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Confocal Spectroscopy in Microstructures

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Abstract

Confocal instrumentation makes it possible to carry out spectroscopic measurements with a very high signal-to-background ratio. The transit of a single fluorescent molecule through the focal point of the light can be monitored with this method. The particle transport can be observed in transparent microchannels. Examples of fluorescence correlation spectroscopy and single molecule handling in microstructures are discussed.

Keywords: Fluorescence correlation spectroscopy; microstructures; single molecule detection; confocal; single molecule sequencing

1. Introduction

We use the term "confocal spectroscopy" to denote the method whereby a sample is probed by the focal point of a laser beam that is generated by illuminating the back of a high NA objective. The same objective collects a fraction of the emitted photons for detection. In most cases fluorescence emission is analyzed, although Raman or scattered light can also be measured to gather information about the particle [1-4]. Confocal spectroscopy should not be confused with confocal microscopy; the purpose of the latter is to obtain high-resolution images of objects [5], whereas the aim of confocal spectroscopy is to extract information from the entirety of a single, fixed volume element. Obviously, both methods can be combined [6].

It will be shown, a confocal setup yields a very high overall detection efficiency and a high signal-to-background (S/B) ratio [7]; this is important for a number of applications such as single molecule detection (SMD) experiments. The laser beam is focused by an immersion objective (NA $\geq$ 0.9,

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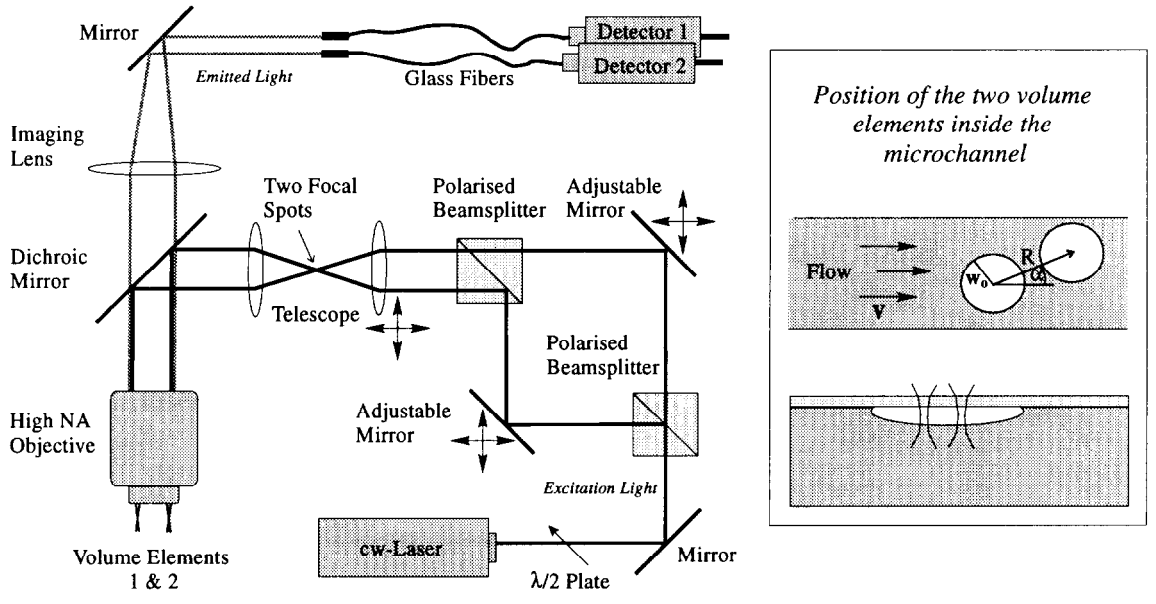

Fig. 1. Experimental setup for two-beam cross-correlation. Two laser beams with equal intensities are generated and coupled into the microscope objective. The two focal spots are imaged onto two glass fibers each leading to a separate detector. The detector signal is then crosscorrelated yielding the time a molecule need to pass both focal points.

magnification $M \geq 40$) and due to the diffraction limited focusing a volume element of approximately a femtoliter is created (i.e. focal radius $w_0 < 1$ μm), thereby decreasing the background noise to a level below the average intensity of the emission of a single fluorophore being in the volume element. High numerical aperture objectives have to be used to collect as much emission light from the fluorophore. For the first time single molecule detection has been shown with a confocal setup by our group [8, 9] and by using an even smaller volume element, a S/B ratio of 1000:1 was obtained [10]. The method is quite convenient because all experiments are done at room temperature in solution.

