Chemistry & Biology
Review

Miniaturization and Parallelization of Biological and Chemical Assays in Microfluidic Devices

Saurabh Vyawahare,[1] Andrew D. Griffiths,[2,3] and Christoph A. Merten[4,*]
[1]Microfluidics Laboratory, Physical Sciences-Oncology Center, Physics Department, Princeton University, Princeton, NJ 08544, USA
[2]Institut de Science et d'Ingénierie Supramoléculaires, Université de Strasbourg, BP 70028 67083 Strasbourg Cedex, France
[3]CNRS UMR 7006, 8 allée Gaspard Monge, 67083 Strasbourg Cedex, France
[4]Genome Biology Unit, European Molecular Biology Laboratory, Meyerhofstrasse 1, Heidelberg, 69117 Germany
*Correspondence: christoph.merten@embl.de
DOI 10.1016/j.chembiol.2010.09.007

Microfluidic systems are an attractive solution for the miniaturization of biological and chemical assays. The typical sample volume can be reduced up to 1 million-fold, and a superb level of spatiotemporal control is possible, facilitating highly parallelized assays with drastically increased throughput and reduced cost. In this review, we focus on systems in which multiple reactions are spatially separated by immobilization of reagents on two-dimensional arrays, or by compartmentalization in microfabricated reaction chambers or droplets. These systems have manifold applications, and some, such as next-generation sequencing are already starting to transform biology. This is likely the first step in a biotechnological transformation comparable to that already brought about by the microprocessor in electronics. We discuss both current applications and likely future impacts in areas such as the study of single cells/single organisms and high-throughput screening.

Introduction

There must hardly be a researcher working at the bench, who while pipetting small volumes into plate wells has not wished on occasion to have, like the Indian deity Ganesha, multiple arms to do work. Perhaps not nearly as dramatic as multiple arms, microfluidics, the technology of plumbing at the micro-scale, enables highly parallelized experiments with very small reagent volumes. Microfluidic systems consist of networks of channels with a diameter of typically ten to several hundred micrometers, into which reagents are injected and processed at the nano/pico liter scale. Many biological and chemical applications are boosted by miniaturization, which both minimizes reagent costs and opens up entirely new experimental approaches. For example, assays on single cells are difficult to perform in macroscopic systems, even though they are absolutely required for measuring population variance and distinguishing between distinct cellular states that are invisible in averaged data from bulk populations. Furthermore, miniaturization enables highly parallelized experiments, thus drastically increasing throughput. The dramatic effect of miniaturization and parallelization using microfluidic systems is seen clearly with next-generation sequencing systems where the time and cost to sequence 3 billion base pairs (the equivalent of the human genome) has been reduced from more than a decade and US $3 billion for the Human Genome Project's reference genome (1990–2003) to less than a month and typically less than US $100,000 using the latest microfluidic sequencing technology (Metzker, 2010).

Historically, the behavior of liquid flow in microscopic systems was already being thoroughly analyzed in the mid-20th century (Taylor, 1953) and comprehensive micro manufacturing knowledge was gained with the introduction of miniaturized chromatographic systems (Terry et al., 1979; Manz et al., 1990) and ink-jet technology (Lemmo et al., 1998). Traditionally, microstructures were patterned into glass or silicon, making use of production techniques developed for the semiconductor industry (such as photolithography and etching). However, this approach is relatively expensive, requires highly specialized equipment, and does not allow rapid prototyping. A real breakthrough was achieved with the advent of soft lithography (Figure 1), which enabled replica molding (Qin et al., 1996). Instead of patterning microchannels directly into expensive and potentially opaque materials (such as silicon), from which only one device could be manufactured, a master mold is used for the fabrication of multiple microfluidic devices made from cheap and transparent elastomers such as polydimethylsiloxane (PDMS). Making use of this approach, custom-made microfluidic chips for a variety of applications can be rapidly manufactured.

Today, microfluidic devices are built out of a number of materials including plastics, elastomers, ceramics, metals, silicon, glass, paper, epoxies, gels, and wax. With each material, there are unique issues (e.g., compatibility with organic solvents, thermal stability, gas exchange, etc.), advantages and disadvantages—and in many applications, a combination of materials is essential (Herold and Rasooly, 2009). Furthermore, the surface chemistry may need to be adapted to specific applications. Besides materials, a variety of forces can be used to drive fluids and objects inside fluids—including mechanical forces, chemical gradients, surface, electrokinetic, and electromagnetic forces. These forces generate a pressure gradient that causes flow through a chip on objects in the fluid to move them. Control, detection, heating, and cooling systems also need to be coupled to a microfluidic chip. We point the reader to several other reviews comprehensively describing the material issues and physics of microfluidics (Beebe et al., 2002; Squires and

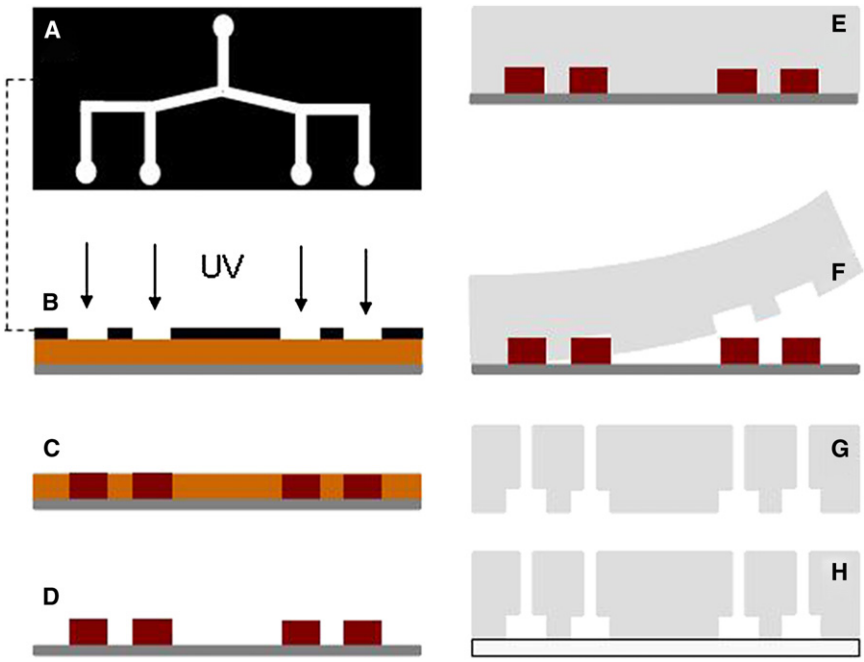

Figure 1. Manufacturing of Microfluidic Devices by Soft Lithography
A photomask is generated using a computer-aided design (CAD) program (A). Subsequently the pattern is projected onto a silicon wafer (gray) coated with a photosensitive polymer (light brown, B). Specific polymerization of the exposed areas (dark brown, C) and development (D) results in a mold that can be filled with an elastomer (E), typically polydimethylsiloxane (PDMS, light gray). After baking, the cured PDMS is peeled off (F) and inlets and outlets are punched into the polymer (thus allowing the connection of syringes via tubing, F). Subsequently, the polymer structure is bound to a surface (typically glass, black lines) by plasma treatment, thus closing the channels (H).

Quake, 2005; Stone et al., 2004; Whitesides, 2006). In this review, we have tried to highlight the essential features of microfluidics relevant to designing highly parallel, multiplexed devices. For detailed information about applications in the analytic and diagnostic field, we also refer the reader to other publications (Lee et al., 2010b; Ohno et al., 2008; Weigl et al., 2008).

