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Current Opinion in Biotechnology

Microfluidics for biological measurements with single-molecule resolution☆

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Single-molecule approaches in biology have been critical in studies ranging from the examination of physical properties of biological macromolecules to the extraction of genetic information from DNA. The variation intrinsic to many biological processes necessitates measurements with single-molecule resolution in order to accurately recapitulate population distributions. Microfluidic technology has proven to be useful in the facilitation and even enhancement of single-molecule studies because of the precise liquid handling, small volume manipulation, and high throughput capabilities of microfluidic devices. In this review we survey the microfluidic ''toolbox'' available to the single-molecule specialist and summarize some recent biological applications of single-molecule detection on chip.

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Current Opinion in Biotechnology 2014, **25**:69–77

This review comes from a themed issue on **Analytical biotechnology**

Edited by **Frank L Jaksch** and **Savaş Tay**

http://dx.doi.org/10.1016/j.copbio.2013.08.013

Introduction

Systems of biological macromolecules exhibit many heterogeneous characteristics. This is the case for dynamic processes [1] as well as equilibrium states [2,3]. Sometimes a distribution of molecular conformational states or transition kinetics deviates normally around a mean value while other times these distributions can be non-Gaussian or multi-modal. Thus when probing the structural characteristics and dynamic behavior of biological macromolecules, ensemble measurements often misinterpret multimodal distributions and rare or short-lived states can go undetected. In such cases a single-molecule approach can be particularly useful. By quantifying population statistics one molecule at a time, single-molecule measurements allow for more detailed reconstruction of the distribution of molecular conformational states, transition dynamics, and interaction kinetics.

Traditional single-molecule detection techniques which use macro-scale sample receptacles and mixers are often limited to measuring equilibrium states and suffer from background noise at physiological concentrations. To address these technical challenges and to expand the repertoire of single-molecule techniques many researchers have turned to microfluidic platforms. Several areas of life science research have benefited from microfluidic technology in the past decade [4–7]. One major reason for this is the ability of microfluidic devices to perform precise manipulation of single cells and single molecules owing to exquisite liquid handling capability at the nanoliter scale and smaller. The precision, control, and reproducibility of microfluidic experimentation have made microfluidic devices an attractive platform for conducting biological measurements with single-molecule resolution.

For over a decade researchers have exploited the union of microfluidic technology and single-molecule detection to achieve more sensitive biological measurements. In an early application, Chou *et al.* reported fast, absolute size-based sorting of single DNA molecules, using fluorescent detection of intercalated dyes [8]. Their microfluidic approach proved to be 100 times faster, consumed significantly less sample – on the order of tens of femtograms – and displayed higher resolution, particularly for longer molecules than pulsed-field gel electrophoresis, the state-of-the-art at the time. In 2001 Lagally *et al.* detected DNA templates at the single-molecule level by employing polymerase chain reaction (PCR) in microfluidic devices and analyzed the product with an integrated capillary electrophoresis detection system [9]. Shortly after, Lipman *et al.* used a laminar mixing device to measure the dynamics of protein folding at the single-molecule level [10]. In a comprehensive 2005 review, Dittrich and Manz surveyed early applications of single-molecule detection on chip and examined the benefits in detail [11]. They posited that single-molecule detection in microfluidic devices might be the ''holy grail'' for micro-total analysis systems (μTAS).

In this review, we look at how microfluidic technology has recently incorporated with single-molecule measurements

in biology research. First we survey the ''toolbox'' that microfluidic devices provide for single-molecule studies by presenting some examples of the major approaches to liquid handling with microfluidic devices, including laminar flow cells, droplet microfluidics, and two-layer devices with integrated valves. We then select a few examples of recent work in which microfluidic tools have been applied to answer biological questions from *in vitro* protein folding studies to gene mapping. Next, we look at examples of how single-molecule studies in live cells have benefited from microfluidic environments. Finally we examine how single copies of DNA can be resolved in chip using molecular amplification like polymerase chain reaction (PCR). In this section we focus particularly on how microfluidics has enhanced digital PCR for absolute DNA and RNA quantification. Our goal is to demonstrate how microfluidic technology can facilitate and in some instances enhance single-molecule studies by presenting the broad range of technologies available for performing single-molecule detection in microfabricated devices and by highlighting the diverse set of applications made possible by combining these two powerful techniques.