Currently, the main application of confocal spectroscopy is fluorescence correlation spectroscopy (FCS). Here intensity fluctuations, caused by fluorophores entering and leaving the focus, are recorded over time and *statistically* analyzed [see the article of P. Schwille, J. Bieschke and F. Oehlenschläger in this issue]. However, because a FCS setup is able to detect the traces of single molecules, the question naturally arises whether it is possible to detect them in an ordered way rather than in a random fashion (due to uncontrollable diffusion), so that one could even handle single molecules. If so, a wide range of applications never anticipated before would open up. Some important examples (SM trapping, SM selection and SM DNA sequencing) will be described below.

Flow is the method of choice to transport a molecule in a controlled way. This cannot be achieved in a free-hanging droplet or in a simple pit-like chamber, as it is done in standard FCS [11]. The submilli-

meter working distance of the objective has to be taken into account. This, and the fact that the molecules are to be observed during the transport process,

electrophoresis (CE), and two-dimensional microstructures. Since the capillaries are quasi one dimensional, they can serve as a purely analytical tool, as is done in CE. Standard CE requires at least nanomolar concentrations of the fluorophore passing by, however in a confocal setup one can detect single molecules within a capillary, as has been shown in our lab and elsewhere [12, 13]. Nevertheless, SM handling is not possible in ordinary capillaries and they cannot be used for our applications. In contrast to these disadvantages, microstructures can be fabricated easily according to the experimental demands, e.g. with channel junctions, crosses, openings or varying channel widths. When designed properly, they demonstrate the potential for SM detection and handling at the same time. In the following sections, we shall outline the work being done in our lab regarding confocal spectroscopy in microstructures.

2. The Setup

The experimental setup (Fig. 1) is basically the same as in normal FCS experiments [P. Schwille J. Bieschke and F. Oehlenschläger in this issue, 7]. In order to accommodate the microstructures, an x-y-z-stage with a 10-nanometer resolution is mounted below the objective. The position of the channels relative to the focus can be monitored in the microscope by using the oculars or a CCD camera.

Instead of a pinhole, the end of a multi-mode glass fiber serves as the aperture that defines the exact dimensions of the volume element (the other end of the fiber is coupled directly to the detector). In this way it is possible to observe not only one but several volume elements by placing two or more glass fiber ends next to each other, each of them leading to its own detector. This is

important for the two-beam FCS and for multielement detection described later. The whole system is designed for maximum detection efficiency. Only the necessary optical components and optimized filters are used and single photon avalanche diodes are employed as detectors. The overall detection yield is about 6 %, i.e. out of 16 photons emitted by the fluorophore one of them generates a detector pulse. This is the highest yield recorded so far [14].

Either the photon signal is correlated or the intensity traces are recorded directly using a personal computer. We have developed a special setup which is able to record up to eight traces simultaneously for several minutes with a channel width below 100 μs. With the same instrument one can observe the signal directly on a screen. The data are analyzed subsequent to the recording.

3. Microstructures

We use two different transparent microchannel structures: thin layer (TL) structures and laser ablation (LA) structures. The TL structures are fabricated by the Industrial Microelectronics Center in Kista, Sweden. Channels of varying sizes are etched on a quartz substrate [15, 16]. The channels are covered with a thin plate of quartz about 2 μm thick (Fig. 2). The diameter of the channels range from 5 to 100 μm. For an experiment, a single-channel structure is put into a holder. Thin platinum wires are integrated into the holder in order to generate an electric field in the channels, causing electroosmotic flow. Hydrodynamic flow can be induced by different cup filling heights, however then the velocity is more difficult to control.

The LA structures are prepared by the research group of Michael Stuke at the MPI for biophysical Chemistry. The substrate of these structures is plexiglas (PMMA). For producing the channels, a silicon mask with the predefined image is placed onto the substrate and rectangular channels are formed by UV laser ablation [17]. After removing the mask a cover slip is glued onto the surface to close the channels. The surface roughness of the channels of both kinds of structures is clearly below 100 nm. In principle, nearly any material can be processed by this method.

One needs an electric field strength on the order of 1 kV/cm to induce an electroosmotic flow of about 1 mm/s in PMMA or glass [18]. Therefore, structures

with a constant channel diameter therefore require a voltage of several kV between the cups. This is undesirable mainly for safety reasons. Problems like this can be avoided by varying the channel structure: the cross section has to be minimized at the location where the detection and sample handling take place. The greatest potential difference is then concentrated at the narrow part, and with moderate voltages (around 100 V) one is able to achieve a field strength of 1 kV/cm in the measurement zone.