Flow in Fluidic Devices

Fluids in microfluidic systems behave in nonintuitive ways that need to be taken into account when miniaturizing a biological assay. Consider the act of mixing sugar cubes in tea: waiting for the sugar to diffuse and mix is too slow, and rapid mixing is achieved by stirring which induces turbulent flow (convectional mixing). If the tea is replaced by a viscous liquid, stirring becomes harder, because viscosity damps any motion reducing turbulence and diffusive mixing dominates. In microfluidic devices, viscous forces dominate. But instead of changing the liquid, the same effect is obtained by reducing size. Scaling a tea cup to less than a millimeter gives an approximation of the fluid physics in a microfluidic system. This fluid flow regimen in microfluidics is laminar and not turbulent. The quantitative description of laminar flow involves the Reynolds number (Re), which is the dimensionless ratio between inertial and viscous forces

$$\mathrm{Re} = \frac{\rho v l}{\mu},$$

where ρ is the density, v the velocity, l a characteristic length scale, and μ the viscosity. In a cylindrical pipe, flows with Re <2300 tend to be laminar. Higher Re results in turbulent flows. Lower Reynolds numbers can be obtained by either increasing the viscosity or decreasing the length scale. Most microfluidic devices have Reynolds numbers less than 5. Viscous "friction" between fluid layers makes the flows streamlined and damps down convectional mixing. Two adjacent flowing fluids mix mainly by diffusion unless special methods are adopted (Figure 2A).

Besides having viscous forces dominate, diffusional mass transport becomes comparable to convection mass transport, and another dimensionless number, Peclet number (Pe) can be used to compare them

$$Pe = \frac{vl}{D},$$

where D is the diffusion constant, v the velocity and l a characteristic length. For Pe <1 diffusion dominates, for Pe >1 convection dominates. In microfluidics both cases are possible. Low Re and Pe ~1 have striking consequences that are exploited in microfluidic devices.

Miniaturization, Multiplexing, and Parallelization

In general, making components smaller enables greater speed, lower cost, and savings in energy usage (Trimmer, 1989). However, the driving pressure scales as the fourth power of the radius of the pipe for low Reynolds number conditions, increasing very rapidly as the radius reduces. Thus in general, the best way to get higher throughput is not to simply increase flow rates, but to parallelize and multiplex.

The ability to multiplex or parallelize requires two things:

(1) Separation: different chemical reactions have to be separated out in time and/or space.
(2) Addressability: there needs to be a way to address or recognize each different reaction. The addressing may use temporal or spatial coordinates or optical or other coding systems.

Single-Phase Devices

Broadly, microfluidic devices are of two types: devices having a single liquid phase, and devices with two or more liquid or gaseous immiscible phases (Figure 2A). The basic element in both types is a channel. With single-phase devices, one simple way to multiplex reactions is to flow reagents in a single channel over a number of different spatially separated entities like

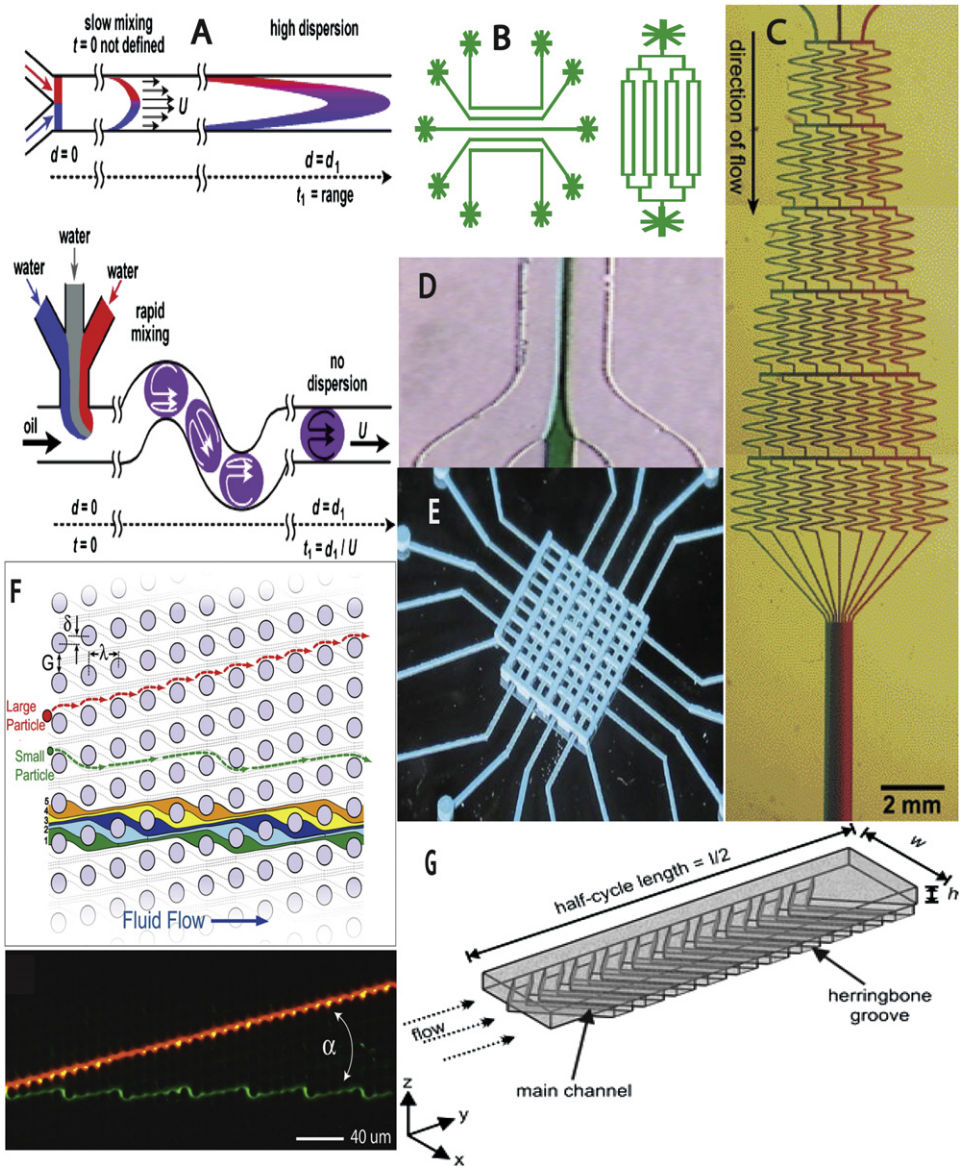

Figure 2. Fluid Flow on the Microscale

(A) Comparison of single-phase and two-phase microfluidic systems (Song and Ismagilov, 2003), Adjacent fluid streams mix by slow diffusion. Reproduced in part with permission of Wiley-VCH Verlag GmbH & Co. KGaA.

(B) Parallel, straight channels compared with channels connected in a binary pattern. Depending on the type of application, it may be advantageous to parallelize in one of the patterns.

(C) Generation of a diffusion gradient using three food dyes in a microfluidic system (Dertinger et al., 2001; Jeon et al., 2000). Reproduced with permission of the American Chemical Society.

(D) Hydrodynamic focusing of green food dye in a 200 μm channel by two side flows containing plain buffer.

(E) A three-dimensional mold made from a 3-D wax printer.

(F) A set of posts arranged at an angle allows the separation of beads of different sizes. The red color is a time trace of a 2.7 μm microsphere, and the green a time trace of a 1 μm microsphere (Morton et al., 2008b). Reproduced by permission of The Royal Society of Chemistry.

(G) Herringbone mixer (Williams et al., 2008) speeds up the mixing process in a microfluidic channel. Reproduced by permission of The Royal Society of Chemistry.

sensors, chambers or molecules attached to surfaces that are monitored for changes externally. A recent high-end example involves flow over fiber bundles etched at one end to create millions of micrometer sized chambers each of which can be filled with a single bead (Tam et al., 2009).

For many applications, this kind of multiplexing is sufficient. It is easy to further parallelize with multiple channels, either separate or connected (Figure 2B). With connected channels, more uniform flows can be achieved with binary tree-like patterns and delay lines. It is even possible to use the third dimension to build more complex forms of channel geometry like vias and overpasses if space considerations prevent high densities of channels (Kartalov et al., 2006a; Martinez et al., 2008; Wu et al., 2002).