The microfluidic toolbox

Microfluidic devices incorporate a range of techniques for fluid and sample manipulation which offer unique advantages for single-molecule detection (Figure 1). In this section we survey the three most commonly used techniques; laminar flow cells, droplet-based devices, and valve-based devices, and give examples of how single-molecule studies have benefited from their implementation.

Laminar flow

One of the most prevalent implementations of microfluidics for single-molecule measurement is the laminar flow cell (Figure 1a). In the low Reynolds number environment of microfluidic channels, parallel streamlines and minimal convection yield a predictable flow profile which is useful for sample handling in single-molecule studies. A microfluidic laminar flow cell can be constructed simply by sandwiching double-sided tape in between two coverslips [12,13•]. Microfluidic flow cells can also involve more advanced fabrication techniques and include multiple inputs with complex mixing architecture [14•]. For a thorough review of laminar flow cell fundamentals for single-molecule studies see Ref. [15].

One of the most important tools for the single-molecule spectroscopist in the microfluidic toolbox is the precise and ultra-fast mixing achieved with hydrodynamic flow focusing (Figure 1b). By reducing the distance over which reactants must diffuse, sheath flow, created at the junction of three microchannels, can yield controlled and thorough mixing in as little as ten microseconds [16]. Advances in 2D [17,18] and 3D [19] hydrodynamic focusing have reduced this mixing time to 1 μs and increased mixing uniformity and positional control. The mixing speeds attainable with laminar mixers make this technology particularly effective for studying protein folding dynamics, see for example Refs [20–23].

Laminar flow cells are popular because they can be straightforward to construct and relatively simple to operate, but they are also extremely versatile and many advanced flow devices have been developed to enhance single-molecule measurement. Lemke *et al.* constructed a laminar mixing device for performing single-molecule FRET measurements with improved photostability by deoxygenating the sample and reactant buffer through gas-phase diffusion between the flow channels and flanking reservoir channels purged with nitrogen [24]. Gambin *et al.* combined a hydrodynamic focusing junction with a deceleration chamber in order to combine the microsecond mixing rates with a flow rate slow enough for FRET detection. In this way they measured protein folding events on the 200 μs to 100 ms timescale [25•]. These advances demonstrate how microfluidic laminar flow devices have the potential to facilitate measurements which cannot be performed with traditional benchtop techniques.

Droplets

Droplets are formed in microchannels when a liquid experiences high shear forces caused by the merging or cross-flow of a second immiscible liquid [26]. In this way droplets can be rapidly generated and their characteristics precisely controlled by the external driving pressure of the fluidic inputs (Figure 1c). The generation of droplets in microfluidic channels combines the ability to form independent and isolated reactions in a high-throughput fashion with small volume confinement [27]. These features make droplet microfluidics a powerful platform for single-molecule analysis. Researchers have demonstrated direct single-molecule counting in droplets using confocal microscopy [28] as well as indirect single-molecule detection using PCR to amplify single copies of DNA in droplets and detecting the amplified product in real time with fluorescent probes [29]. Droplet arrays have also been used in high density enzymatic activity assays for the detection and counting of single enzymes [30,31]. Arayanarakool *et al.* measured the activity of single β-glucosidase molecules in micrometer-scale droplets created in a microfluidic device [32]. The droplets provide an ideal environment for single-molecule activity assays because the small volume (≈10 fL) confined by the droplets allows for high effective enzyme concentrations, on the order of nanomolar, without increased background noise. This approach has also been used with agarose droplets for single copy DNA [33–35] and RNA [36] detection. Agarose droplets can transition into solidified agarose beads preventing cross contamination between droplets and facilitating manipulation and allows storage. The