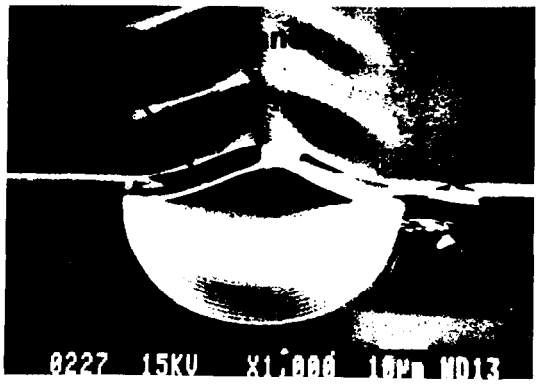

Fig. 2. Electronmicroscopic photograph of a cross section of a quartz wafer (with friendly approval of Industrial Microelectronics Center, Kista Sweden)

Both kinds of microstructures are quite robust and easy to handle. One can use them for a few experiments but beyond a certain stage it is not possible to refill them without the help of an external pressure. For the SMD experiments they can be used only once because some molecules from the previous experiment always adhere to the surfaces.

4. Single Molecule Detection in Microstructures

In the past few years, numerous advances have been made in SMD [19-24]. In the introduction a brief outline was given why it is advantageous to use a microstructure for detecting single molecules with a confocal setup. Basically, the fluorescent analyte has to be guided into the detection focus.

The detection efficiency of our confocal setup decreases considerably when using microstructures. In the free droplet one reaches signal-to-noise ratios of up to 1700:1 if the Raman bands of water are properly suppressed [7]. Under normal working conditions one achieves a signal-to-noise ratio in the order of 100:1. This is very different within microstructures because the background increases (intrinsic fluorescence of the bulk and scattering effects on surfaces). This is minimized by using transparent materials. In our experiments SiO_2 and PMMA were tested and found to be suited equally well with respect to background minimization. Figure 3 shows the intensity signal as a function of the relative position of the laser focus in the channel. In this experiment the channel had a depth of 15 μm.

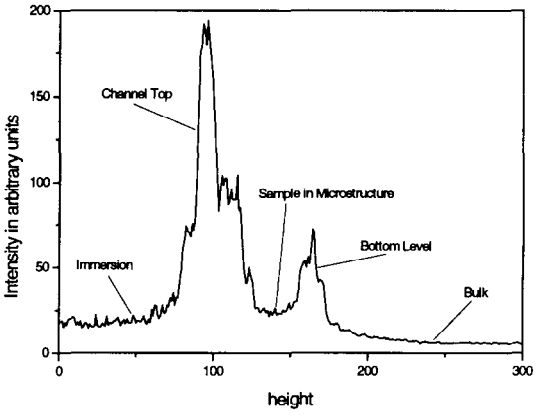

Fig. 3. Intensity as a function of the relative position of the focus in a SiO_2 wafer (height and intensity in arbitrary units)

A number of wall effects, especially adhesion, are also present in microstructures.

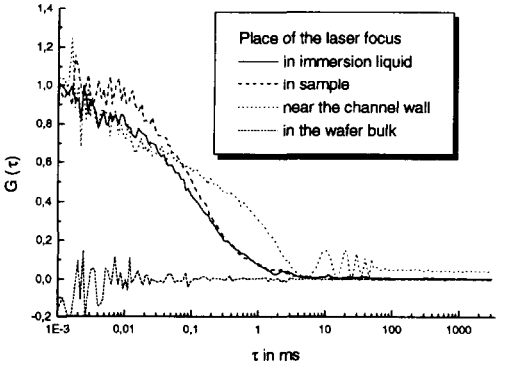

Fig. 4. Normalized autocorrelation function at different focal positions. The shift towards longer diffusion times occurs if the focus is positioned near the channel walls.

A distinctive indication of such a phenomenon is the much longer diffusion time for molecules near the channel walls as determined by FCS (Fig. 4). Diffraction spots of single molecules can be seen by eye to remain for fractions of seconds in the laser focus indicating that the molecules diffuse slower close to the wall than in free solution.