Mixing and Concentration Gradients

The real power of the microfluidic approach begins to reveal itself when flow channels are combined with diffusion to create multiplexed mixing ratios inside chips. A popular example is the diffusion mixer designed by the Whitesides group (Jeon et al., 2000) (Figure 2C). Even more sophisticated spatial and temporal chemical patterns can be generated (Campbell and Groisman, 2007; Lin et al., 2004; Xie et al., 2008). These devices enable multiplexed experiments that probe a large spatial chemical phase space in one chip (Lee et al., 2010a). Hydrodynamic focusing experiments initiated by Knight et al. (1998) and further refined by others (Song and Ismagilov, 2003) combine the flow of three channels into a single one-flow channel. The central channel flow can be squeezed into a narrow stream (as small as 50 nm)—a consequence of laminar flow. As one traverses down the channel, convectional flow competes with diffusional mixing and the narrow stream acts like a timeline for probing reaction kinectics, i.e., temporal chemical phase space.

In many experiments, rather than creating a concentration gradient, components need to be well mixed. Mixing by diffusion alone is often too slow, and therefore microfluidic mixers are necessary. Herring bone type mixers (Stroock et al., 2002) and rotary peristaltic mixers (Unger et al., 2000) are two examples of mixer design (Nam-Trung and Zhigang, 2005). On the flip side, instead of mixing, complete separation of components may be required. Again, there are single-phase microfluidic methods: channels containing a set of posts arranged at an angle have been used to separate out micron-scale particles based on size (Figure 2F) (Morton et al., 2008b).

It must be noted that in all these microfluidic chips, a critical element is accurate, long-term control of flow rates, which requires connecting macroscale plumbing to the chip (like syringe pumps or solenoid-based air pressure controllers). Inevitably, this increases the bulk and complexity of systems, and presents a big hurdle to widespread use of fluidic chips. Thus, methods that bypass macroscale plumbing or miniaturize it will have a big impact.

Compartmentalization Using Active Valves

Valves allow the transient creation of small, separated chambers, increasing the power as well as the complexity of microfluidic devices (Oh and Ahn, 2006). They can be made in several different types: normally open, normally closed, check valves (which allow flow only in one direction), and latch valves (which need only a pulse signal to change state). Besides stopping flow, valves can be used in other ways: for example, they may be adjustable; partially closed valves can filter out particles or create a reversible bead absorption column on chip (Figure 3D); three valves in a row can be operated in a peristaltic motion to move liquids (Figure 3E).

Since valves need energy, each must be connected to a control line that supplies it (Figure 3A). If they are operated simultaneously, then all of them can be connected to one control line (Figure 3B). Several applications use this to create independent reaction chambers, since one control line can control any number of valves that are operated simultaneously. Multilayer soft lithography is a particularly versatile method to create thousands of simultaneously controlled valves (Melin and Quake, 2007).

If the valves on a chip are not operated simultaneously, then the number of external control lines needed can be prohibitive. As in electronics, this problem can be overcome by multiplexing. Valves can be combined together to create addressable channels in the forms of a multiplexer. There are two types of multiplexers: combinatorial and logarithmic (Hua et al., 2006; Thorsen et al., 2002) (Figure 3F). Grover et al. (2006) have designed two way valves that use pressure and vacuum and have multiple states, reducing even further the number of control lines required (Figure 3G).

The reader may wonder in which direction all this extra complexity is headed. From the parallel in electronics, one possibility is the creation of more general purpose programmable fluidic controller chips similar to microcontrollers. For instance, all microcontrollers have an instruction set which includes an instruction to add two numbers in a register; similarly, a general purpose microfluidic chip may contain an instruction to combine two plugs of reagents located in specific chambers (Urbanski et al., 2006). Some early work in the direction has been in the form of multiplexers, which can be used to run a set of valve combinations that function as a chip's state or instruction set, in a device called a deconvolver (Vyawahare et al., 2008), and the combining of a cascade of fluidic logic gates, using a combination of pressure levels to generate gain and compensate for line losses (Weaver et al., 2010).

Applications of Single-Phase Microfluidics

Hundreds of examples exist of using valves and multiplexers to create complicated chips that can perform multiple tasks. An increasing number are also commercially available from companies such as Fluidigm (http://www.fluidigm.com/), Caliper Lifesciences (http://www.caliperls.com), and Dolomite (http://www.dolomite-microfluidics.com/). Researchers can custom-design devices in microfluidic foundries located at Caltech (http://kni.caltech.edu/foundry) and Stanford University (http://www.stanford.edu/group/foundry/). Here, three broad areas of single-phase microfluidic applications will be described: nucleic acid biology, biochemical screens, and cell-based assays.

Nucleic Acid Biology

All of the next-generation sequencing techniques use microfluidic flow cells or chambers (Holt and Jones, 2008; Metzker, 2010; Shendure and Ji, 2008) (Figure 4A). Although the technologies for template preparation, sequencing, and imaging differ, all rely on tethering a template to a solid support in a flow cell, to which nucleotides/oligonucleotides and other reagents are iteratively applied and washed away. The DNA template may be clonally amplified before sequencing by bridge PCR (Illumina/Solexa) or emulsion PCR (Roche/454, Life/APG and Dover Polonator) or single DNA molecules can be sequenced directly (Helicos). In each cycle, sequencing can be performed by measuring the incorporation of unlabeled dNTPs by the polymerase by detecting pyrophosphate release using chemiluminescence (Roche/454). Alternatively, the incorporation of fluorescently labeled modified nucleotides, which function as cyclic reversible chain terminators, is measured (Illumina/Solexa, Helicos) (Figure 4B). In this case, after each cycle, the fluorophor is removed, and the nucleotide can be extended in ensuing cycles. DNA ligase can also be used in place of polymerase to sequentially incorporate fluorescently labeled

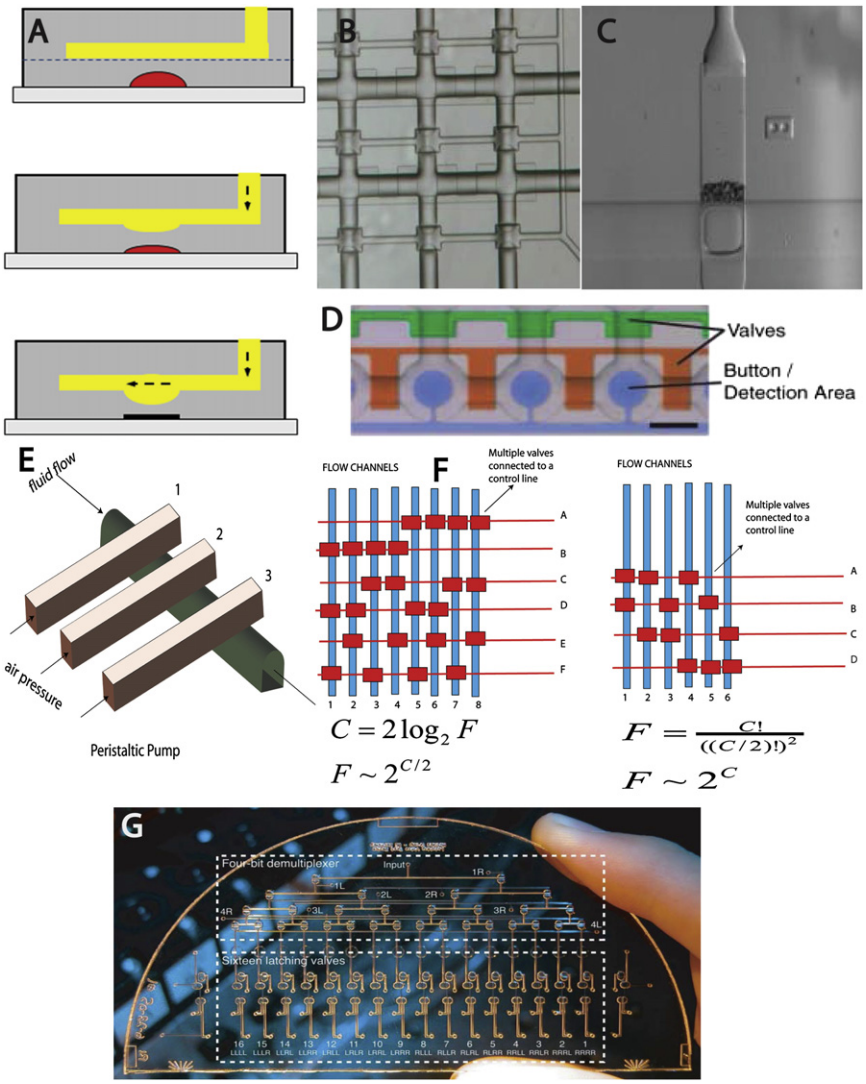

Figure 3. Components of Continuous-Phase Microfluidic Circuits

(A) Schematic showing a pneumatic valve in operation. Air pressure from the control line shown in yellow deflects the membrane closing the flow channel shown in red.

