Seeing single molecules

Andrew J. de Mello describes recent research focussed on detecting single molecules within microfluidic systems

†It is fair to say that the development of microfluidic systems for use in the physical and biological sciences has progressed at a significant rate since the early 1990's. The potential benefits afforded by system downsizing are documented and have provided persuasive reasons for the transferral of instrumentation from the macroscale to planar chip formats.[1,2,3] For example, huge leaps in the application, flexibility and efficiency of separation techniques have been realised by miniaturising column dimensions and creating monolithic fluidic networks on planar substrates. Indeed, today almost all separation techniques based on electrophoretic or chromatographic discrimination have been successfully transferred to chip-based formats. Nevertheless, although system downsizing affords many performance gains, it also generates many new problems and challenges. Most of these are due in large part to the very small volumes encountered on the microscale. For example, the efficient delivery of fluids into microfluidic systems relies on the creation of high fidelity macro-to-micro interfaces. These are often both difficult and complex to engineer.[4] In addition, the high surface area-to-volume ratios typical of small volume environments mean that internal surfaces play an important role in defining the achievable efficiencies in both separation and reaction systems.[5] Another significant challenge arising directly from the adoption of small volume systems is the ability to efficiently detect analyte molecules. When performing capillary electrophoresis within chip-based microfluidic systems, injection volumes are commonly no larger than 50 pL. This means that for an analyte concentration of 10^{-8} mol L^{-1}, only 3×10^5 molecules are available for separation and detection. This simple calculation demonstrates that detection is undoubtedly one of the primary issues determining the practicality and application of microfluidic systems.[6]

†In this article SMD refers to detection of single fluorescent molecules in solution, and reference will not be made to single molecule detection techniques incorporating alternative optical phenomena.

Indeed, it has long been accepted that the minimum size limits for most microfluidic devices are chiefly determined by capabilities of the system detector.

A cursory survey of the literature indicates that small volume detection *within* microfluidic systems is most commonly performed using one of the following techniques: laser-induced fluorescence (LIF), electrochemistry, UV-visible absorption, indirect fluorescence, refractive index variation, Raman spectroscopy, chemiluminescence, and electrochemiluminescence.[6] All of these, apart from electrochemical methods, involve electromagnetic radiation. The widespread adoption of optical methods is not surprising. Early examples of chip-based analytical systems generally utilised glass as the substrate material of choice. Silica-based glasses are chemically similar to silicon, and thus can be processed or machined using standard lithographic techniques. Importantly, glasses are transparent in the visible, near-UV and near-IR regions of the electromagnetic spectrum and thus allow sample contained within to be probed *via* the absorption, emission or scattering of radiation. Furthermore, more recent developments in polymer micromachining have made available a new range of substrate materials with similar optical properties[7]. Of the optical techniques listed, LIF methods are the most prevalent for a variety of reasons. The exquisite sensitivity and selectivity of emission spectroscopy is ideally suited to non-invasively probing the small volumes and low analyte concentrations that are associated with microfluidic systems. Furthermore, much of the early interest in chip-based systems was driven by the need to create high-throughput analytical instruments for nucleic acid analysis[8]. Importantly, established methods for fragment sizing, sequencing and DNA amplification all incorporate fluorescent chemistries to facilitate detection, making their transferral to planar chip formats facile. In addition, developments in laser technologies over the past two decades have expanded the availability of low cost light sources that span the visible and infrared regions of the electromagnetic spectrum.

As stated, the ability to perform high sensitivity measurements is extremely valuable in many applications, including DNA analysis, immunoassays, environmental monitoring, and forensics. Over the past few years a number of optical techniques with sufficient sensitivity have been developed to detect single molecules. Scanning probe microscopies (most notably scanning tunnelling microscopy, atomic force microscopy and other near field microscopies) have been hugely successful in the analysis of surface bound species, but for single molecule detection (SMD) in fluids optical methods involving the measurement of absorption or emission processes have proved most successful. This article is intended to briefly introduce some of the basic concepts behind these techniques and provide some examples of the application of SMD within microfluidic systems.

