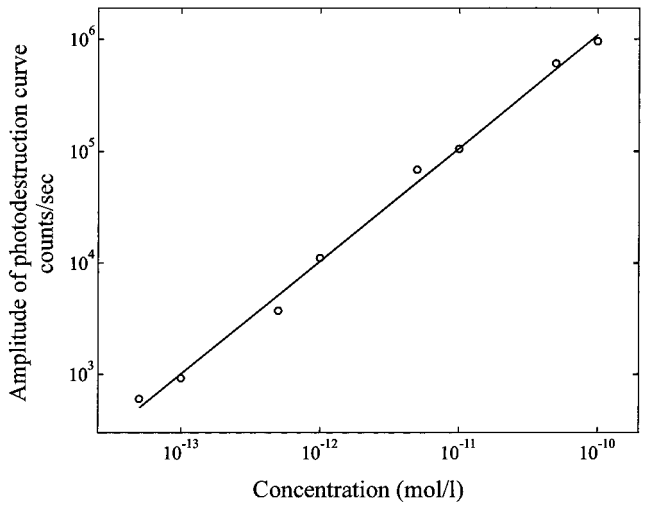

Figure 8. Dependence of fluorescence upon analyte concentration, when the fluorescence signal is determined by superposing the measured signal of 1000 spots and fitting the photobleaching decay as described in the main text. The straight line represents a least-squares fit of the experimental values (open circles). The slope of the line has a value of 1.01, very close to the ideal value of 1.0.

illuminated spots. At lower concentrations, the character of the measured signal changes: only a few spots show some increased signal at the beginning of a measurements, which, after a few milliseconds, suddenly drops back to background level. We interpret these signal drops as the result of sudden photodestruction of single Cy5 molecules, indicating that indeed single molecules are detected.[26] At a concentration of 10^{-14} mol/L, only very few spots are showing a nonbackground signal at all. If our interpretation is correct, the presented results are the first successful fluorescence detection of single molecules that has been obtained by only collecting the forbidden light.

For extracting quantitative information about surface concentrations of bound fluorophores, two different approaches have to be used: at very low concentrations, where the probability of finding more than a single molecule can be neglected, directed counting of molecules can be applied. At higher concentrations, a different method of evaluating the measured fluorescence data has to be applied. In that case, the data evaluation procedure was as follows: A monoexponential decay curve plus a uniform background was fitted to the accumulated signal of all spots. The amplitude of the exponential decay was then taken as a measure of the fluorescence intensity of all spots. By applying such a procedure, the photodestruction of the fluorescent molecules is explicitly used to discriminate between fluorescence and background. It should be emphasized that such an approach is only possible due to the very small excitation region and high laser intensities, causing rapid photobleaching of the fluorescent dyes. The advantage of explicitly using photobleaching for quantitating the fluorescence is that such a method is independent of any prior knowledge of background levels. Moreover, dust particles or surface defects may cause false signals, which can be easily discriminated against true molecule fluorescence due to the absent time decay of their signal. Figure 8 shows the dependency of the determined fluorescence amplitude upon Cy5-antibody concentration of the solution used during cover slip preparation. In the concentration range between 10^{-10} and 5×10^{-14} mol/L, one finds a linear correlation between signal and concentration. The depicted line represents a least-squares fit to the data and has a slope of 1.01, very close to the expected value of 1.0. With this method of data evaluation, concentrations down to 10^{-13} mol/L can be reliably determined.

At lower concentrations, direct counting of single molecules is the preferred method of data evaluation. In that case, for every spot, the presence of a molecule was judged according to whether the fluorescence signal of the spot exceeded a preset threshold value. A molecule was counted when the count rate exceeded 1.7 times the average background. In Figure 9, the ratio of positive detection events to total number of measured spots is plotted against concentration of Cy5-antibodies in the solution used for cover slip preparation. As can be seen, the molecule-counting method works reasonably well only if, on average, there is much less than one molecule present per spot. At concentrations higher than 10^{-13} mol/L, the probability of finding more than one molecule per spot is not negligible and the probability to find at least one molecule approaches the value of 1. However, for lower concentrations, one finds indeed a linear dependency of the number of registered molecules versus concentration. The slope of the least-squares fit (done for the lowest four concentrations) is 1.21, which still comes close to the expected value of 1.0, regarding the rare occurrence of positive events at concentrations

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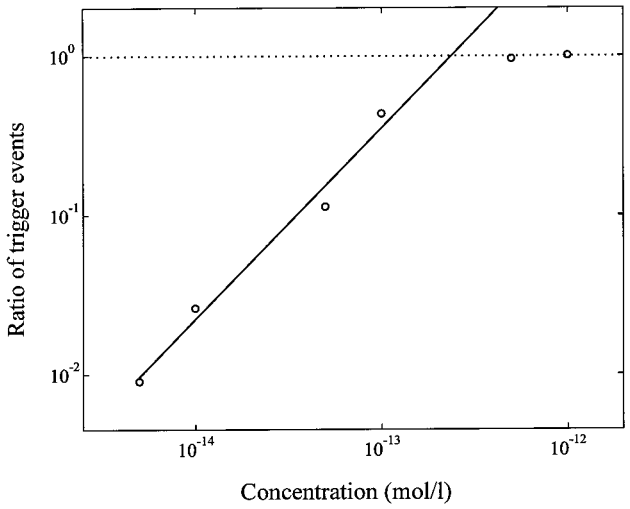

Figure 9. Dependence of signal upon analyte concentration, when the signal is determined by counting single molecules as described in the main text. A single molecule was counted when the photon count rate exceeded the background by a factor of 1.7 for at least 12 ms (see Figure 4b). The straight line represents a least-squares fit of the experimental values (open circles). The slope of the line has a value of 1.21.