(B) Pneumatic valves cordon off 100 μm wide chambers in a microfluidic chip.

(C) Partially closing valve allows fluids through but stops 6 μm diameter microbeads creating a temporary affinity column.

(D) Button valves allow cordoning of a circular area on the surface, removing any proteins that are not chemically bound to the surface (Maerkl and Quake, 2007). Reproduced with permission of The American Association for the Advancement of Science.

(E) Schematic of peristaltic pump with three valves in a row.

(F) Schematic of logarithmic and combinatorial multiplexers, where C is the number of control lines, and F the number of flow lines (Vyawahare et al., 2008).

(G) Example of a 4 bit multiplexer circuit that uses vacuum and pressure pulses to operate latching valves (Grover et al., 2006). Reproduced with permission of The Royal Society of Chemistry.

oligonucleotides in the sequencing by ligation approach (Life/APG and Dover Polonator). This way, tens of millions of gene fragments can be sequenced in parallel: for example, the Solexa GAII uses a microfluidic flow cell split into eight lanes, allowing eight independent experiments (of up to 8 million reads each) in a single run (Figure 4A).

Microfluidic chips can also be used to perform sample preparation upstream of sequencing, for instance, to quantify amounts of DNA in a DNA library using digital PCR (Dube et al., 2008; Pushkarev et al., 2009; Vogelstein and Kinzler, 1999). Digital PCR relies on dividing target DNA samples into small enough volumes such that they contain, for the most part, only one or zero DNA molecules of interest, and then performing PCR in each volume to quantitate initial copy number. This method allows absolute quantitation without the need to compare to a standard. Microfluidic chips can also be used to prepare multiple library samples for sequencing: the Fluidigm Access Array chip allow the amplification and bar-coding of 48 samples in parallel, and all 48 samples can be multiplexed at the sequencing step.

Biochemical Screens

Multiplexing by surface patterning of proteins can be combined with multiplexing by microfluidic architecture to obtain large data sets, for example, in experiments to measure protein binding kinetics (Bates and Quake, 2009; Maerkl and Quake, 2007). In this particular case, the devices used a further innovation, the "button valve" (Figure 3C), which is essentially a membrane valve used to squeeze out unbound protein and isolate a surface. Microfluidics can also be used to pattern the proteins themselves (Delamarche et al., 2005), and one device can then be used to both generate the protein pattern and perform the subsequent assay.

In diagnostics, moving toward a microfluidic approach can provide significant advantages due to portability and low reagent use (microliters to picoliter). Commercially, blood diagnostic devices made by Abbott laboratories called i-STAT use microfluidic cartridges with a handheld reader. Another example of this approach involves multiplexed immunoassays on human blood serum using a system of bar-coded proteins combined with microfluidic channels (Fan et al., 2008). Several immunoassays benefit from flowing reagents multiple times over a reactive region by pumping reagents continuously in a closed loop (Kartalov et al., 2006b) A singularly promising approach for low cost, multiplexed, and disposable diagnostics uses sheets of patterned paper and adhesive tape as the main subtrate, moving liquids by wicking (Martinez et al., 2008). The nonprofit organization "Diagnostics for All" (www.dfa.org) is commercializing this approach.

For protein crystallization, precise dispensing of picoliter volumes of liquids (Hansen et al., 2002) can be followed by using

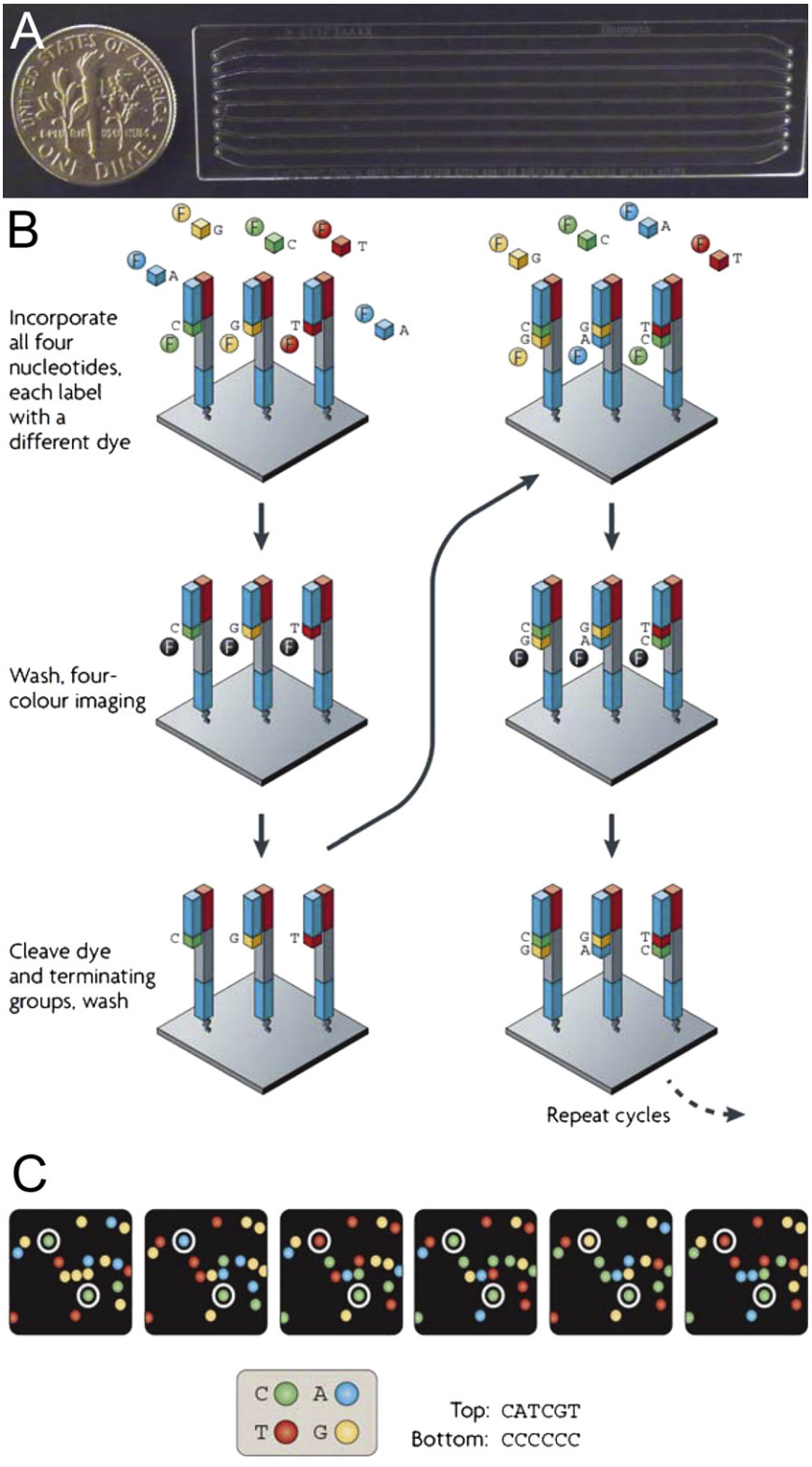

Figure 4. Next-Generation Sequencing Using the Illumina/Solexa System

(A) The microfluidic sequencing flow cell, which is divided into eight lanes.

