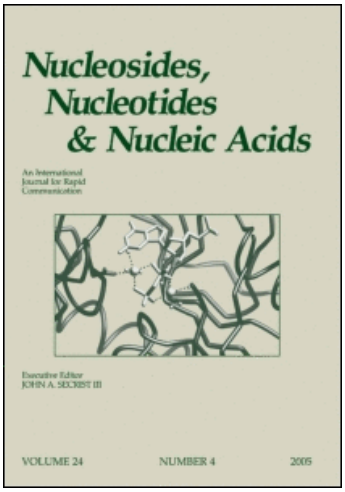

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Single Molecule Detection in Microstructures

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SINGLE MOLECULE DETECTION IN MICROSTRUCTURES

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ABSTRACT
Identification and manipulation of molecules on the single molecule level is best done in microstructures. The capability of observing single dye molecules in microstructures is demonstrated. Single molecules of DNA (106 bp) labelled with the intercalating dye TO-PRO-1 could also be observed.

Progress in single molecule detection in solution is an important step in analytical biotechnology. The ability to detect and identify single molecules offers the possibility to study molecular mechanisms without averaging and to reach ultimate specifity in chemical analysis. During the last few years, efforts of different groups (1-5) to push the sensitivity for single molecule detection in solution to higher levels have created a basis for further developments. In our work two-dimensional resolved detection of laser-induced fluorescence will allow single molecule trajectories to be followed in larger than diffraction limited volume elements. Spatially resolved detection of small molecules in solution will open up quite a number of new applications such as selection and sorting of suitable molecules in drug sreening, spatial resolution of biochemical reactions at the single molecule level and manipulation of single molecules with electric and hydrodynamic fields. A special detector for spatially (128 x 128 pixels) and temporally resolved (ns gated, 4 MHz single photon counting) single molecule detection is currently being developed and has reached the test phase. The detector has the potential of measuring cross-correlation functions in parallel. To achieve single molecule manipulation in combination with this detector, microstructures are well suited as sample compartments. Microstructured sample compartments allow the construction of

microreactors for biochemical reactions and open a means to develop micro-manipulators such as sorters and traps.

A setup with a zero-dimensional detection volume was used to observe single molecules of DNA (double-stranded, down to 106 bp) that were labelled with the intercalating dye TO-PRO-1 (Molecular Probes). Single molecules could also be observed in microstructured reactors. The reactors were made of silicon, in which channels with a diameter in the micrometer range were etched. In addition microstructures with a thin glass base were produced using special microstructuring techniques (6).

Observation of single molecules of DNA

Upon intercalating into DNA, TO-PRO-1 fluoresces intensely and this property has been used to identify single molecules of DNA with low background fluorescence from unbound dye molecules. In former studies, other groups were able to observe single molecules of DNA labelled with an intercalating dye. Matsumoto et al. stained different phage DNA with DAPI (7). Using the dye acridine orange, Houseal et al. identified single DNA molecules with a length of ≥4400 basepairs (8). Nie et al. (9) observed single molecules of λ-phage DNA by staining with YO-YO. Castro et al. used the bis-intercalator TO-TO-1 with DNA fragments of 24kb (10) and Petty et al. predicted a lower limit of 1.5 kbp for detection of single molecules of DNA with TO-TO-1 (11). The bis-intercalating dyes have a higher association constant for binding to DNA. This turns out to be an advantage in that the higher probability of finding DNA molecules stained with several bis-intercalating dye molecules gives higher fluorescence intensities. On the other hand, bis-intercalating dyes are known to interfere seriously with DNA replication. As we want to observe single molecules of DNA that can still be active in an evolutionary process, we decided to use the less mutagenic mono-intercalating dye TO-PRO-1 in our studies, taking into account a lower binding affinity of dye molecules to DNA.