Single molecules yield multiple photons

The crucial concept underlying most optical approaches to single molecule detection is that a single molecule can be cycled repeatedly between its ground electronic state and an excited electronic state to yield multiple photons. Inspection of Fig. 1 illustrates this idea in a simplified form. In condensed phases, excitation from a ground electronic state to an excited state is followed by rapid (internal) vibrational relaxation. Subsequently, radiative decay to the ground state is observed as fluorescence emission and is governed by the excited state lifetime. Under saturating illumination, the rate-limiting step for this cycle is dictated by the fluorescence lifetime, which is typically of the order of a few nanoseconds. If a single molecule diffuses through an illuminated zone (normally the focus of a laser beam) it may dwell in that region for several milliseconds. The rapid photon absorption–emission cycle can therefore be repeated many times during that time, resulting in the production of a 'burst' of fluorescence photons as the molecule transits the beam. Notionally, the number

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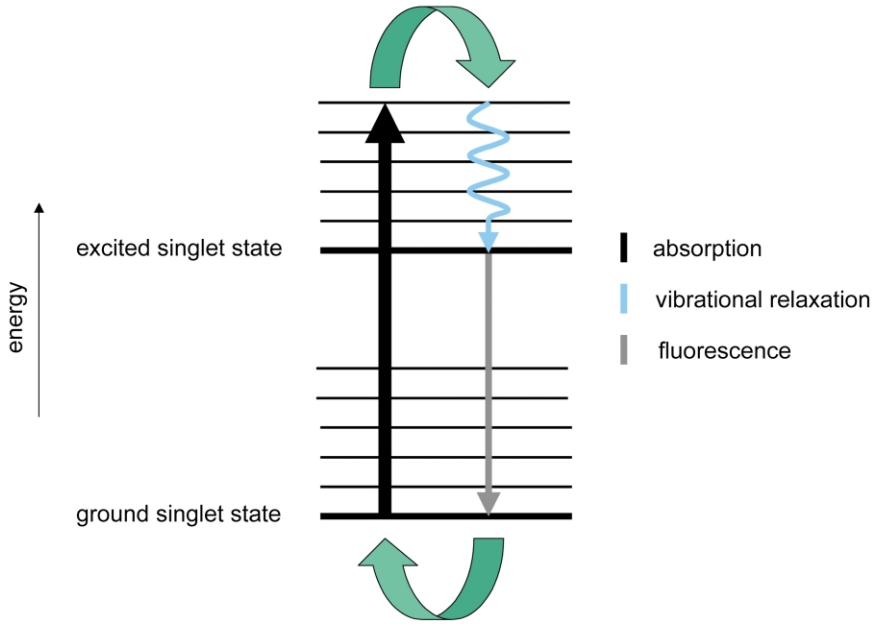


Fig. 1 Schematic illustration of the molecular absorption–emission cycle in condensed phases. Competing processes that may reduce the ultimate photon yield are not shown.

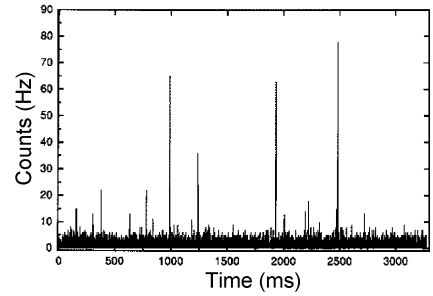


Fig. 2 Photon burst scan for a tetramethylrhodamine biomolecule solution (0.01 pM) in a hanging droplet using confocal fluorescence microscopy. Probe volume = 1 fL. (Adapted with permission. Copyright 2000, The American Chemical Society).

of emitted photons (or the burst size) is limited by the ratio of the beam transit time and the fluorescence lifetime. This means that a single molecule (with a fluorescence lifetime of 5 ns) spending 5 ms in a laser beam may emit up to 1 million photons.

In reality, this situation is never achieved as the combination of non-unity fluorescence quantum efficiencies and photodegradation processes limit the photon yield to about 10^5 photons. The next problem is that it is impossible to detect all of the emitted photons. Since spontaneous emission generates photons travelling in all directions (4π steradians) only a small fraction may be collected by the best optical system. In addition, photon detectors are non-ideal and will only register a certain fraction of all incoming photons. Fortunately, recent advances in optical collection and detection technologies enable registration of about 1% of all photons emitted. This results in a fluorescence burst signature of up to 1000 photons or photoelectrons. It should also be realised that only certain species can be detected at the single molecule level. Specifically a molecule is well suited for SMD if it possesses a large molecular absorption cross-section at the wavelength of interest, has a high fluorescence quantum efficiency (*i.e.* favours radiative deactivation of the excited state), has a short fluorescence lifetime, and is photostable. Ionic dyes (such as the xanthene dyes Rhodamine 6G and Rhodamine 101) are ideal for SMD applications as fluorescence quantum efficiencies can be close to unity and fluorescence lifetimes below 10 ns. Importantly, larger (non-fluorescent) macromolecules (such as proteins and nucleic acids) can be probed at the single molecule level by extrinsic labelling with appropriate fluorophores. For example, double stranded DNA units can be loaded with multiple dyes *via* intercalative binding. This is highly advantageous for SMD applications since a duplex containing n base pairs can normally accommodate between $n/4$ and $n/2$ dye molecules, and thus will afford significantly increased photon yields when compared to signals from single fluorophores.