(B) Individual DNA molecules are immobilized on the surface of the flow cell and amplified by bridge PCR to form DNA colonies (polonies). Subsequently modified nucleotides, labeled with four different colored fluorescent dyes, which function as cyclic reversible chain terminators, are incorporated in the newly synthesized strands by polymerase and imaged. After each cycle, the fluorophores are removed, and the 3′-hydroxyl group of the nucleotide deprotected to allow extension in ensuing cycles.

(C) Images highlighting the data obtained during six cycles of the sequencing process (Metzker, 2010). Reprinted by permission from Macmillan Publishers Ltd.

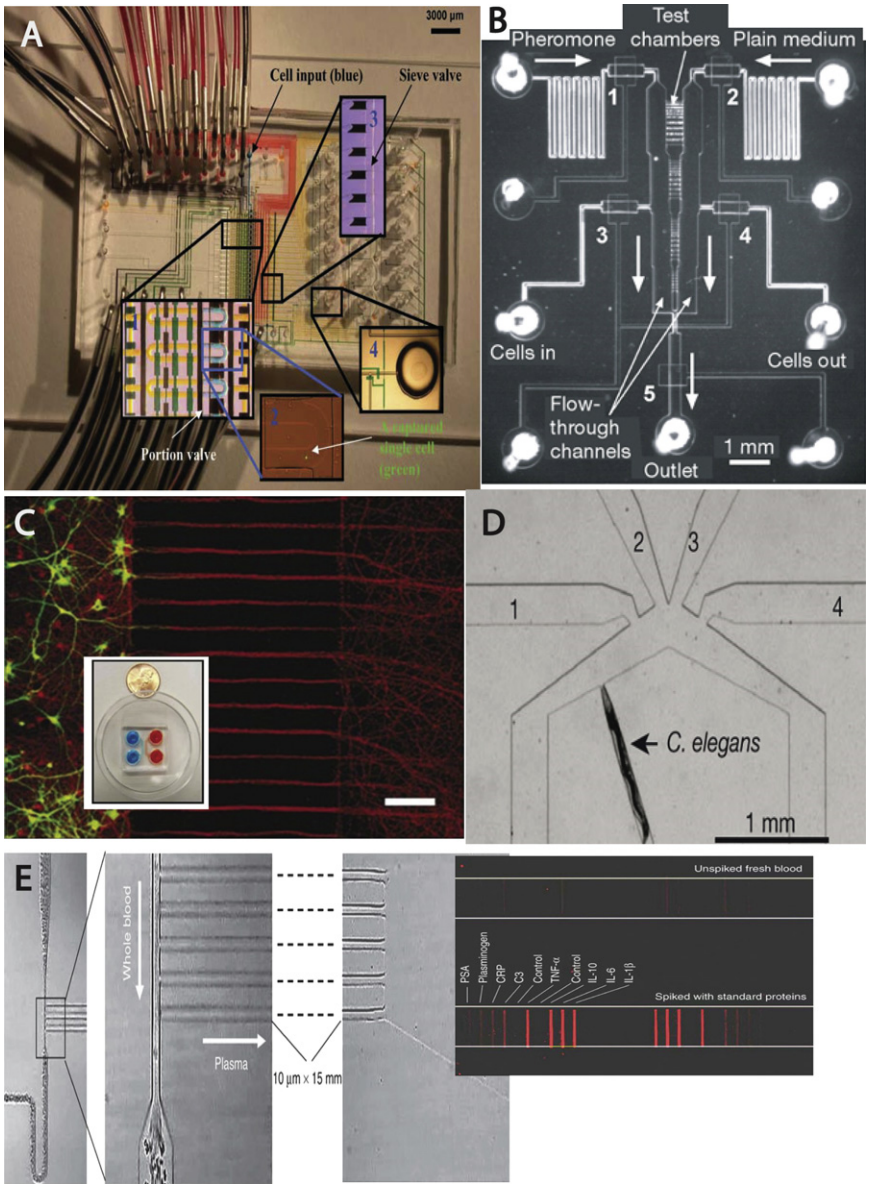

Figure 5. Examples of Continuous-Phase Microfluidic Applications

(A) A single-cell processor to extract mRNA from stem cells, capable of performing 32 simultaneous single cell experiments (Zhong et al., 2008). Reproduced with permission of The Royal Society of Chemistry.

(B) Fluidic chip to study MAPK signaling network in yeast cells (Paliwal et al., 2007).

(C) Neuronal culture chip which consists of two large channels connected by narrow slits. The inset shows the chip, and the main figure shows axons growing into the slits (Park et al., 2006).

(D) *C. elegans* trapped in a microfluidic channel and exposed to odors (Chalasani et al., 2007).

(E) Human blood protein analysis performed using a bar-coded chip (Fan et al., 2008).

(B–E) Reproduced with permission of Nature Publishing Group.

free-interface diffusion to generate of a vast chemical phase space with different reagent concentrations (Li and Ismagilov, 2010). This approach uses low reagent volumes and is particularly suited for cases where only a small amount of target protein is available. Commercially, systems made by Fludigm under the brand name Topaz use this technology.

Cell-Based Assays

The small volumes in microfluidics are especially well matched for single-cell analysis, which is often the only route for studying cells that cannot be cultured. Typically, sophisticated chips are needed for single-cell analysis, and the number of single-cell experiments that can be performed in parallel currently run in the low two-digit range. Work has been done on TM7 bacterial cells from the human oral cavity (Marcy et al., 2007), immune cells (Huang et al., 2007; Toriello et al., 2008; Wheeler et al., 2003), mammalian cells (Coupland, 2010; Marcus et al., 2006), termite gut bacterial cells (Ottesen et al., 2006), insect cells (Hellmich et al., 2005), yeast (Taylor et al., 2009), stem cells (Zhong et al., 2008) (Figure 5A) among many others. In many of these devices, a system of valves and multiplexers is used to isolate single cells of interest, followed by cell lysis and extraction of genetic material or proteins for analysis. The use of microbead columns allows part of the subsequent steps, like cDNA conversion, to be performed on-chip (Zare and Kim, 2010). However, coupling of PCR and RT-PCR (Zhang et al., 2006), i.e., the miniaturization and integration of heating and cooling or of sequencing methods, would also be extremely powerful, but has not been demonstrated yet in a single-cell device. Besides cell analysis, another exciting possibility is the use of microfluidic chips to study dynamics of genetic networks at the single-cell level (Bennett and Hasty, 2009). For instance, Cai et al. (2006) were able to isolate *Escherichia coli* cells in picoliter size chambers to analyze stochastic protein expression using a chemiluminescence-based assay.

Three further single-phase microfluidic cellular applications are worth briefly mentioning: separation, culturing, and chemotaxis. First, separation: an arrangement of posts in a channel has been demonstrated to separate cells in blood (Inglis et al., 2008) and the same chip can also be used to wash, lyse, and label them (Morton et al., 2008a). Posts coated with antibodies have also been shown to separate out circulating tumor cells in blood (Nagrath et al., 2007). The operating principle of these devices relies on microscale fluid physics and would be impossible at the macroscale. Further cell separation applications are discussed later in the multiphase device section of this review.