A zero-dimensional confocal set-up was used to approach single molecule detection. Fig. 1 shows a scheme of this setup. The exciting laserbeam (514.5 nm from an argon-ion laser, model 2080, spectra physics) is focused by a lens and then reflected by a dichroic mirror. A microscope objective (Zeiss Plan-Neofluar, 63x, NA 1.25, oil, ∞)

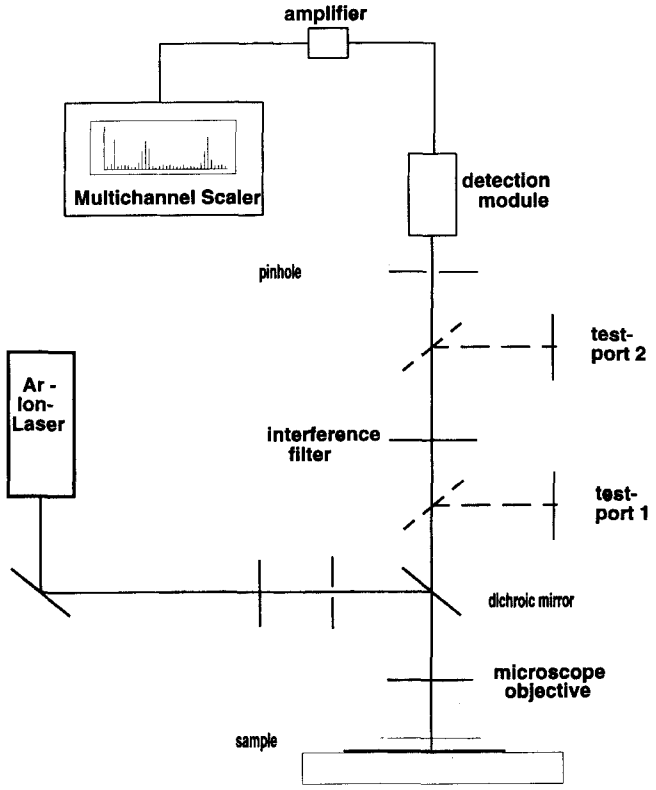

FIG.1: Setup for zero-dimensional single molecule detection with confocal microscopy.

focuses the beam on the sample which is deposited on a microscope table. Fluorescence is collected through the same microscope objective and focused on a pinhole (90 μm). The pinhole rejects stray light that does not originate from the direct focal spot of the laserbeam. Stray light is blocked by an additional interference filter (Omega Optical 565DF50). Two replaceable mirrors are used for aligning the optical system. The output pulses from the detector module (EG&G SPCM 200 PQ) are amplified and are then fed into a multichannel scaler. The observation of single molecules of a test dye, rhodamine 6G (Rh6G) in water at different concentrations, served as a tool to optimie the optics. A statistical analysis of the measured data confirmed that the observed signals originate from individual molecules (12).

Single molecules of DNA with different lengths (plasmide, supercoiled, 8813 basepairs and double-stranded DNA with 106 basepairs as in (13)) were stained with the intercalating dye TO-PRO-1, deposited on glass substrate and observed in zero-dimensional detection volumes. The concentration used was 10^{-9} M DNA and 10^{-6} M TO-PRO-1. Fig. 2 and 3 display the results of the measurements. DNA molecules with a length of 8813 basepairs are identified by large photon bursts (Fig. 2). These bursts originate from single DNA molecules that enter the focus and emit fluorescence until they leave the focus. As large DNA molecules have smaller diffusion constants than smaller DNA fragments their average time spent in the focus differs drastically. This results in shorter bursts for DNA molecules with a length of 106 basepairs, as displayed in Fig. 3. The bursts are reduced to small peaks indicating the presence of DNA molecules in the focal plane. Despite the large background noise of about 200 counts/2 ms single events can be clearly distinguished as they consist of more than 300 counts / 2 ms. These peaks are missing when the pure solvent is used as a sample (data not shown). To our knowledge this is the first time such short single DNA molecules labelled with an intercalating dye could be observed.