A typical example of a photon burst scan is shown in Fig. 2. Here the mean number of molecules within the probe volume at any time is much less than one and photon bursts (indicative of single molecules moving through the illuminated region) can be seen above the background signal. The width and height characteristics of the bursts are variable due to the stochastic nature of the measurement and the range of possible molecular trajectories through the probe volume. It can also be seen that SMD depends critically on the optimisation of the fluorescence burst size and the reduction of background interference from the bulk solvent and impurities. To illustrate the enormity of this challenge, in a 1 nM dye solution each solute molecule occupies a volume of approximately 1 fL. However, within this same volume are contained in excess of 10^{10} solvent molecules. Although scattering cross-sections for an individual solvent molecule may be small, the collective scattering signal from the solvent can easily swamp the desired fluorescence signal. Fortunately, the solvent background signal can be drastically reduced by minimising the optical detection volume (since the signal from a single molecule is independent of probe volume dimensions, whilst the background scales proportionally with the probe volume).

The small volumes within which single molecules can be detected are generated in a variety of ways.[9] Picolitre volumes can be created using mutually orthogonal excitation and detection optics focussed in a flowing stream.[10] Femtolitre probe volumes are generated using confocal methods.[11] Confocal detection techniques are versatile and have been widely adopted for SMD in a variety of systems. Another common approach to performing SMD in solution involves the physical restriction of single molecules within microdroplets.[12,13] With droplet volumes less than 1 pL imaging of the entire droplet enables single molecule detection to be realised with good signal-to-noise ratios. More recently, spatial confinement of molecules in capillaries and microfabricated channels has been used to create probe volumes between 1 fL and 1 pL, and thus facilitate single molecule measurements.[14] Accordingly, it is important to realise that microfluidic systems provide ideal environments in which to perform SMD, whilst conversely the small volumes and low concentrations typical in microfluidic systems often require detection methods that offer the ultimate limit of detection. It is this synergy that has initiated interest in performing SMD within microfluidic

systems. A brief description of some recent studies in this area is now provided. Due to the considerable interest in single fluorescent molecule detection, a vast number of publications have appeared over the past decade. The reader is directed to a number of excellent review articles that detail these fundamental studies;[9,15–17] our focus here is the application of SMD within microchannel systems.

SMD in microstructures

Carlo Effenhauser and colleagues at Novartis reported one of the first examples of SMD in microfabricated chip structures in 1997.[18] In the same publication, the authors also provided the first report of electrophoresis microchips structured in PDMS using soft lithographic techniques. Injection and detection of full length λ DNA molecules in free buffer yielded results similar to those shown in Fig. 3,

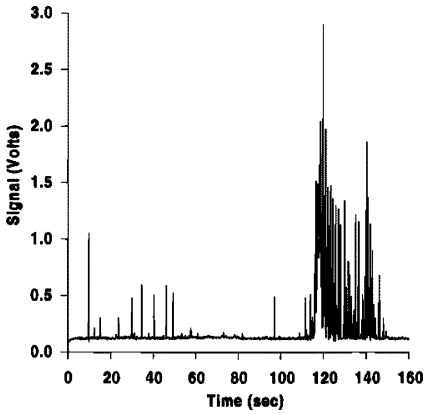


Fig. 3 Injection and detection of λ DNA molecules in free buffer solution. Channel cross section, 50 μm (width) × 20 μm (height); E = 86 V cm^{-1}; L = 20 mm. DNA concentration, 41 pM; injection volume, 150 pL; injected amount, 6.1 zmol (~3700 molecules). (Adapted with permission. Copyright 1997, The American Chemical Society).

where each fluorescence burst was attributed to the passage of a single molecule through the detection volume. High signal-to-noise ratios were achieved, primarily due to the fact that each DNA molecule was loaded with approximately 5000 intercalating dye molecules. At a similar time Harald Mathis and colleagues at the Institut fœr Molekulare Biotechnologie, Jena described SMD of Rhodamine 6G in simple silicon/glass microstructures.[19] Although, the authors demonstrated bursts with good signal-to-noise ratio, scattering from the silicon substrate generated extremely high background levels.