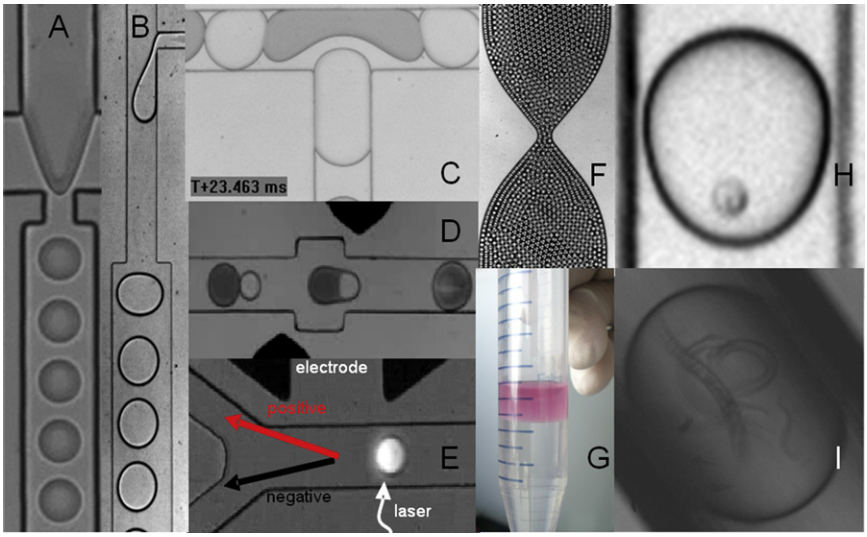

Figure 6. Generation and Manipulation of Droplets

Droplets can be generated by flow-focusing (A) or by using a T-junction configuration (B). Subsequently they can be split (C), fused (by applying electric fields (Frenz et al., 2008). Reproduced with permission of Wiley-VCH Verlag GmbH & Co. KGaA. (D) Sorted (according to their fluorescence, E), and incubated on- (F) or off-chip (G). Cells (H) and multicellular organisms (I) can be cultivated.

For cell culturing, microfluidic chips allow precise control of the growth conditions and reduce the amount of reagents required. While *E. coli* and yeast are easily cultured, it has proved harder to culture mammalian cells like neurons (Park et al., 2006) (Figure 5C) continuously in fluidic chips, due to the more stringent conditions required. Cell-culture chips can be used to analyze how an aggregate of cells responds to various stimuli and conditions. Environmental stresses like food scarcity and toxins are easily simulated in chips, even in a time-dependent manner (Keymer et al., 2006). Several designs to study cell aggregates have been successfully demonstrated including the Tesla chemostat (Cookson et al., 2005), chemostat chips to study genetic population circuits in *E. coli* (Balagadde et al., 2005), and protein network dynamics in yeast (Paliwal et al., 2007; Rowat et al., 2009).

Finally, in chemotaxis assays, microfluidic chips can play two roles: one, proteins can be patterned precisely on a surface (or as a spatial chemical gradient) and two, the chips also serve as containers for the cell of interest and cell-growth media. A variety of cells have been studied for chemotactic response including: cancer cells (Saadi et al., 2006), bacterial cells (Kim and Breuer, 2007), neutrophils (Li Jeon et al., 2002), neural crest cells (Cornejo et al., 2010), neurons (Taylor et al., 2006) to name just a few. Ibidi (http://www.ibidi.com/) sells a line of disposable microfluidic slides for chemotaxis studies.

It should also be mentioned that besides cells, high-throughput microfluidic experiments on small multicellular organisms such as *Caenorhabditis elegans* have also been progressing rapidly (Chronis, 2010; Figure 5D). For small organisms like *C. elegans*, planaria, and zebra fish, microfluidic channels are at the right size scale to allow screening experiments inside chips (Lockery, 2007). Furthermore, chips can be used to precisely expose trapped single organisms like *C. elegans* or parts thereof to odors (Chalasani et al., 2007; Figure 5D).

Two-Phase Devices

The compartmentalization of assays is obviously one key element for obtaining large data sets in biological experiments. Besides using valves for the compartmentalization of individual reactions on chip, aqueous droplets in an immiscible carrier phase (such as oil or air) can be generated for the stable separation of chemicals, biomolecules, and even cells. The first devices for the microfluidic generation of droplets were based on capillary systems (Umbanhowar et al., 2000). Today, drops are usually generated in PDMS chips, either by injecting an aqueous phase orthogonally into a flow of the immiscible phase (T-junction configuration) or by hydrodynamic flow-focusing (with the immiscible phase) through a narrow nozzle (Anna et al., 2003; Christopher and Anna, 2007; Song et al., 2003; Thorsen et al., 2001) (Figures 6A and 6B). In both cases, the aqueous phase is dispersed into distinct droplets, each of which can function as an independent microreactor. In contrast to continuous flow microfluidics, chemicals encapsulated in drops are not subjected to diffusion or dispersion and can be mixed in milliseconds by chaotic convection (Song et al., 2003). Microfluidic drop makers allow for the generation of highly monodisperse droplets (±3% in terms of the volume) at kilohertz frequencies, making them attractive for high-throughput applications. Drops can be generated either using a stabilizing surfactant, in which case the drops do not coalesce upon contact (they can be stored off-chip in form of an emulsion), or in absence of any surface active molecules. In the latter case, to prevent coalescence between the individual drops they permanently have to be kept in contact with the channel walls in form of extended plugs. This way, the oil spacers in between the plugs cannot drain, and a contact between the plugs is excluded during "segmented flow" of the system.

Alternatively droplets can also be generated by electrowetting (digital microfluidics) and droplet on demand (DOD) technologies. For electrowetting, multiple electrodes are embedded into the device and used to change the contact angle between the droplets and the surface of the chip, thus enabling controlled movement, splitting, and merging (Srinivasan et al., 2004). However, due to the complex nature of the system, only a few droplets (typically <100) can be processed at the same time for which reason they have mainly been used for analytical or preparative applications (Miller and Wheeler, 2009), which are not described further in this review.

Various techniques have been developed for the generation of droplets on demand. For example, this can be achieved using piezoelectric (Bransky et al., 2009) or acoustic (Lee and Lal, 2004) actuators. As a general feature, these devices couple an

electronic control unit with droplet production, thus allowing the discontinuous generation of droplets. A potential benefit of this approach is the possibility of synchronizing the generation of droplets.

To exploit the potential of droplet-based microfluidics for high-throughput applications, further modules for the controlled manipulation of droplets at very high frequencies (up to kilohertz) have been developed. Droplets can be split, fused, incubated, and sorted (Ahn et al., 2006; Link et al., 2004, 2006; Song et al., 2003) (Figures 6C–6E). The splitting of droplets can be achieved by flushing them through a T-junction, where the size of the resulting droplets can be determined by the relative flow rates in the outgoing channels. Droplet fusion can be achieved by applying opposite charges to the drops, by destabilizing uncharged droplets in electric fields or simply by generating droplets without surfactants or with transiently low concentrations of surfactants at the interface of one of the droplets being fused (passive droplet fusion) (Li et al., 2007; Mazutis et al., 2009; Niu et al., 2008). Any unfused droplets can be removed by passive hydrodynamic size separation, generating highly monodisperse emulsions comprising >99% pairwise fused droplets (Mazutis and Griffiths, 2009). For incubation purposes, droplets can be flushed through long delay lines or collected in reservoirs (on and off-chip) (Figures 6F and 6G) in the absence of any flow. Due to the fact that droplets can be reinjected into further devices subsequent to an incubation period off-chip (Clausell-Tormos et al., 2008), almost unlimited incubation times can be realized. However, one has to keep in mind that mass transport through the oil phase occurs over time, either based on diffusion of molecules dissolved directly in the oil or by micellar transport. This is of particular importance for small hydrophobic molecules which might exchange between the droplets, thus resulting in cross-contamination of the samples (Bai et al., 2010; Courtois et al., 2009). The use of perfluorinated oils, in which nonfluorinated molecules are highly immiscible and insoluble (Hudlicky and Pavlath, 1995; Li et al., 2006), mitigate against transport of molecules directly dissolved in the oil but does not eliminate micellar transport.