Zero-dimensional detection in microstructures

Measurements with samples of a test dye (Rh 6G in water) deposited in microstructured sample compartments were performed. Detection in microstructures is a precondition for single molecule control *en route* to molecular information processing. In microstructured sample compartments only minimal sample volumes are needed. The small structures enable us to observe and influence flowing samples on the single molecule level, so that they can be fully controlled when observed through a microscope. Different microstructures were tested (Fig. 4). A, C and D are completely made of silicon, in B the bottom is made of glass. Diameter and depth of the microstructures vary between millimetres and micrometers. The microstructure in B shows a broad channel and the bottom is made of glass. It is most similar to the object slide originally used. The version in A has a surface of reflecting silicon. The structure is broad, therefore no influences from the walls have to be expected under the microscope. The structures in C and D have a low depth and are very narrow. Stronger reflections here are anticipated. All structures

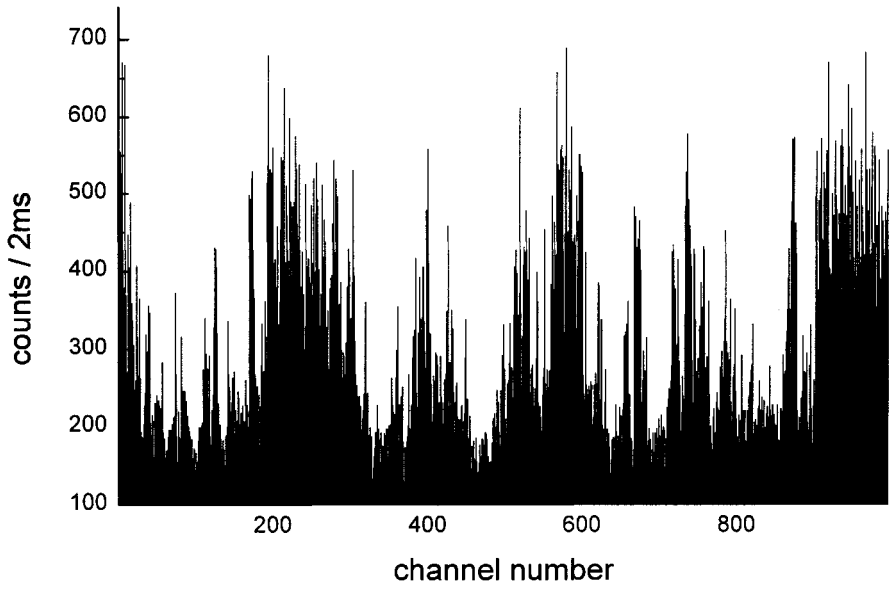

FIG. 2: Detection of single molecules of DNA with a length of 8813 basepairs. Each channel represents a time interval of 2 ms, so the x-axis is effectively a time scale.

