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Fast Quantitative Single-Molecule Detection at Ultralow Concentrations

Philippe Haas,[†] Patrick Then,[‡] Andreas Wild,[†] Wilfried Grange,[§] Sylvain Zorman,[§] Martin Hegner,[∥] Michel Calame,[⊥] Ueli Aebi,[⊗] Josef Flammer,[○] and Bert Hecht*[,‡]

National Center of Competence for Research in Nanoscale Science (NCCR), Institute of Physics, Klingelbergstrasse 82, CH-4056 Basel, Switzerland, Nano-Optics & Biophotonics Group, Physikalisches Institut, Wilhelm-Conrad-Röntgen-Center for Complex Material Systems (RCCM), Experimentelle Physik 5, Am Hubland, 97074 Würzburg, Germany, Institut Jacques Monod, 15 Rue Hélène Brion, 75013 Paris, France, Centre for Research on Adaptive Nanostructures and Nanodevices, College Green, Dublin 2, Ireland, Department of Physics, Klingelbergstrasse 82, CH-4056 Basel, Switzerland, Biozentrum, Klingelbergstrasse 50-70, CH-4056 Basel, Switzerland, and Universitätsaugenklinik, Mittlere Strasse 91, CH-4031 Basel, Switzerland

The applicability of single-molecule fluorescence assays in liquids is limited by diffusion to concentrations in the low picomolar range. Here, we demonstrate quantitative single-molecule detection at attomolar concentrations within 1 min by excitation and detection of fluorescence through a single-mode optical fiber in presence of turbulent flow. The combination of high detectability and short measurement times promises applications in ultrasensitive assays, sensors, and point-of-care medical diagnostics.

The possibility to detect single fluorescent dye molecules and quantum dots in solution using fluorescence techniques is widely exploited in fluorescence correlation spectroscopy[1] as well as in assay applications.[2−4] To achieve single-molecule detection, it is important to minimize background signals by using a small active volume and to optimize the overall photon detection efficiency. For single-molecule assays in solution, considering free diffusion only, the time between two successive single-molecule detection events increases inversely proportional to the concentration. We estimate that the average time it takes between two individual molecules in a 1 fM solution to reach a ∼10 fL-detection volume, assuming 3D Brownian motion, is already on the order of several tens of minutes (part 1 of the Supporting Information). This poses a practical lower limit of workable concentrations for real-time single-molecule detection assays based on diffusion in solutions in the low picomolar range and necessitates long incubation times for surface bound assays.[5] One way to overcome this diffusion limit is to vigorously stir the sample such that conditions for turbulent flow are created. Here, we demonstrate that it is possible to detect single molecules in solutions with nominal concentrations down to the attomolar range by excitation and detection of single-molecule fluorescence through a cleaved single-mode optical fiber in the presence of turbulence. Because of the resulting fast transport and rapid mixing within the sample solution, typical measurement times in this study are as low as 1 min. While the detection of fluorescence through the optical fibers of marked ligands immobilized to the fiber end has been demonstrated previously,[6−8] detection of single molecules has not been achieved so far although it was proposed earlier.[9] Our approach combines for the first time the power of single-molecule detection with the ability to quantitatively detect ultralow analyte concentrations in short times. In combination with suitable fluorescent probes, this opens up a plethora of possible applications in molecular biology and medical diagnostics. In particular, the small dimensions and flexibility of optical fibers and the short measurement time suggest applications in restricted geometries, point-of-care diagnostics, and in time-critical assays. Specifically this may include direct, on-site, and real-time detection of viruses by means of their specific DNA, the detection of other pathogenes including proteins, real-time monitoring of rare but important marker molecules in medical applications, or mobile environmental ultrasensing.