Figure 1

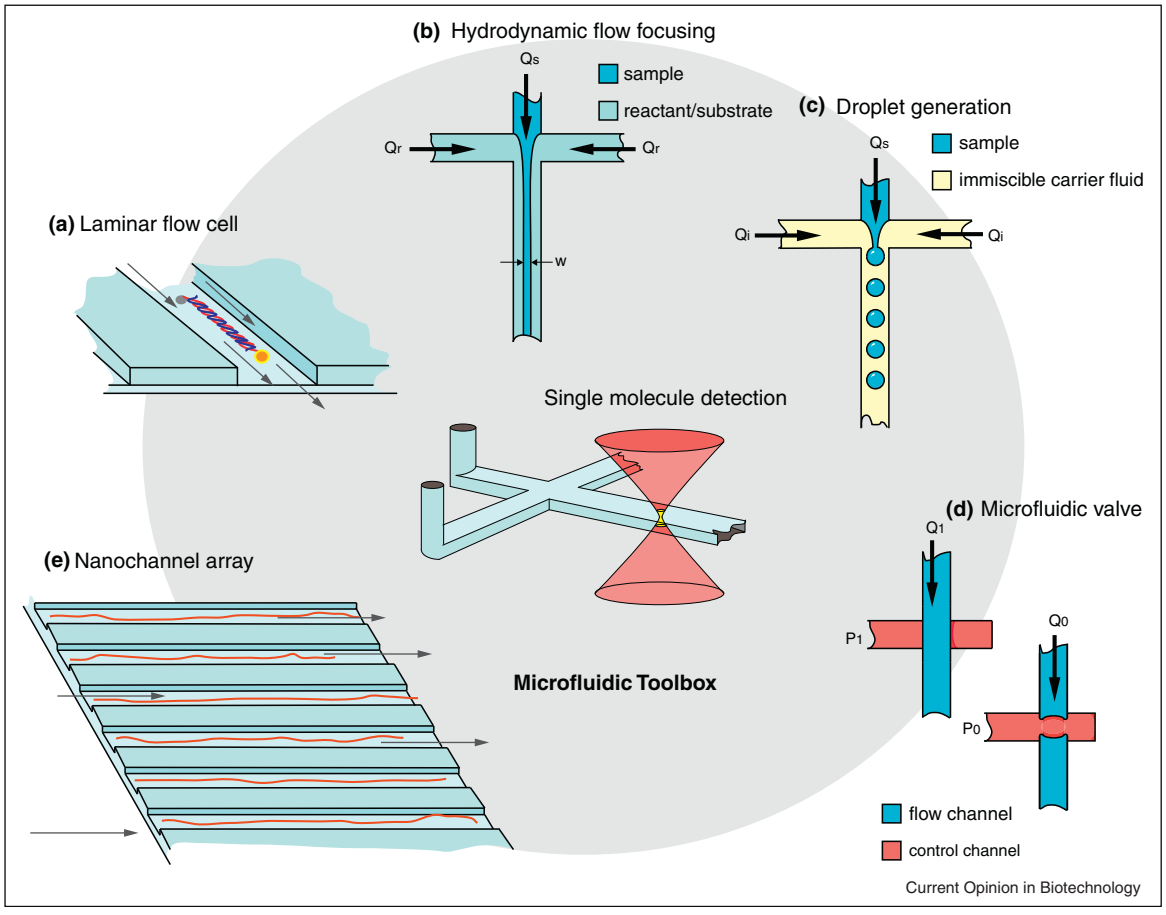


Diagram of the microfluidic toolbox. **(a)** Single channel laminar flow cell. Constructed either with microfabrication or by sandwiching double sided tape or paraffin between glass coverslips. A labeled DNA molecule is immobilized on one end to the coverslip and is being stretched by flow. **(b)** Hydrodynamic focusing of an input sample (dark blue). The width of the sample sheath (w) is determined by the ration of the input flow rate of the reactant (Qr) to the input flow rate of the sample (Qs). **(c)** Droplets of sample solution (dark blue) generated in an immiscible fluid (yellow). The droplet size and generation rate are functions of the flow rates Qi and Qs as well as the channel dimensions. **(d)** A diagram of the microfluidic valve. The flow rate of the sample through the flow channel (blue) is either Q1 or Q0 (no flow) for control channel (red) input pressures of P1 (typically 1 atm) or P0, respectively. **(e)** Nanochannel arrays are use in Refs [56–60,63,64] to stretch long DNA molecules for optical mapping. Nanofabrication techniques allow construction of channels with widths as small as 50 nm in order to confine and stretch single DNA molecules. **(f)** Schematic of confocal illumination and detection in a microfluidic channel, a common approach to single-molecule detection on chip.

high-throughput nature of droplet microfluidics makes it a powerful technology for systematic genomic investigation, particularly for DNA quantification with digital PCR which will be discussed in the section *Single copy measurements*.