In the following year Michael Ramsey and co-workers at Oak Ridge National Laboratory used SMD for analysing separations of single chromophore molecules in microfabricated channels.[20] Separations of Rhodamine 6G (at 15 pM) and Rhodamine B (at 30 pM) were detected by counting fluorescence bursts from individual molecules. As can be seen in Fig. 4 the two dyes are clearly

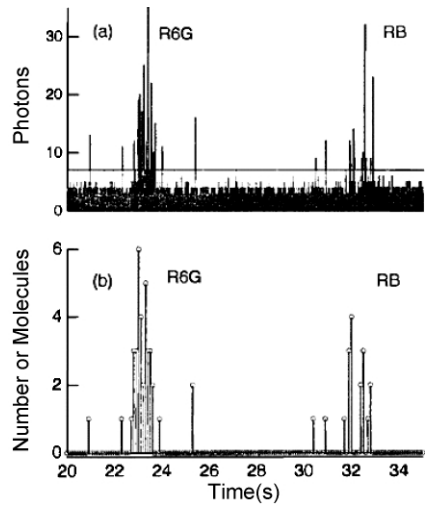


Fig. 4 Electrophoretic separation of Rhodamine 6G and Rhodamine B along a 13.5 mm microchannel (10 μm deep and 40 μm wide). (a) Raw data from a 1.25 s gated injection of a solution containing 15 pM R6G and 30 pM RB. Solid line represents a molecular detection threshold of 7 photons per bin. (b) Histogram of the number of molecules detected in 100 ms-wide intervals. (Adapted with permission. Copyright 1998, The American Chemical Society).

discriminated by virtue of their migration times, however for very small population numbers the sampling noise masks the forms of the underlying peaks. Despite this problem, the authors demonstrated that parameters such as migration time, peak standard deviation and amplitude can be estimated from the distribution of detected molecules. In addition, they noted that although the molecular detection efficiency was low (~1.75%), reductions in channel dimensions and electrokinetic focusing of sample streams could be used to theoretically increase detection efficiencies to approximately 75%. The reality of this idea was elegantly demonstrated in 1999 by Brian Haab and Richard Mathies at the University of California, Berkeley who reported SMD of DNA fragments separated by capillary gel electrophoresis (CGE) within microfabricated channels.[21] The authors realised that in order to detect a larger fraction of the intra-capillary sample either the detection volume has to be increased or more sample should pass through the detection volume. As stated previously, an increased detection volume is generally undesirable since background interferences increase. Consequently, the authors adopted an approach incorporating both a physical narrowing (or tapering) of channel dimensions and electrodynamic focusing to deliver more sample through the detection volume. The structures and operation are shown in Fig. 5 Both designs

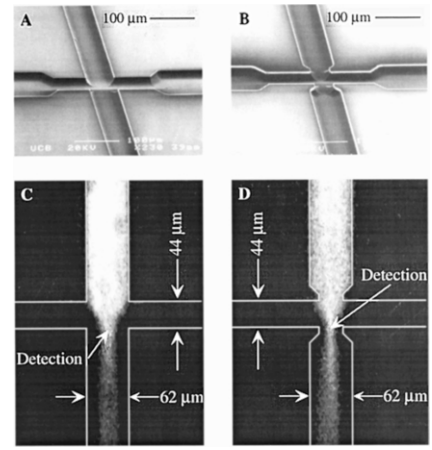


Fig. 5 Scanning electron micrographs of straight (A) and pinched (B) detectors for microfabricated electrophoresis chips. Fluorescence images of continuously injected fluorescein electrophoresing through the straight (C) and pinched (D) detectors. The electrophoresis medium was 3% linear polyacrylamide and 1X TAE. The separation channel current was 3.2 A, and each cross channel carried a focusing current of 1.6 A. (Adapted with permission. Copyright 1999, The American Chemical Society).