At subnanomolar concentrations bursts of single molecules are clearly observable and can be distinguished from the background. Figure 5 shows a statistical analysis of one single volume element in a PMMA-channel where the background is the pure Raman signal of water. The fluorescence tail of photons stands out strongly. As it has been pointed out by Goodwin [25] and by us [9] the photon count distribution curve decays exponentially if the focal point is smaller then the dimension of the sample stream.

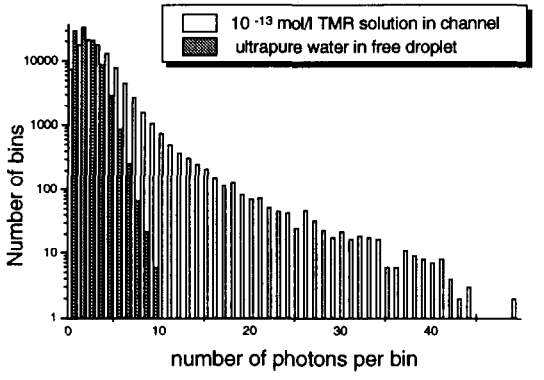

Fig. 5. Statistical analysis of single fluorescent molecules (10^{-13} mol/l) compared with pure Raman background from water. The number of photons in one time channel is taken as the x-axis and the number of bins with that amount of photons is the y-axis

A special setup for complete detection of all fluorophores is discussed below.

5. Two-Beam FCS

The flow profile within a channel depends on various parameters: the nature of the flow, the flow velocity (or, more specifically, the Reynolds number) and the geometry of the channels [26]. Thus, it is necessary to know the flow profile under given experimental conditions. This has been determined by "two-beam FCS" [14, 27, 28]. In standard FCS, one usually measures the time for a particle to diffuse through the volume element. In principle, it is also possible to measure the flow velocity [29] but this can be done effectively only if the flow time through the focus is shorter than the diffusion time. Also, the direction of the flow cannot be determined by autocorrelation FCS when the focus has the usual circular shape.

In two-beam FCS, two focal points are generated by two parallel laser beams, both placed within the channel (Fig. 1). The focal points are adjusted to the same focal plane but separated in the xy-position, so that they can be observed separately by different conjugated fiber ends. The intensity fluctuations of the volume elements are fed into a correlator card which cross-correlates the signals. By this method, a

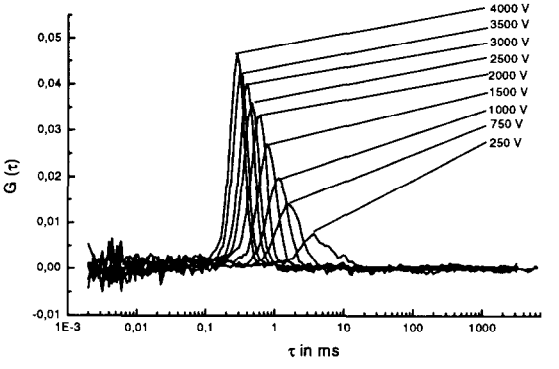

Fig. 6. Cross-correlation curves at different electroosmotic velocities

correlation curve is calculated that has a distinct maximum (Fig. 6). The position of the maximum yields the average time for the particle to flow from one focus to another. By using the mathematical model of a flow-diffusion system for a cross-correlation fitting function one obtains the values for flow, diffusion and system geometry with an accuracy of ≈ 10 %. The mathematical model is discussed further in the literature [14, 28]. The flow direction can also be determined because the correlation amplitude of the fluctuations depends on the trajectory of the particles through *both* focal points.

The time to measure a two-beam FCS experiment is on the order of 10 seconds; the spatial resolution depends on the size of the focal points, i.e. 0.5 - 2.5 μm. As an example, we have measured the flow profile through a cross section of an LA structure (Fig. 7). The flow was induced by electric fields (generating a piston-like profile), but as can be seen from the parabolic dip in the profile, some hydrodynamic flow also occurred in this case [18, 30].