Microfluidic large-scale integration

The microfluidic valve is equivalent to a transistor in an integrated electronic circuit. It is a three-terminal component in which an input signal on the control channel determines the fluidic current through the flow channel (Figure 1d). Multilayer soft lithography [37] enables monolithic fabrication of thousands of valves on a single microfluidic chip allowing for large-scale integration of fluidic components in order to achieve complex liquid routing schemes [38]. Integrated valves have thus enabled researchers to perform high-precision, high-throughput, and automated single-molecule titration studies in microfluidic devices. Ridgeway *et al.* constructed a microfluidic bioreactor capable of automatically mixing combinations of up to eight reactants for fluorescent analysis [39]. The critical circuit component was a microfluidic mixing ring capable of precise injection and mixing of reagents using peristaltic microfluidic pumps [40]. With this device the authors measured long time scale RNA-protein interaction kinetics with single-molecule sensitivity and in a fully automated fashion. We extended this approach in order to perform

single-molecule FRET measurements of single-stranded and double-stranded DNA conformation over a multi-dimensional chemical space [41]. In this work, single-stranded DNA was labeled on either end with a FRET pair and mixed with varying concentrations of complementary molecules and ionic solutions, in automated serial experiments. FRET intensities of thousands of labeled single DNA molecules were measured for hundreds of sequential reactions autonomously in order to map out a conformational 'phase-diagram' of DNA.

The primary advantage of the single-molecule microfluidic formulation device is the precision and reproducibility of chemical titration made possible by microvalve pumps combined with automated protocol execution. With this device hundreds of serial single-molecule experiments can be performed with precision of over three orders of magnitude better than traditional pipetting and without the need to manually change samples between experiments.

Another approach to generating multi-parameter titration diagrams was presented by Vandelinder *et al.* They used a multilayer device to create a high density, two-dimensional temperature–concentration gradient for wide-field fluorescent imaging of the conformation of FRET labeled α-synuclein [42]. The measurements made in this report did not have single-molecule resolution but the device was capable of making tens of thousands of fluorescent measurements and could be very useful for high-throughput single-molecule studies. Microfluidic large-scale integration has also been advantageous in manipulating single-cells for *in vivo* single-molecule detection, as well as for array based digital PCR devices. We discuss both of these technologies in later sections.

In addition to the techniques discussed above, our group and many others have been developing a growing list of optofluidic components that have promise to enhance applications of florescence detection within microfluidic devices including tunable optofluidic compound lenses [43] and integrated liquid core PDMS cladding waveguides [44]. The combination of the technologies presented in this section along with the integration of a diverse set of optofluidic components, microstructures and nanostructures yields a large toolbox with a variety of techniques and devices available for single-molecule specialists.

Biological measurements with single-molecule resolution *in vitro*

Microfluidic-based single-molecule detection broke ground early in protein folding with a study in 2003 by Lipman and colleagues in which a laminar flow mixer was used to study nonequilibrium states in the denaturing of a cold shock protein (Csp) [10]. After labeling the Csp with donor and acceptor dyes at key residues, they probed the conformation of single-molecules undergoing this two-state transition by making measurements at different points along the mixing channel. Further points corresponded to later delay times after mixing the protein with a denaturant allowing temporal resolution of the folding phenomenon. Microfluidic devices have since been regularly used to study protein folding at the single-molecule level.

Hofmann *et al.* used an improved rapid mixer [45] to measure the fast time scale kinetics of chaperone assisted protein folding with single-molecule FRET [46]. They labeled a substrate protein, rhodanese, with donor and acceptor fluorophores in order to characterize the folding pathway with and without binding inside the cavity of the GroEL/GroES chaperonin. Using traditional macro-scale mixing and rapid mixing in a microfluidic device, rhodanese folding was monitored on timescales from milliseconds to hours. This work is a good example of how microfluidic technology can be used in conjunction with benchtop techniques to thoroughly examine a system. The broad dynamic range in kinetic timescale allowed the authors to assemble a complete picture of rhodanese folding dynamics which led to a more accurate description of the rate limiting effects of chaperonin binding. A similar multi-pronged approach was used by Soranno *et al.* to characterize the roughness of the energy landscape during refolding of disordered proteins [47•].