Both charged and uncharged drops can be actively sorted using electrical fields (Ahn et al., 2006; Link et al., 2006), however, for practical applications, the sorting of uncharged droplets by dielectrophoresis is most useful. Drops can be sorted, triggered on fluorescence, at rates of up to 2000 per second and with false positive rates of <1 in 10^4 droplets (Baret et al., 2009). Microfluidic fluorescence-activated droplet sorters combine many of the advantages of microtiter-plate screening and traditional fluorescence-activated cell sorting (FACS). As with microtiter plates, the reactions are compartmentalized (in drops rather than wells) allowing a wide range of assays to be performed: the fluorescent marker(s) do not need to remain either inside or on the surface of the cells being sorted. However, as with traditional FACS high-throughput fluorescence-activated sorting is possible.

Applications of Two-Phase Microfluidics

Nucleic Acid Biology

Single-molecule PCR is of significant importance for many sequencing applications, as it allows the unbiased amplification of templates of different sizes, such as fragments of a cellular genome (Margulies et al., 2005). Initially the template and the PCR mix were encapsulated into droplets of in bulk water-in-oil emulsion using nonmicrofluidic emulsification systems. However, this approach results in highly polydisperse emulsions and hence varying efficiencies of the amplification. The monodisperse emulsions created using microfluidic systems can be used to overcome this problem and more. For example, droplet-based microfluidics has been used for sample preparation for targeted DNA sequencing (Tewhey et al., 2009). Despite the substantial reductions in costs with next-generation sequencing systems, whole-genome sequencing is still an expensive endeavor and targeted sequencing of specific regions of the genome, for example, genes implicated in a specific disease is a powerful strategy. However, PCR bias makes it impossible to simply amplify thousands of genes in a single pool. To avoid this problem, Tewhey et al. (2009) described the simultaneous amplification of 3976 products using a droplet microfluidic system which enables 1.5 million amplifications in parallel. Each droplet containing the target DNA and PCR reagents was electrocoalesced on-chip with a droplet containing a pair of forward and reverse primers specific for a single target. The fused droplets were then thermocycled off-chip and the amplified DNA recovered and used for sequencing. They reported an 84% capture efficiency with 90% of the targeted bases showing uniform coverage with either the Roche/454 or Illumina/Solexa platform. This approach is now exploited as the very first commercial application of droplet-based microfluidics (www.raindancetechnologies.com).

Droplet-based systems can also be used for the quantification of DNA or RNA. In general, droplet-based PCR or RT-PCR can be performed by either thermocycling the whole chip (or any off-chip droplet reservoir) (Beer et al., 2007), or by flushing the droplets through different temperature zones (Figure 7) (Dorfman et al., 2005; Kiss et al., 2008; Schaerli et al., 2009). Beer and co-workers demonstrated the amplification of single-copy viral genomes at high efficiencies. Diluting the template to statistically less than one molecule per droplet also allows precise and sensitive DNA quantification using digital PCR, as already described for valve-based compartments.

Biochemical Screens

A further application of droplet-based microfluidics is protein crystallization. The microfluidic approach allows simultaneous testing of multiple crystallization conditions using only minimal quantities of protein. Plugs of different chemicals potentially enhancing nucleation and or growth of the crystals can be generated manually (e.g., by aspirating compounds and oil in an alternating fashion into a length of tubing) before multiple dilutions of each compound are generated on chip. This way, 1300 different crystallization conditions were tested starting with only 10 μl of protein solution (Li et al., 2006). Furthermore, the droplet-based technology allows the conditions for crystal nucleation and growth to be adjusted separately (Gerdts et al., 2006), and samples can be concentrated by transfer of water from one plug to another via a water-permeable carrier oil by osmosis. In a similar approach, the system was used for the titration of the anticoagulant argatroban within whole blood or plasma (Song et al., 2006).

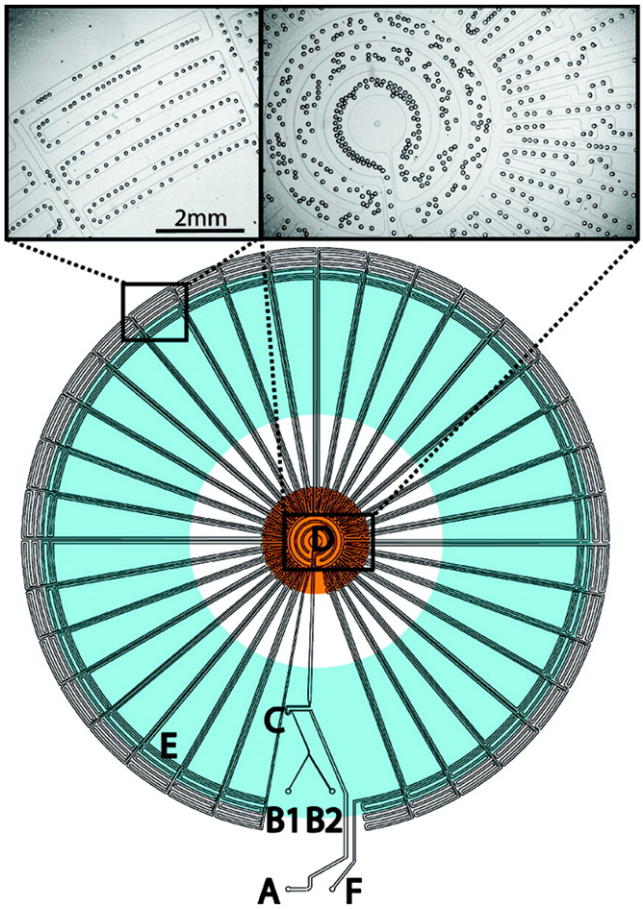

Figure 7. Droplet-Based PCRT
The template and the PCR mix are encapsulated into picoliter droplets that subsequently pass through different temperature zones on a chip. A heating rod (orange) is embedded in the center of the device while the outer parts (blue) are cooled using a Peltier element (Schaerli et al., 2009). Reproduced with permission of The American Chemical Society.

One limitation for the screening of chemical compounds in droplet-based microfluidics is the rate at which they can be injected onto the chip. While chemically identical droplets (or dilutions of the same compound) can be generated very rapidly (up to kilohertz frequencies), the loading of different compounds from external sources such as microtiter plates is obviously several orders of magnitude slower. While first studies on compound screening were based on manually preloaded cartridges (Chen and Ismagilov, 2006), automated systems have also been developed (Chabert et al., 2006; Clausell-Tormos et al., 2010). For example, compounds can be aspirated from microtiter plates using an HPLC autosampler, before being injected into a length of tubing using oil as the carrier phase. This way, the compounds are compartmentalized within aqueous plugs and can be subjected to further manipulation such as droplet fusion or splitting. Noteworthy, once a parental array of plugs has been generated, multiple small volume copies can be obtained by flushing its contents through symmetrical branching channels. Hence the time-consuming step of loading the compounds (>30 s per compound) has to be performed only once for several independent screens.

Cell-Based Assays

Two-phase microfluidic systems have also become a very attractive tool for cell-based assays, particularly for studies on single cells or organisms. Already at an early stage of the technology cells were encapsulated into droplets to obtain and cultivate individual bacterial clones starting from bulk suspensions (Martin et al., 2003). Integrating laser-based fluorescence spectroscopy into such systems allowed quantitative protein expression measurements in single GFP-expressing bacteria and the determination of population variance (Huebner et al., 2007). While assays based on intracellular or membrane bound fluorophores (e.g., GFP, fluorescence-labeled antibodies) can also be performed using conventional flow cytometry, droplet-based approaches uniquely facilitate the use of soluble fluorescent markers that are not directly linked to the cell and enable the co-compartmentalization of further assay components. Joennson and co-workers exploited this conceptual advantage for the analysis of low abundance cell-surface biomarkers that are below the detection limit of conventional methods (usually several hundred protein molecules per cell). In particular, they stained the cells with enzyme-linked (β-galactosidase) antibodies and coencapsulated a fluorogenic substrate for the enzyme (fluorescein di-β-D-galactopyranoside, FDG). Consequently each bound antibody generated high numbers of fluorophores, thus allowing the quantitative detection of the poorly expressed surface markers CD19 and CCR5 on human U937 cells (Joensson et al., 2009).