incorporate a cross-channel to deliver a focusing current near the detection volume. In addition, one design features a restriction in the separation channel width near the cross-channel intersection. It can be seen (Fig. 5C and D) that both designs effectively focus the sample stream (which attains a minimum width immediately after the channel intersection), however combination of both a physical taper and electrokinetic pinching affords superior sample stream restriction. This single molecule counting technique was subsequently used to detect electrophoretic gel separations of a DNA sizing ladder and yielded a greater than three-fold enhancement in detection efficiencies with respect to normal chip-based separations. Interestingly, it was noted that higher

levels of focusing could be achieved provided that detection electronics are able to detect molecules moving with high velocity through the probe volume. This combined with background suppression techniques could yield enhancements in molecular detection efficiency of up to ten-fold. More recently, Stephen Soper's group at Louisiana State University reported the detection of individual double-stranded DNA molecules confined within microchannels structured in polycarbonate and poly(methylmethacrylate).[22] X-Ray lithography allowed the production of channels with high aspect ratios, and the use of near-IR intercalating dyes significantly minimised autofluorescence from the substrate material. Initial experiments demonstrated efficient detection of single DNA molecules and molecular detection efficiencies of up to 73% were reported for probe volumes of 1 pL and channel widths of 10 μm.

The efficient functioning and integration of processes within microfluidic systems is almost always closely related to the precise control of flow velocities and flow profiles within microchannels. Although recent developments in computational fluid dynamics have presented opportunities to use fluidic simulations in the design and optimisation of microfluidic systems, the ability to accurately monitor flow velocities and flow profiles with high spatial resolution in experimental systems is a highly powerful tool. A number of approaches for such measurements have been documented. Elegant studies by Rudolf Rigler and associates at the Karolinska Institute demonstrated high-spatial resolution flow profiling in microchannels using fluorescence correlation spectroscopy (FCS).[23] In FCS, fluorescence emission from individual molecules is collected as they move through a diffraction-limited detection volume. Autocorrelation analysis of the emission subsequently yields information relating to molecular diffusion coefficients, flow velocities and reaction rate coefficients.[24] By scanning microchannels with a diffraction-limited laser focus the authors reported the detection of single tetramethylrhodamine labelled biomolecules at flow velocities as high as 25 mm s^{-1} in channels 50 μm wide and 50 μm deep. Subsequent autocorrelation analysis of the resulting burst scans demonstrated a parabolic flow profile for hydrodynamically pumped samples in both dimensions (*i.e.* Poiseulle laminar flow). Importantly the approach generates a complete two-dimensional flow profile both quickly and easily. More recently, Pierre-François Lenne and colleagues at the Institut Fresnel have described similar studies in which FCS was used for hydrodynamic and electrophoretic flow profiling in both rectangular and cylindrical capillaries.[25] Flow profiles resulting from electrophoretic motivation demonstrated a clear dependence on capillary diameter. As expected from theory, small diameter capillaries yield a flat flow profile, whilst larger diameter capillaries exhibit parabolic flow due to more dominant thermal effects. Moreover, Antonie Visser and collaborators have reported the use of FCS to calculate flow profiles and velocities of fluorescent microspheres, bacteria and molecules within microcapillaries.[26] Interestingly, the authors find that when particle diameters are greater than 200 nm, the recovered flow velocities are dependent on the laser intensity used for measurement, indicating that particle motion is retarded by optical forces. Researchers at Imperial College, London have also reported the use of single particle confocal fluorescence detection and subsequent analysis of fluorescence burst scans to estimate particulate flow velocities in microchannels.[27] The authors show that under hydrodynamic flow conditions the mean burst frequency is proportional to volumetric flow rate and also that the Poisson recurrence time and width of recovered autocorrelation curves are inversely proportional to volumetric flow rate.

DNA fragment sizing is an important tool in a wide range of diagnostic applications. For example in restriction fragment length polymorphism (RFLP) analyses restriction enzymes are used to cleave or cut DNA at specific recognition sites (normally 4–6 base pairs in length). The digested DNA, consisting of fragments of varying length, is then sized using gel electrophoresis. Differences in the resulting electropherograms, can then be used for fingerprinting or diagnostic purposes, since variations in fragment sizes are a result of modifications within the DNA sequence. Interestingly, DNA fragment sizing can be performed without recourse to electrophoresis by performing detection at the single molecule level.[28,29] The basic idea behind SMD approaches to DNA sizing is that the amount of intercalating dye bound to a fragment is proportional to the fragment length. Thus if single DNA molecules are flowed at constant velocity through an excitation volume, the resulting photon burst size should be proportional to the fragment size. Although these 'direct' SMD methodologies for DNA fragment sizing have been progressed in flow cytometry environments they can be effectively transferred to chip-based formats. For example, in 1999 Stephen Quake and colleagues at the California Institute of Technology described the fabrication and testing of a microfluidic device for the sizing and sorting of DNA molecules.[30] A simple T channel device, fabricated from a silicone elastomer using soft lithography was used to size a *Hin*dIII digest of λ DNA and a λ DNA ladder (Fig. 6).