Microfluidic single-molecule detection has also been effective for studying DNA-protein interactions [48]. Forget *et al.* integrated dual optical trapping and fluorescent detection with a multi-channel microfluidic flow cell in order to examine the effects of double-stranded DNA conformation on RecA assisted homologous pairing *in situ* [14•,49••]. Cipriany *et al.* combined fluorescent detection with electrokinetically driven flow to construct a fluorescence activated single-molecule sorter in order to distinguish and select methylated DNA with high efficiency [50•]. Kim *et al.* used a simple single channel flow cell to study allostery in DNA-protein interactions [13•]. Elting *et al.* further expanded the microfluidic toolbox by introducing two new approaches to minimize background fluorescence in single-molecule interaction studies that require high molecular concentrations. They used linear zero-mode waveguides and so-called ''convex lens induced confinement'' to image the movement of single molecular motors along actin filaments and demonstrated the potential to perform these measurements at up to micromolar concentrations [51].

The linear zero-mode waveguide demonstrates how microfluidic and nanofluidic channels not only aid in fluidic manipulation and optical analysis of solutions containing single molecules, but also how these small length scale features can facilitate manipulation of the

molecules themselves. For example, nanochannels can be used to stretch DNA when the width of the channel approaches the persistence length of the DNA molecule (50 nm for double-stranded molecules). These nanofluidic stretching devices have proven to be particularly useful both for making biophysical measurements at the single-molecule level [52–55] and for optical mapping of sequence information along large DNA molecules [56–60] (Figure 1e).

Yeh *et al.* performed an elegant experiment using the interface between microscale and nanoscale chambers to examine entropy-driven phenomena in single biopolymers. By stretching a large DNA molecule through a nanochannel between two microchannels, they were able to study the effect of confinement on the entropic force associated with polymer coiling [61•]. Lam *et al.* used a graded micro-structure array [62] to guide many long (>100 kb) DNA molecules into a nanochannel array for optical genome mapping [63••]. They mapped the 4.7 Mb human major histocompatibility region, a complex genomic loci, and demonstrated the ability to construct scaffolds for *de novo* sequence assembly. Marie *et al.* designed a device which used a combination of on-chip chromosomal DNA extraction with nanochannel confinement and hydrodynamic flow stretching in order to achieve mapping with single molecules of greater than 2 Mb in length [64]. Molecules were then recovered for off-chip genetic analysis with sequencing and fluorescent *in situ* hybridization for more thorough assessment of genetic structural variation. Development of microfluidic and nanofluidic devices for optical mapping of DNA has advanced steadily over the past ten years with many research groups actively incorporating and improving upon previous reports. This field provides a good example of optimal collaboration between device engineers and biologists in order to produce platforms that go beyond proof of principle and directly address biological questions.