The experimental setup is depicted schematically in Figure 1A (for further details see Methods in the Supporting Information). To minimize the background, we optimize the system for fluorescence detection in the near-infrared (750−830 nm), as few naturally occurring compounds emit fluorescence above 600 nm.[2] The excitation light (635 nm, 2mW at the fiber exit) is transmitted through a 20−30 cm length of optical fiber and emitted via a 90°-cleaved fiber end into the liquid sample volume. Here it excites single molecules to saturation whose fluorescence is coupled back into the fiber if they reside in the detection volume. The fiber end's collection efficiency is estimated to be about 10× smaller

* To whom correspondence should be addressed. E-mail: hecht@physik.uni-wuerzburg.de.
[†] NCCR, University of Basel.
[‡] Universität Würzburg.
[§] Université Paris 7 et CNRS.
[∥] Trinity College.
[⊥] Department of Physics, University of Basel.
[⊗] Biozentrum, University of Basel.
[○] Universitätsaugenklinik, University of Basel.

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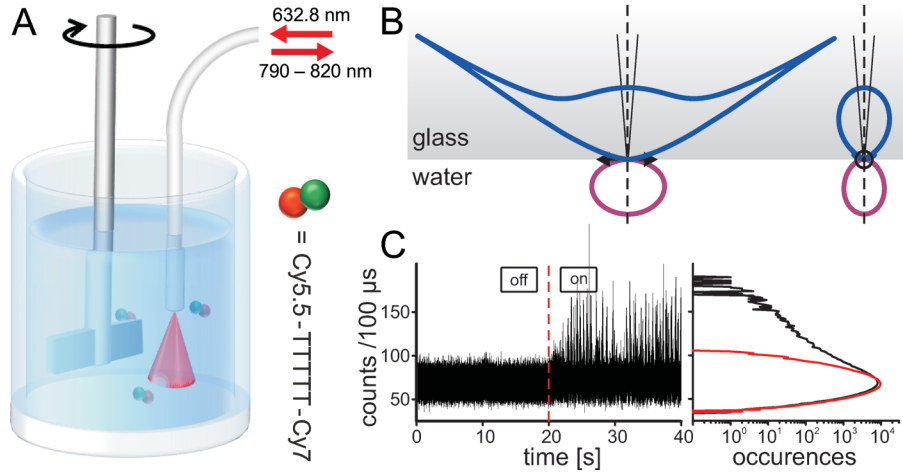

Figure 1. Principle of quantitative single-molecule detection at ultralow concentrations using excitation at 635 nm and fluorescence detection at 790−820 nm: (A) schematic experimental setup showing the analyte container with the optical fiber positioned close to the rotor blades and some analyte molecules (green-red dots, see the text and Methods in the Supporting Information). (B) Blue line: cuts through the distorted emission pattern of a single dipole emitter oriented parallel and close to the water/glass boundary at the cleaved fiber end (gray box) along the dipole and perpendicular to the dipole indicated by the black arrows. Only photons emitted into a small angular range (as indicated by the two thin lines next to the dashed surface normal) are transmitted to the detector. (C) Fluorescence time trace recorded from a 10 pM solution showing the difference between rotor off (0−20 s) and on (20−40 s), respectively. Left panel: corresponding histograms calculated separately for rotor on (black trace) and off (red trace) showing a deviation from the Poisson distribution only for the rotor on case.

than that of a 0.5 NA microscope objective. We take into account that in proximity of a plane interface, like the water−glass boundary at the fiber end, the emission pattern of dye molecules changes from the well-known dipolar pattern of a free dipole to an emission pattern which directs the majority of photons into the optical denser medium.[10] Representative emission patterns for the main dipole orientations are displayed in Figure 1B. However, only photons radiated within the angular range suitable for total internal reflection in the fiber are transmitted to the detector. For the present geometry and assuming the molecule to be close to the fiber end, this accounts for about 0.3% of the total emission by the molecule[11] (part 2 of the Supporting Information).