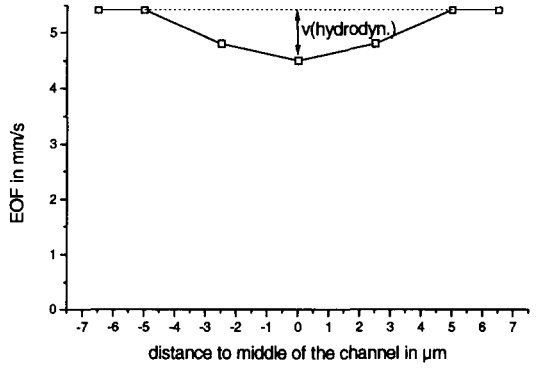

Fig. 7. Electroosmotic flow profile in a 15 μm wide channel at 600 V/cm

6. Applications

As mentioned above, the ability to guide particles in a controlled way through microchannels (and thereby extracting information from them) opens up the new field of single molecule technology. Since the experimenter is no longer restricted to sampling statistically large numbers of single events which are not resolvable individually, it is possible to perform procedures similar to batchwise macroscopic events, such as trapping, selecting or counting. Some future applications will be described below.

6.1. Rapid DNA Sequencing based on Single Molecule Detection

In the last few years the interest in the rapid analysis of genetic material, especially for clinical diagnostics, has increased tremendously. Classical techniques are orders of magnitude too slow and also too expensive to solve many problems [24, 31]. The present level of research opens up the possibility of developing new, faster methods that require less material and time for routine diagnostics [19, 32].

One promising approach concerns single molecule techniques using only a single strand of DNA for each analysis. The detection principle is based on the fluorescence spectroscopy of single fluorophores in microchannels as described above [25, 33]. For this method the DNA-fragment must be labeled with fluorescent dyes. The genetic material of interest is amplified in a standardized procedure via polymerase-chain-reaction (PCR) in the presence of fluorescently labeled nucleoside triphosphates [34], such as the rhodamine or cyanine derivatives.

One primer in the PCR is biotinylated, that the DNA can be linked to a streptavidin-coated bead. This procedure is necessary to select a single molecule out of the solution and to handle it inside a microchannel. Details of this technique are described below. Once a bead that is loaded with one DNA strand has been put into the right position in the microreaction system, a solution of exonucleases is pumped through the channel. In most cases the proofreading function of a DNA polymerase serves as a 3'-5'-exonuclease The enzyme degrades the single strand gradually, releasing labeled mononucleotides according to the given sequence.

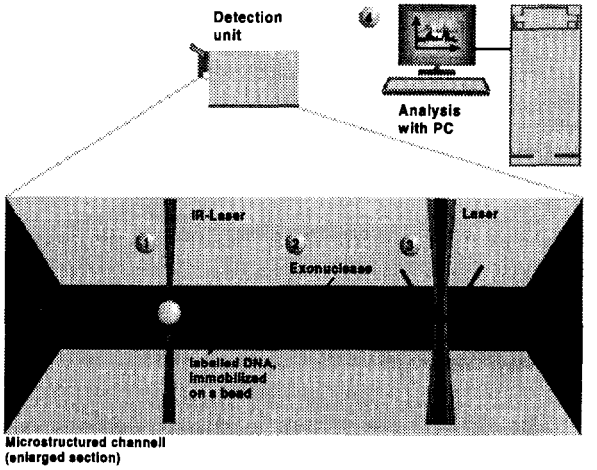

Fig. 8. Schematic setup for single molecule sequencing in microstructures

By applying an electroosmotic flow, the released mononucleotides all move into the same direction where they pass the elliptical volume element of the laser beam.Here the photons are detected that are emitted from each passing molecule (Fig. 8). The speed of sequencing is determined by the cleavage rate of the enzyme, about 100 bases per second.

The nature of the detected base can be differentiated according to the fluorescence signal because each base has been labeled with a dye specific for each nucleotide and each dye has different spectral properties [37].The signals are recorded with a special PC-controlled instrument and the sequence of the investigated fragment of DNA is thus determined.

One basic problem in sequencing a single DNA molecule is the handling of this molecule. In our approach we link the DNA to a bead of 2 μm diameter. This solution containing the beads is introduced into reservoir I in the microstructure (Fig. 9). Between reservoirs I and III a flow is created, forcing the beads to move continuously until the selection point is reached. If exactly one bead is at the selection point, a pulse of 100 to 300 V between reservoirs II and IV is applied for two seconds inducing

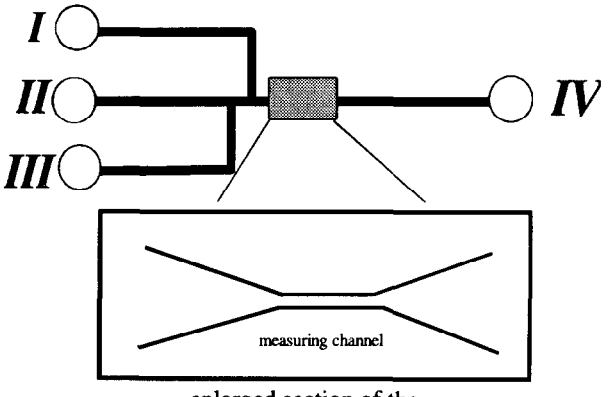

Fig. 9. Schematic picture of a PMMA-microstructure

a strong electroosmotic flow towards the measuring channel. The selected bead is then optically trapped by a 400 mW Nd:YAG infrared laser (1064 nm) and transported to the narrow part of the channel. There it is fixed either by adhesion to the walls or by trapping.