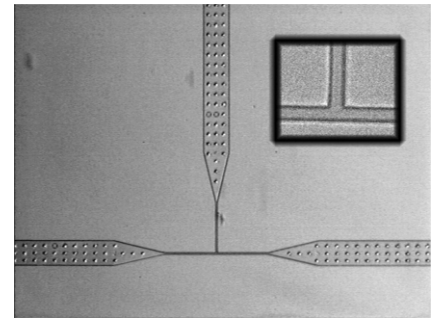


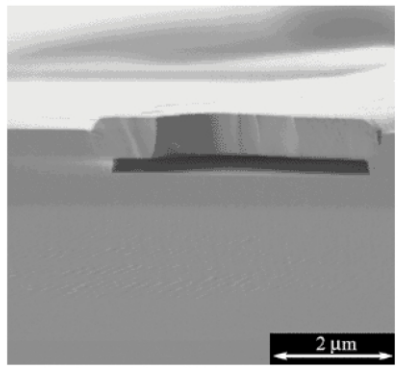
Fig. 6 Optical micrograph of an elastomeric T-channel device. The large channels have lateral dimensions of 100 μm, which narrow down to 5 μm at the T junction. The depth of all channels is 3 μm. Support pillars are used prop up the large channels and prevent bowing. (Inset) Magnified view of T junction. The channels are 5 μm wide at this point. (Adapted with permission. Copyright 1999, National Academy of Sciences, USA).

Introduction and motivation of about 3000 molecules through the microchannel and the use of a confocal probe volume, yielded burst height distributions with distinct peaks representing the major fragments of the digest. The authors demonstrated efficient sizing of DNA molecules ranging from 2000 to 200,000 base pairs and also noted that resolution improves as fragment size increases (unlike gel electrophoretic methods). Although this approach is clearly successful, channel dimensions are still relatively large with respect to confocal probe volumes. This normally leads to reduced detection efficiencies (*i.e.* the fraction of analyte molecules sampled). If detection volumes are increased to more closely matched channel dimensions additional issues arise. These include increased background noise and limitations on flow velocities and molecular concentrations (to ensure instantaneous occupation probabilities in the sparse-

molecule or single-molecule regime). The obvious remedy to this conundrum is to reduce channel dimensions to sub-micron scales, thereby allowing the vast majority of molecules to be detected with high sensitivity. To this end, Harold Craighead and co-workers at Cornell University have reported significant advances in DNA fragment sizing *via* SMD by fabricating sub-micron channel systems for sample delivery.[31] Microchannel circuits were fabricated using sacrificial layer methods, allowing the creation of complex structures with a high degree of structural precision (Fig. 7A). Specifically, DNA fragments (ranging from 564 to 27490 base pairs) were electroosmotically driven through a channel (290 nm deep and 1 μm wide) and directed through a diffraction-limited excitation volume. The resulting photon burst scans were then analysed to yield burst width distributions (as shown in Fig. 7B). As can be seen, the method can be used to size DNA since burst size is linearly proportional to fragment size at a given flow velocity. Significantly, DNA sizing and quantitation could be performed with as little as 10^4 molecules in only a few seconds. In a more recent study Craighead's group have utilised zero-mode waveguides (created by defining sub-wavelength holes in a metal film) to circumvent the problems associated with diffraction-limited optics and have performed single molecule measurements at micromolar concentrations and with microsecond temporal resolution.[32]

A similar approach to molecular confinement was reported for capillary based systems in 1997. William Lyon and Shuming Nie at Indiana University utilised sub-micron detection capillaries for extended observation of single molecules in solution.[33] Sub-micron dimensions were formed by pulling standard electrophoresis capillaries to create tapered regions with an internal diameter of between 500 and 600 nm. Electrokinetic pumping of dye molecules through these tapered regions yielded photon burst data very different to that observed in larger capillary systems. In particular, clusters of emission were observed over extended periods of time, indicating dynamic confinement of molecules (or a reduction in the effective diffusion coefficient of the confined species).