The use of a single-mode fiber as an optical transducer is hindered by background generated due to inelastic scattering processes in the fiber exhibiting a spectrum extending ~100 nm beyond the excitation wavelength. The fiber background is proportional to the intensity coupled into the fiber as well as to the length of the fiber. For fiber lengths of 30 cm, this generic background could already prevent successful single-molecule detection using standard dye molecules with their typically rather small Stokes shift. However, the Stokes shift can be increased by using a covalently linked donor−acceptor pair of dye molecules exhibiting efficient fluorescence resonant energy transfer (FRET) or suitably chosen colloidal semiconductor quantum dots. Here we use a Förster pair consisting of five thymidine bases connecting a Cy5.5 molecule at the 3′ end and a Cy7 molecule at the 5′ end (Cy5.5-TTTTT-Cy7, Genelink and Microsynth). The Förster pair is excited at a wavelength of 635 nm while the emission maximum for Cy7 occurs at 767 nm, well within the setup's detection bandwidth (see part 3 of the Supporting Information).

Figure 1C displays a time trace of fluorescence with a binning time of 100 μs recorded from a 10 pM dye solution with the rotor initially turned off. The trace shows a constant level of background counts with a Poissonian distribution due to background generated in the fiber but no fluorescence bursts due to diffusing molecules. As soon as the stirring is switched on (after 20 s), fluorescence bursts appear at a high rate and the histogram deviates from a Poissonian distribution. It may be noted that the signal-to-noise ratio seems to be unusually small compared to conventional single-molecule experiments. However, because of the fact that the fluorescence bursts can be clearly distinguished from the Poissonian background, detection of single-molecule events is possible with high efficiency (see ref 12). Small improvements of the signal-to-noise ratio will lead to a further increased detectability. Since molecules can only be efficiently collected if they reside within a small volume close to the fiber end (10 fL, see part 2 of the Supporting Information), diffusion is no longer sufficient at low concentrations (picomolar and below) to have a sufficient number of molecules passing the detection volume per unit time. In addition, excitation of molecules takes place in a much larger illuminated volume due to the Gaussian light beam emerging from the fiber end. Therefore, even for moderate concentrations, stirring is needed to ensure that enough molecules reach the detection volume before being bleached (Figure 1C, rotor off). We find that stirring at a rate of 20 000 rpm yields the best results if at the same time the relative position between the fiber and rotor is optimized (see part 4 of the Supporting Information). For the characteristic dimensions of the rotor (some 10^{-3} m) and the rather large velocities imposed by the stirring, Reynolds numbers of several thousands are easily achieved. The result is a highly turbulent flow in the sample volume which ensures rapid mixing.[13] We suggest that the fiber end acts as a discontinuity in the flow profile, which leads to the formation

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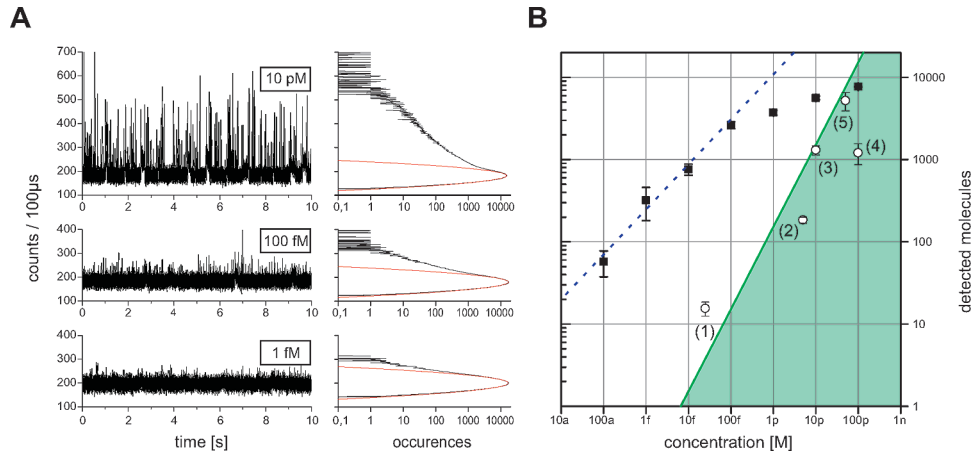