Biological measurements with single-molecule resolution *in vivo*

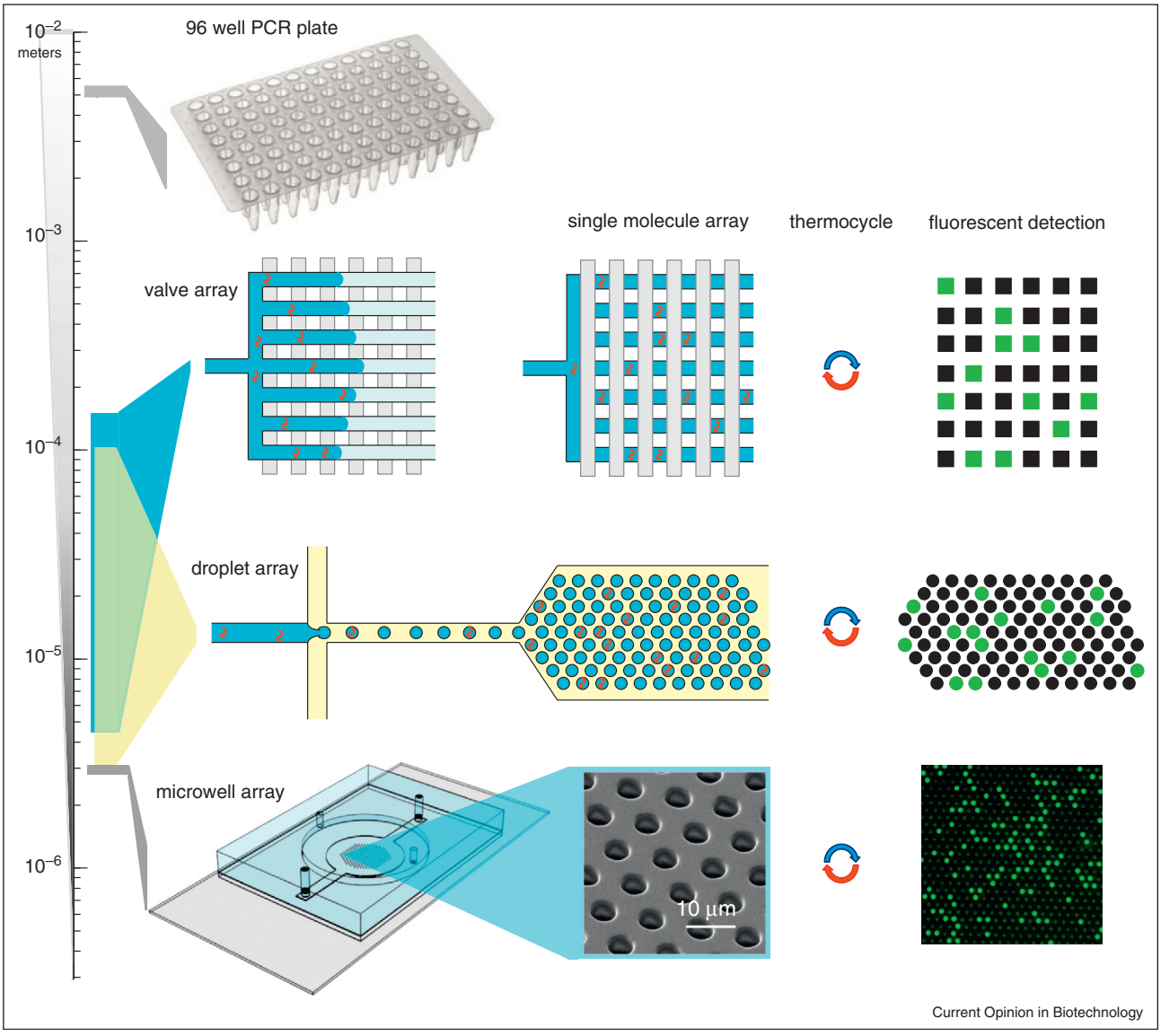
Microfluidic devices have proven to be particularly suitable for single cell manipulation and analysis [4,6]. Naturally, there has been the emergence of microfluidic platforms for single-molecule detection within single cells. The ability of microfluidics to isolate, manipulate, and optically analyze single cells is especially advantageous for the quantification of protein expression in single cells with single-molecule resolution and has enabled the characterization of the stochastic nature of gene expression [65], direct counting of scarce protein molecules in single cells [66], and whole single-cell transcriptome quantification [67]. Ullman *et al.* used a microfluidic platform to cultivate and track bacteria cells in order to quantify protein expression through thousands of cell cycles [68]. In another application of protein tracking in single live cells Zhang *et al.* monitored nerve growth factor transport in the axons of live neurons which were cultured and guided in microfluidic channels [69]. Juul *et al.* isolated single cells in droplets and used rolling circle amplification to detect enzymatic activity at the single-molecule level [70]. In their study, single human cells were lysed in droplets containing DNA sensors and fluorescent probes to detect amplification product. Many droplets containing single-cells or no cells were organized in a capture array where amplification and detection were performed in parallel. The small volume confined by the droplets effectively increased the concentration of the amplification product enabling detection of rare events. This strategy of detecting single molecules using molecular amplification is a powerful technique for molecular quantification in microfluidic systems and is the basis of digital PCR. In the next section we will see how this approach is used to detect single copies of DNA on chip.

Single copy measurements

The small volumes which can be attained with microfluidic chambers facilitate the amplification and detection of single DNA molecules with PCR [9]. Digital PCR [71] is a technique in which a solution of DNA molecules is partitioned into isolated chambers where PCR is performed in parallel, amplifying the contents of each chamber (Figure 2). The DNA template molecules are distributed at a concentration such that there are only one or no molecules per chamber before amplification. Amplified product is detected with a fluorescent probe and only chambers which initially contained a template molecule will yield positive signal whereas chambers that were initially empty will show a negative signal. Thus the chambers provide a digital readout that can be used to quantify the initial starting material in absolute terms without suffering from amplification bias.