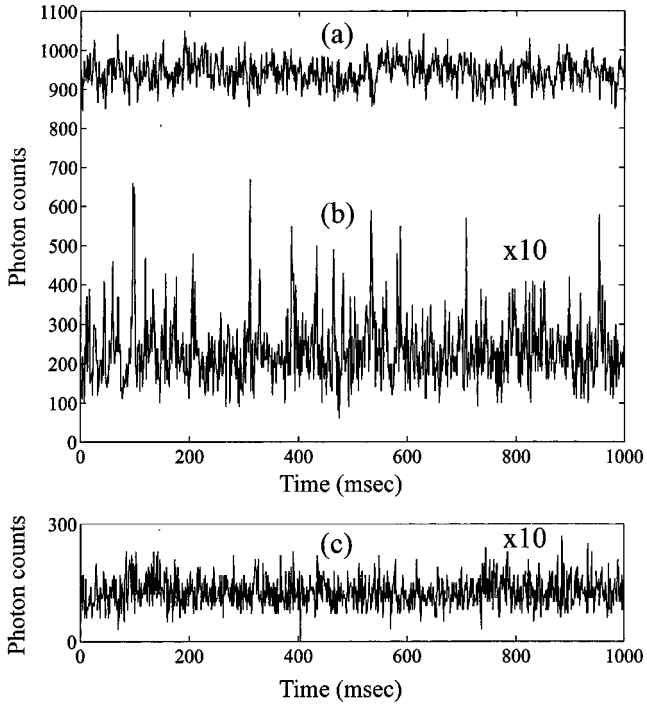

Figure 10. Comparison between a conventional epifluorescence microscope and the paraboloid detector. (a) Signal of the epifluorescence microscope. (b) Signal of the paraboloid detector. For better visibility, the paraboloid signal is multiplied by a factor of 10. (c) Background signal (no fluorescing dye) as detected with the paraboloid detector. Again, signal is multiplied by a factor of 10.

of 10^{-14} mol/L and below. The lowest measured concentration of 5×10^{-15} mol/L is not the detection limit of the system. To determine reliable values at lower concentrations, one has only to increase the number of spots to be measured (at 5×10^{-15} mol/L we found 9 molecules in 1000 spots).

As was explained at the end of the introduction, by detecting the forbidden light only, one achieves an extreme restriction of the detection volume to the direct vicinity of the surface of the cover slip. To demonstrate this experimentally, a measurement was performed at a fixed spot for a long time, without having molecules directly immobilized on the cover slip but having a drop of a 5×10^{-9} mol/L water solution of free Cy5 on it. For comparison with a conventional detection system, the same measurement was repeated with a standard confocal epifluorescence microscope as described in the last section. All conditions of the experiments were held as similar as possible. The measurement results for both systems are shown in Figure 10. The detection volume of the epifluorescence microscope has a value of several femtoliters. At a concentration of 5×10^{-9} mol/l, the number of Cy5 molecules simultaneously present in such a detection volume is clearly beyond 1. Therefore, the epifluorescence setup is not able to resolve single-molecule events. In contrast, the paraboloid detector is practically "blind" to dye molecules in solution far from the surface, and its detection volume has a value of only 10^{-17} L. Single-molecule transits near the surface (<100 nm) and adsorption events onto it can be clearly discerned within the time track of the signal. It may seem somehow unexpected that no long-lasting fluorescence bursts, indicating strong adsorption of molecules to the surface, are seen in Figure 10b. However, the discernible fluorescence bursts are longer than one would expect for pure diffusion, and as was already pointed out by Hansen and Harris,[27] adsorption/desorption kinetics depends critically on a multitude of conditions such as pH value, solvent polarity, and temperature.

DISCUSSION

The experiments presented here demonstrate the potential of the paraboloid collector in respect to detecting fluorescent molecules immobilized on a surface. Usually, single-molecule detection is by either confocal microscopy setups or by using wide-field microscopy in connection with high-sensitive camera systems. Besides the partially high costs of such systems, these detection techniques have long reached their theoretical limits concerning fluorescence collection efficiency. In this respect, the presented detection system offers new possibilities, combining an exclusively high fluorescence collection efficiency with an extremely low detection volume. An additional advantage is the exceptional simplicity of the core optical element. Both aspects may be prove useful when building low-cost high-sensitivity fluorescence readers for analytical applications.

The paraboloid detector redirects the collected fluorescence into a ring of parallel light rays by only one loss-free reflection (via total internal reflection). The only optical losses caused by this element can occur at the exit of the light off the element and by optical absorption within the glass. Within the parallel ring of collected light, the distance from the optical axis is directly connected with the polar angle into which the fluorescence was emitted by the molecules. This allows one to choose for detection certain angular ranges of emission by blocking of all light outside the desired range. In the experiments presented in this paper, we detected only the forbidden light by blocking of all allowed components. It was shown that it is possible to detect single molecules by exclusively collecting the forbidden light.

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By detecting the forbidden light only, the detection volume was reduced to 10^{-17} L, which is ~2 orders of magnitude smaller than the typical detection volumes of a confocal epifluorescence microscope. The small detection volume made it possible to detect single-molecule events within a dye solution even at bulk concentrations where a confocal microscope setup is no longer able to discern single-molecule events. This might be advantageous if one is interested in observing, for example, binding or enzymatic reactions at the glass surface at high bulk concentrations of free dye molecules.

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