Next, a buffer solution with an exonuclease is pumped electroosmotically to the position of the bead. Since beads with one DNA on them are still difficult to isolate, beads highly loaded with DNA have been used so far (approx. 10^4 DNA-strands). For actual sequencing of course the beads have to be loaded with only a single DNA strand. To solve this problem, promising approaches have been demonstrated [38].

A basic problem in this context is the faultless detection of every fluorescently labeled mononucleotide. In our approach we use several volume elements instead of a large single focus. The cw-laser beam is shaped elliptically by a cylindrical lens and focused by the microscope objective. The elliptical volume element is imaged onto a glass fiber bundle where seven fibers are aligned in a row. Each fiber act as a pinhole to suppress the background, and each fiber is connected to its own separate detector, like in two-beam FCS.

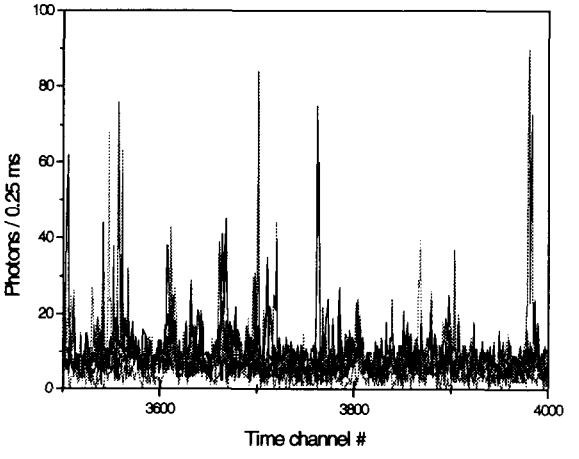

Fig. 10. Typical bursts from single TMR molecules in water detected from an elliptically shaped volume element (flow 500 μm/s; bin 250 μs

So every fiber filament "sees" only a small part of the illuminated volume. Thus the background is reduced by a factor of seven, and single molecules can be distinguished well from Raman noise and scattering effects (Fig. 5).

So far, we are able to decrease the background to approximately 20.000 photons per second. At the moment we are working on a computer algorithm to

carry out real time measurements of fluorescence photons from single molecules. Other groups are collaborating with us to combine this conical setup with a pulsed excitation and gated detection [39]. This would result in a drastic decrease in the background signal and concomitandly in an improvement of the S/B ratio.

6.2. Single Molecule Trapping

The number of photons which can be extracted from a single fluorophore depends mainly on the residence time of the molecule within the volume element or its photobleaching time, whichever is shorter. In solid state SM experiments, where fluorophores are embedded in a rigid matrix at cryotemperatures, both values are practically infinite and therefore various experiments regarding the electronic properties can be pursued [40]. However, these experimental conditions are not useful for biotechnological applications. In aqueous solutions at room temperature, there is no way to ignore diffusion. In addition, the oxygen dissolved in the solution can react with the fluorophore in the excited long-lived triplet state causing irreversible photodestruction [41, 42]. It is assumed that a dye molecule like rhodamine 6G can cycle 10^4 to 10^5 times before decomposing [25]. Until now only an indirect estimate of the turnover number can be made because the bleaching lifetime is usually longer than the diffusion time. This evaluation yields only the short-lived part of the bleaching time distribution resulting in a large error range. If bleaching occurs faster than the diffusion of the particle out of the focus, either the volume element [44] or the laser intensity must be made larger [42] than usual - resulting in an increased background signal and therefore a smaller S/B ratio. One method to overcome this problem is to increase the diffusion time artificially by increasing the viscosity of the solvent. Of course, the resulting liquid has, due to a different polarity, a strong influence on the emission properties of the dye. One has to interpret the data with caution if one wants to extrapolate to pure water samples. To avoid diffusion we intend to use active trapping [19]; instead of having just one volume element, seven are arranged in a flower-like 2D-stacked manner (Fig. 11). Again, every volume element is linked to its own detector so that seven intensity traces with the usual high S/B ratio can be measured. In this way the actual position of a single molecule can be monitored. The molecule is supposed to be held the in the center position. For this purpose, a cross-like microstructure is needed.