The ability to create high density arrays of small volume chambers in microfluidic devices makes for an ideal platform for digital PCR applications. A valve based digital PCR device was commercialized by Fluidigm Corporation and has been used for many applications of molecular counting and mutation detection with a sensitivity high enough to asses single cell quantities of DNA [72,73] including detection of fetal aneuploidy [74,75] and quantification of point mutations in leukemia [76]. Heyries *et al.* expanded this technique and used a novel valving method to create a device that contained one million reaction chambers in a high density array [77]. Our group demonstrated digital PCR in an array of femtoliter chambers allowing for a reactor density of 20 000 mm^{-2} [78]. Another approach to partitioning chambers for digital PCR is achieved with the so-called ''slip-chip'' [79] which uses progressively sized reaction chambers to increase the effective dynamic range of detection.

Figure 2



A schematic diagram of microfluidic digital PCR and size comparison chart. The microfluidic valve array uses a common sample input (blue) to fill microfluidic chambers with diluted DNA sample. When the array valves (gray) are closed, chambers become isolated and a thermocycling protocol executes the PCR. Fluorescent probes report product through fluorescent imaging. Chambers which contained a single DNA molecule show product (green) while empty chambers report no signal (black). The droplet array uses the same detection scheme but DNA sample is partitioned into droplets which are stored in a large chamber for thermocycling and imaging. The microwell array reported in Ref. [78] uses wells of 3 μm radius for increased reactor density.

Droplet microfluidics has recently proved to be a powerful platform for digital PCR as well [80–82]. Because droplets can be generated on the fly, the number of potential reactions is not limited by array design parameters. Instead millions of droplets can be rapidly produced yielding a significantly increased dynamic range. Both Bio-Rad Laboratories and RainDance Technologies now offer commercial droplet-based digital PCR platforms. Droplets have also been used for isothermal amplification of single DNA molecules [83] as well as reverse transcription PCR on single RNA molecules [36,84].

Conclusion

It is clear that the union of single-molecule detection and microfluidic technology has much to offer the life scientist. This potential has been realized in many areas of research and is only just becoming apparent in others. Promising directions include further applications in optical genome mapping, and high-throughput digital

realtime PCR. The high sensitivity that microfluidic devices offer for single copy measurements also facilitates single cell measurements with single-molecule resolution, including applications of single cell transcriptome analysis. Additionally, microfluidic cell culture platforms are already providing much more systematic approaches to single-cell stimulation and signaling investigations. It is likely that single-molecule detection will begin to take advantage of these platforms as well.

Some of the applications reviewed here, like the protein folding investigations, genetic mapping, and digital PCR, have undergone successive rounds of technology development and have endured interdisciplinary collaboration to produce optimized, robust devices with versatile biological application and in some cases commercial distribution. Yet while much of the technology described in this review provides a powerful platform for the investigation of heterogeneous biological systems, the question now is, will these techniques remain as niche applications in technology oriented research groups, or will we see broader adoption of these techniques in more traditional biology labs and even clinical facilities? While the less field-tested microfluidic platforms presented here show significant potential to aid important biological investigation, the proof will be in the pudding so to speak. In order for the proof-of-principle technologies to become useful, there will have to be more fluent and regular transfer of technology between groups and disciplines. This may require more detailed and reproducible descriptions of methods in publications, including chip design CAD files, control software, part's lists, and descriptions of optimized reagents and protocols. An emphasis on experimental methods, engineering parameters and general attention to technical detail is particularly important for work like those presented in this review as they all require expertise in a broad range of techniques. Fluid collaboration between labs in fields ranging from engineering to biology is the other ingredient for the promotion of this kind of interdisciplinary work. From the examples presented here, the field seems to be trending in this direction. The future looks bright for single-molecule detection on chip.

Acknowledgements

This work was supported by the Ministry of Science and Technology of China (2011CB809106) and the National Natural Science Foundation of China (21222501). AMS was supported by the Whitaker International Biomedical Engineering Fellowship.

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- •• of outstanding interest

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