Figure 2. Dilution series: (A) Representative 10 s portions of 60 s time traces for different concentrations of dye molecules as indicated as well as respective histograms (black) with best fits to the Poissonian background (red) as needed for burst detection (part 1 of the Supporting Information). (B) ■, counted bursts above threshold as a function of the concentration measured within a 60 s integration time. A linear dependence is obtained between a concentration of 100 fM down to 100 aM (blue dashed line as guide-to-the-eye). Above 100 fM, the number of counted bursts deviates from linearity since bursts start to overlap in time (see ref 12 and part 5 in the Supporting Information). ○, Experimental data for diffusion-based assays taken from the literature (1,[14] 2,[15] 3,[16] 4,[3] and 5[17]). Note that ref 14 relies on active transport of analyte molecules through a capillary. All values have been scaled to a detection value of 10 fL. Solid green line and shaded area, theoretical upper limit and accessible area of the number of molecules entering the detection volume during 60 s considering Brownian motion only, see part 1 of the Supporting Information.

of additional vortices at the fiber end, which ensure the rapid exchange of fluorescent molecules in the detection volume at the fiber facet close to the core. The fact that detection of molecules for too low stirring rates is impossible indicates a sudden onset of vortex formation at the fiber end at rotor speeds of ~5000 rpm. The onset of vortex formation is also supported by an autocorrelation analysis of recorded time traces, which shows a broadening of the fluorescence bursts instead of a narrowing for higher stirring rates (see part 4 Figure 5b in the Supporting Information). These additional vortices do not appear in the vicinity of an infinitely extended boundary between liquid and glass which is encountered in detection schemes based on microscope objectives. In addition, the typical 200−300 μm working distances of high-numerical-aperture microscope objectives would limit the accessible detection range to low-velocity surface-bound flow sheets.

In order to demonstrate the possibility to detect ultrasmall concentrations of dye molecules within short measurement times of 60 s, a series of measurements have been performed for solutions with analyte concentrations varying over 6 orders of magnitude between 100 pM and 100 aM. Each acquired 60 s time trace is subjected to an analysis by a burst counting algorithm to determine the number of fluorescence bursts (see ref 12 and part 5 of the Supporting Information). Figure 2A shows typical 10 s portions of fluorescence time traces obtained from solutions containing analyte molecules in concentrations as indicated together with the respective histograms. For the higher concentrations (≥1 pM) the mean number of molecules that enter the detection volume per bin time is larger than one, causing occasional large count rates. In the corresponding histograms this leads to a pronounced deviation from the Poisson distributed background with a rather broad range of large amplitude bursts. With decreasing concentration, the number of detected fluorescence bursts decreases and the peak count rate above background observed in the histograms tends toward ~50 counts/100 μs limited by the saturation emission rate of single Cy7 molecules and the maximum overall detection efficiency. At a concentration of 100 aM, the number of detected fluorescence burst is still on the order of several tens. This number does not only allow a reliable detection of single molecules at this concentration but lets one reasonably envision an extension of the technique to even lower concentrations. Quite remarkably, 100 aM is already about 1000× smaller than the smallest concentration reported in the literature for diffusion-based single-molecule detection.[3,15−17] Figure 2B summarizes the results obtained by measuring a dilution series with our fiber-based setup. As a reference we also plot values for conventional diffusion-based single-molecule detection experiments in solution as reported in the literature. Furthermore, we plot the expected number of molecules that enter the detection volume within 60 s due to Brownian motion only as a function of the concentration (solid green line) according to the discussion in part 1 of the Supporting Information. This line describes the upper limit for the number of detected molecules during 60 s in a conventional diffusion-based single-molecule assay while the shaded area designates the regime accessible to conventional single-molecule detection assays. In conclusion, we clearly demonstrate that by combining turbulent flow and detection through a finite-sized optical fiber the fundamental detectability limit of diffusion-based single-molecule detection assays can be overcome. As a result, we are able to report fast quantitative single-molecule detection down to concentrations of 100 aM.

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SUPPORTING INFORMATION AVAILABLE

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