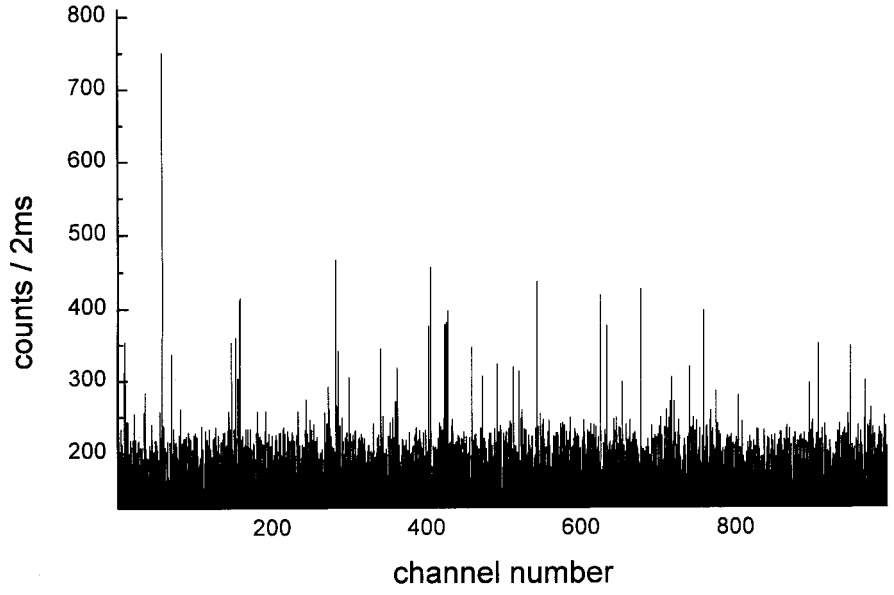

FIG. 3: Detection of single molecules of DNA with a length of 106 basepairs.

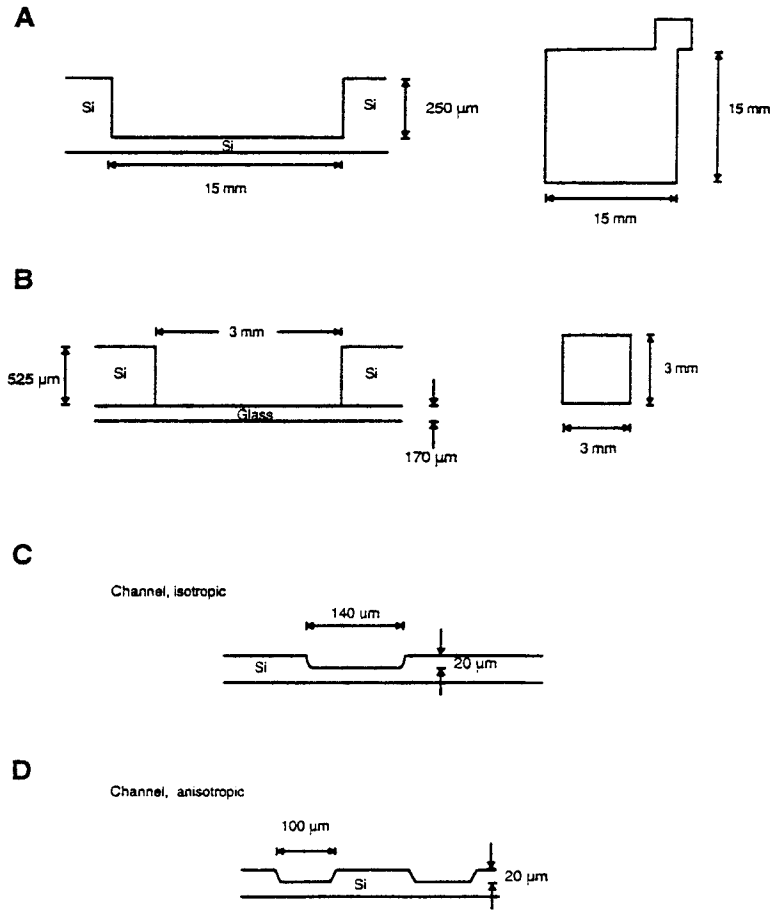

FIG. 4: Schematic of the microstructures used.

were filled with solutions of the test dye. A and B were covered with a cover slip. Structures of type C and D are closed by bonding glass to the silicon wafer. Fig. 5 and 6 display the results of measurements with structures of type B and D. All measurements were done with a concentration of 10^{-11} M. The background noise is found to be about 120 counts/ 2 ms in structure B and events with more than 350 counts/ 2 ms are detected. In smaller microstructures (D) the background increases (here: 180 counts/ 2 ms) but in burst events the number of detected photons is also higher. In the smaller microstructures more burst events are observed although the concentration is constant for all measurements. As the surface-to-volume ratio changes drastically when comparing