The four channels are linked to electrodes. Whenever the molecule is moving, e.g. to the right, a short electric pulse between the left and the right electrode is applied in order to push the molecule back to the center point. The electronic setup described above has the ability to record not only the traces but also to trigger real-time output pulses. The reaction time of the electronics is faster than the diffusion time of the molecules (50 - 1000 μs).

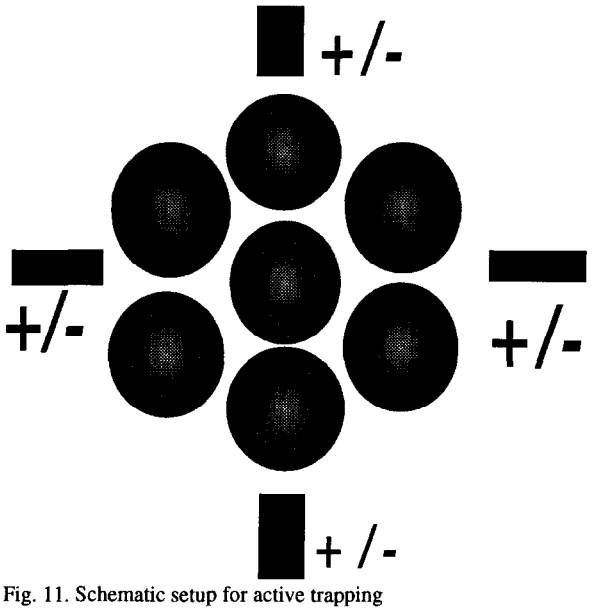

Fig. 11. Schematic setup for active trapping

So far, we have installed the electronics and designed the structures for trapping. Once the time development of the flow profile during pulsed electric fields has been examined, we can start to trap single particles; first large ones, such as 10 nm latex beads, then we can gradually reduce the scale to single fluorophores with diffusion constants of $\approx 3 \cdot 10^{-6}$ cm^2/s. If it was possible to increase the residence time to infinity, one could directly count the number of fluorophores emitted prior to photodestruction. Furthermore, one could carry out numerous electronic state experiments similar to those per-

formed in solid state SMD and, most interesting, the ergodic principle of statistical mechanics could be checked. From this point of view, SM trapping will be an exciting basic research topic in physics.

6.3. Single Molecule Selection

The principle of the Darwinian model of evolving species is well known: the individuals which have the optimal fitness with respect to given environmental conditions posses the highest chance to reproduce. This phenomenon has found to be true not only for living systems, but also for self-replicating macromolecules such as RNA [45]. Several approaches have been taken to use "Evolution in the test tube" for optimization of Enzymes or drug design [see the article of A. Koltermann and U. Kettling in this issue]. In Manfred Eigen's department several machines for the evolution of chemical substances have been constructed [see the article of G. Strunk and T. Ederhof in this issue]. These machines cannot select single individual molecules, but rather check whether one *pool* of molecules (i.e. a large number of molecules) is adjusted better to the boundary conditions than another pool. This does not suppress evolution completely, but decreases its effectiveness remarkably - it takes more time and material to evolve molecules. If it was possible to pick out single molecules from a large pool, the method would work more effectively, creating a new type of molecule after a few generations.

Confocal spectroscopy in microstructures provides a method to screen a large number of molecules. The following simple system is an example: an RNA oligomer is searched for that has the highest affinity to a certain protein. The RNA is labeled with a fluorophore. As long as the RNA is bound just to the fluorophore the label is quenched remarkably; as soon as a protein is attached to the RNA molecules the quantum yield rises drastically. A Y-like structure is needed for the selection process. The RNA-protein mixture is filled into the stem cup and pumped from the upper to the lower branch in Fig. 12. The focal point of the probe light is placed at the branch intersection. Whenever a bound RNA molecule passes the volume element, it emits a photon burst whereas free RNA molecule would yield remarkably less photons. If a certain treshhold is reached an electric field pulse is triggered selecting this molecule into the upper branch. In this way those RNA molecules that bind more strongly to the protein are concentrated in the upper branch cup, from where one can extract them and amplify them by PCR, 3SR or a similar method. After a few cycles one obtains RNA molecules with high affinities to the protein.