The development of SMD methods over the past decade has opened up a number of fascinating opportunities within the field of analytical science. Perhaps the most exciting is the concept of single molecule DNA sequencing. At a simplistic level, if a single strand of DNA is fixed within a flow stream the terminal nucleotide may be sequentially cleaved through the action of an exonuclease. Consequently, as long as the nucleotides can be detected in the order that they are released, sequence determination can be performed in extremely short times (which are only limited by the rate of enzymatic digestion). Although the reality of efficient single molecule sequencing has yet to materialise a number of feasibility studies have been reported.[11,34,35–37] An elegant approach towards single molecule sequencing in microstructures has been described by Susan Brakmann and co-workers.[38] The method involves the sequential enzymatic degradation of fluorescently labelled DNA molecules within a microfluidic channel. As noted, it is essential to detect all cleaved nucleotides in the order that cleavage occurs. This is achieved by illuminating the entire cross section of a microchannel at a given point, and imaging fluorescence photons onto a linear array of seven adjacent multimode glass fiber tips (that act as confocal pinholes) coupled to seven avalanche photodiode detectors. This arrangement, in effect, generates a number of overlapping femtolitre probe volumes across the channel, and ensures that no molecule flowing through the channel passes undetected. This set-up was used to demonstrate efficient two-colour excitation and detection of two fluorescent dye molecules (tetramethylrhodamine and 5-*N*,*N*′-diethyltetramethylindodicarbocyanine) flowing through a microfluidic channel (7 μm wide and 10 μm deep).



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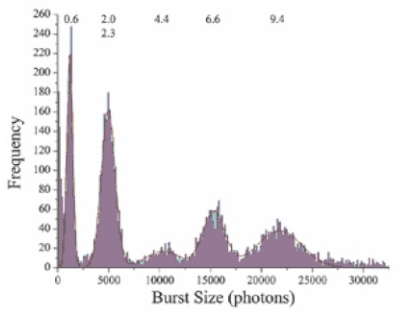

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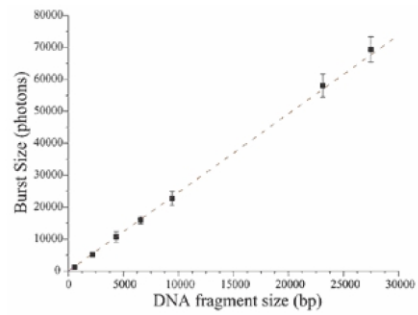

(C)

Fig. 7 (A) Scanning electron micrograph showing the cross section of a wide region (10 μm width, 270 nm height) of the channel. The ridges visible in the ceiling are an artefact due to cutting of the microstructure prior to SEM imaging. (B) Photon burst histogram obtained for a mixture of several DNA fragments; the peak positions depend on fragment size and the peak areas are proportional to the relative concentration of each fragment. (C) Plot of burst size as a function of the (known) fragment size. (Adapted with permission. Copyright 2002, The American Chemical Society).

Outlook

It is evident from this cursory analysis that, although far from routine, single molecule detection can be performed to good effect within microfluidic systems. A key advantage of performing analytical operations within microfluidic environments is the ability to efficiently process extremely small volumes of

sample. This means that detection will always be a key issue in defining the applicability of any chip-based system. Accordingly, SMD will prove to be immensely useful in many applications. Conversely, it should be realised that the potential use of SMD methods in applications such as DNA sequencing relies on the capacity to individually detect all molecules within a given sample. As has been seen, this is an immense challenge due to the requisite small probe volumes. Fortunately it is well known that mass transport in micron-sized channels generally falls into the laminar flow regime where viscous forces dominate over inertia. This characteristic affords precise manipulation of flow streams and also allows sample to be focussed (*via* electrokinetic or hydrodynamic forces) into extremely narrow flow streams. Consequently, microfluidic systems will prove to be useful tools for sample handling and delivery that are compatible with SMD techniques.

As a final thought, it is realised that both single molecule detection and lab-on-a-chip technology are emergent research fields at an early stage of development. Although far from maturity, they offer (in different ways) the tantalising prospect of high-throughput, high-sensitivity measurements in a wide variety of situations. Nevertheless, it may turn out to be the case that the true potential of each technology will only be unlocked by integration of key features from the corresponding field. Only time will tell.

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