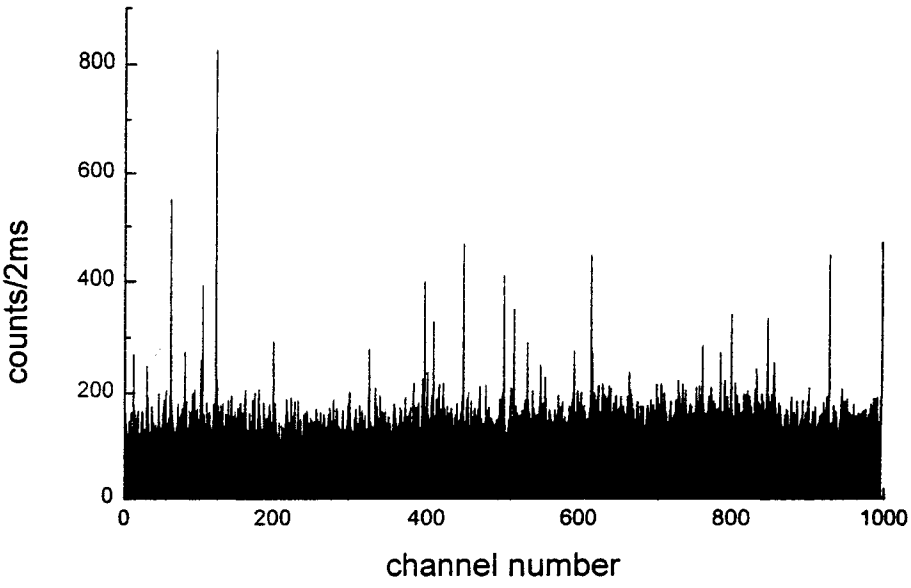

FIG. 5: Detection of single molecules in microstructures of type B. Again each channel represents a time interval of 2 ms.

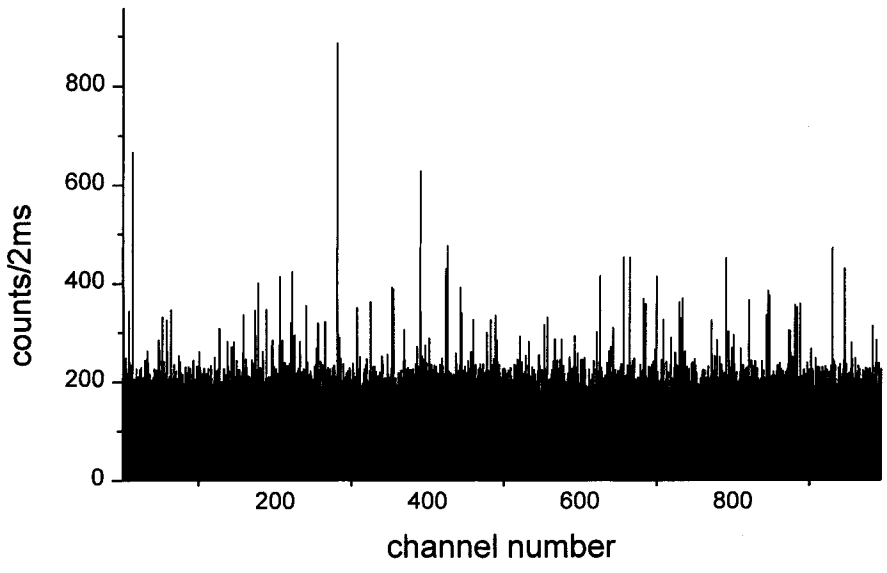

FIG. 6: Detection of single molecules in microstructures of type D.

structure B to structure D, surface effects could be responsible. If the affinity of the dye molecules to silicon surfaces is lower than to glass an apparent higher concentration of dye in the vicinity of the glass surface is expected.

The signal-to-noise ratio ($S/\sqrt{N}$) of 70 achieved in microstructures allows the identification of single molecules in the case of zero-dimensional detection volumes and is a basis for further developments towards spatially resolved single molecule detection in microstructures.

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