This method works only with substances that can be amplified such as RNA or DNA. In order to broaden the applicability, it would be a big advantage to couple the phenotype (protein) and the genotype (DNA) into a single entity [46]. As soon as this is achieved, the possibility to induce a change in the fluorescence properties and therefore in the selection criteria, increases dramatically. For example, it would be interesting to evolve new red fluorescent proteins from the available GFP molecules. At present, this is just speculation.

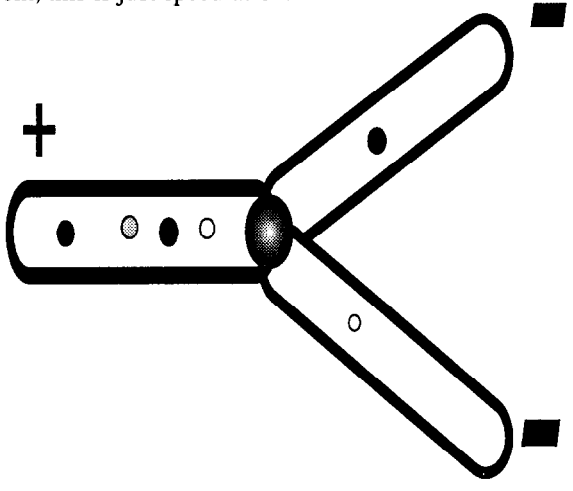

Fig. 12. Y-like structure for single molecule selection or continuos flow kinetics. By applying different voltages the flow velocity or the direction can be varied. The filled circles represent the strongly fluorescent molecules and the open circles show the quenched labels.

6.4. Continuous Flow Kinetics Revisited

By reversing the direction of the flow in Fig. 15, the components of the two branches can be mixed. This resembles the first simple kinetic experiments: two components react in one of the branches with a given flow velocity v. The reaction begins at the branch point and proceeds with time as the mixed

components flow along the branch. Assuming that the fluorescence properties of the reaction product differ from that of the educts (via intensity, color, S^1 lifetime, triplet state etc.), one can monitor the fluorescence at a certain distance d from the intersection. The fluorescence changes with time along the direction of flow as a function of the distance according to $t = d/v$, enabling one to determine the kinetics of the reaction and eventually to draw back conclusions about the reaction mechanism. The drawback of this method has been the poor lateral resolution (≥ 5 mm) which limited the time resolution to 50 ms even at a flow velocity of 10 cm/s. Even worse, the faster the flow, the more sample is wasted during the experiment. To overcome this, the method of "stopped flow" mixing experiments was developed [47]. It is still an important tool for measuring the kinetics of interactions between e. g. nucleic acids and proteins.

However with continuous flow mixing experiments within microstructures it becomes immediately obvious that one is dealing with a completely different order of magnitude. The size of the volume element is about 1 μm. Since it is also possible to achieve flow velocities on the order of 1 cm/s in the microchannels, one can obtain a time resolution of 100 μs for nonequilibrium reactions. The amount of sample needed is a few μl of a nanomolar solution. Just by this simple estimation it is clear that the macroscopic setups for measuring kinetics can be scaled down to the microstructure sizes.

Our aim is to measure the exonucleic digestion of a DNA strand. For this an intercalating dye is added to the DNA solution. The dye is nonfluorescent when it is not intercalated. Therefore the intensity of the fluorescent signal with respect to the distance d yields the length of the uncleaved DNA strand and thereby the cleavage rate. One big advantage over the macroscopic setups is that the average intensity and additionally the fluctuations can possibly be measured by FCS. This means that the time resolution for measuring kinetics with FCS could be scaled down from a few seconds in standard FCS by four to five orders of magnitude. One could even measure the causal dependence between two reaction steps at time t_1 and t_2 by cross-correlating the fluctuations of the volume elements positioned at d_1 and d_2 via two-beam FCS.

Concluding Remarks

This paper shows the prospects of confocal spectroscopy in microstructures. No doubt, there is a need to downsize the conventional detection and preparation techniques. Obviously, microstructures offer a great potential for handling small sample sizes down to single molecules. Unfortunately at the present the availability of microchannel structures is limited because techniques for the mass production of such structures must still be developed. Most probably, future structures will be manufactured of plastic by injection molding techniques. This will reduce the price per wafer to a reasonable level, thus enabling single molecule detection and handling on a large scale.

Acknowledgments

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