In addition to analytical applications, droplet-based microfluidics also allow the sorting of cells according to specific enzymatic activities, as long as specific fluorogenic substrates are available (Baret et al., 2009). This is of major interest for directed evolution approaches and protein engineering. For example, individual variants of an enzyme library can be displayed on yeast cells and subsequently be selected for improved catalytic activity. Agresti and co-workers demonstrated the screening of 10^8 horseradish peroxidase mutants and selected a variant exhibiting catalytic rate more than ten times faster than the wild-type (Agresti et al., 2010). This experiment, which took only ~7 hr and cost only $2.50, would have taken ~2 years and cost $15 million using a conventional robotic microtiter plate system.

Even the long-term cultivation of cells and organisms within droplets is possible (Figures 6H and 6I). A crucial factor for this purpose is the biocompatibility of the oil and (if used) the surfactant, the availability of nutrition and sufficient gas exchange. Many droplet-based microfluidic systems use fluorinated oil as the carrier phase since it shows good compatibility with PDMS, low solubility of nonfluorinated molecules and high solubility of respiratory gas. However, few fluorinated surfactants are commercially available for which reason a number of new fluorosurfactants have been synthesized and tested for their biocompatibility (Holtze et al., 2008). Clausell-Tormos et al. (2008) identified novel fluorosurfactants enabling human adherent and suspension cells to survive for several days inside 660 pl droplets. Furthermore, it was possible to quantitatively determine the expression level of cellular reporters (LacZ and green fluorescent protein) after an off-chip incubation period of 16–24 hr, which enabled the generation of a dose-response profile for the nuclear receptor agonist 20-hydroxyecdysone at the

single-cell level (Clausell-Tormos et al., 2008; Baret et al., 2010). The encapsulation and incubation of individual cells in drops has also been exploited for determine the cytotoxicity of compounds (Brouzes et al., 2009). Furthermore, droplets have even been used for the cultivation of small cell populations (~3000 cells) and multicellular organisms. By increasing the droplet size approximately 1000-fold (nanoliter volumes), a full life cycle of C. elegans within droplets could be demonstrated (Figure 6I) (Clausell-Tormos et al., 2008).

Droplet-based microfluidics has also been exploited to analyze cellular responses to chemical stimuli at high spatial and temporal resolution. Cells on a solid substrate such as a microscope slide can be brought in contact with chemicals encapsulated in droplets. Factors released by the cells are then captured in these droplets and can be subjected to parallelized analysis using different readout systems (e.g., fluorescence spectroscopy/microscopy and MALDI-MS). This so-called chemistrode setup allowed measuring the secretion of insulin from single Langerhans cells at a frequency of 0.67 Hz (Chen et al., 2008). In an alternative approach, small lipid coated oil droplets (1–4 μm) have been brought into close contact with specific regions of the plasma membrane of individual cells (using optical tweezers), allowing the extraction of membrane proteins at high spatial resolution (Lanigan et al., 2009).

The possibility of analyzing molecules secreted from individual cells might also enable entirely new approaches for the screening of antibodies. Koster et al. (2008) showed that single hybridoma cells release detectable amounts of soluble antibodies after just 6 hr of incubation. Hence clonal expansion prior to the screening of antibodies (as performed for conventional screening of hybridoma cells) is not required, potentially even allowing the direct screening of nonimmortalized B cells.

Conclusions

The miniaturization, parallelization, and integration enabled by microfluidic systems is somewhat analogous to miniaturization and integration of electronic components in microprocessors, which has already transformed society. Similarly, microfluidics is starting to open up completely new opportunities in biology. It has been lamented that microfluidics is a solution looking for a "killer" application (Blow, 2007). However, it could now be argued that next-generation sequencing, of which microfluidics is an integral (but not the only) part, constitutes the first such revolutionary application. Other applications related to upstream processing of DNA samples for next-generation sequencing and systems for genomic and trascriptomic analysis are already commercially available. The reduction in the amount of reagents of up to 1 million-fold makes experiments that were previously prohibitively expensive feasible and allows assays to be performed on systems which are not available in large quantities (such as primary or stem cells). The ability to use microfluidics for "digital biology" in which many assays are performed at the single-molecule, single-cell, or single-organism level are likely to be particularly important. However, as with electronics, miniaturization using microfluidics system is also likely to have many unforeseen and exciting applications.

ACKNOWLEDGMENTS

S.V. gratefully acknowledges support from the Physical Sciences-Oncology Center, Princeton University, funded by Award Number U54CA143803 from the National Cancer Institute. C.A.M. thanks the Fonds der Chemischen Industrie for their support in form of a Liebig Fellowship. We thank Linas Mazutis for kindly providing Figures 6B and 6F and Jean-Christophe Baret for kindly providing Figure 6C. The content of this review is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute or the National Institutes of Health. The authors are inventors on several patents related to technologies mentioned in this review, and could potentially benefit from royalty payments. A.D.G. is a co-founder of RainDance Technologies, Inc.

REFERENCES

Agresti, J.J., Antipov, E., Abate, A.R., Ahn, K., Rowat, A.C., Baret, J.C., Marquez, M., Klibanov, A.M., Griffiths, A.D., and Weitz, D.A. (2010). Ultra-high-throughput screening in drop-based microfluidics for directed evolution. Proc. Natl. Acad. Sci. USA *107*, 4004–4009.

Ahn, K., Kerbage, C., Hunt, T.P., Westervelt, R.M., Link, D.R., and Weitz, D.A. (2006). Dielectrophoretic manipulation of drops for high-speed microfluidic sorting devices. Appl. Phys. Lett. *88*, 024104-1–024104-3. 10.1063/1.2164911.

Anna, S.L., Bontoux, N., and Stone, H.A. (2003). Formation of dispersions using "flow focusing" in microchannels. Appl. Phys. Lett. *82*, 364–366.

Bai, Y., He, X., Liu, D., Patil, S.N., Bratton, D., Huebner, A., Hollfelder, F., Abell, C., and Huck, W.T. (2010). A double droplet trap system for studying mass transport across a droplet-droplet interface. Lab Chip *10*, 1281–1285.

Balagadde, F.K., You, L., Hansen, C.L., Arnold, F.H., and Quake, S.R. (2005). Long-term monitoring of bacteria undergoing programmed population control in a microchemostat. Science *309*, 137–140.

Baret, J.C., Miller, O.J., Taly, V., Ryckelynck, M., El-Harrak, A., Frenz, L., Rick, C., Samuels, M.L., Hutchison, J.B., Agresti, J.J., et al. (2009). Fluorescence-activated droplet sorting (FADS): efficient microfluidic cell sorting based on enzymatic activity. Lab Chip *9*, 1850–1858.

Baret, J.C., Beck, Y., Billas-Massobrio, I., Moras, D., and Griffiths, A.D. (2010). Quantitative cell-based reporter gene assays using droplet-based microfluidics. Chem. Biol. *17*, 528–536.

Bates, S.R., and Quake, S.R. (2009). Highly parallel measurements of interaction kinetic constants with a microfabricated optomechanical device. Appl. Phys. Lett. *95*, 73705.

Beebe, D.J., Mensing, G.A., and Walker, G.M. (2002). Physics and applications of microfluidics in biology. Annu. Rev. Biomed. Eng. *4*, 261–286.

Beer, N.R., Hindson, B.J., Wheeler, E.K., Hall, S.B., Rose, K.A., Kennedy, I.M., and Colston, B.W. (2007). On-chip, real-time, single-copy polymerase chain reaction in picoliter droplets. Anal. Chem. *79*, 8471–8475.

Bennett, M.R., and Hasty, J. (2009). Microfluidic devices for measuring gene network dynamics in single cells. Nat. Rev. Genet. *10*, 628–638.

Blow, N. (2007). Microfluidics: in search of a killer application. Nat. Methods *4*, 665–670.

Bransky, A., Korin, N., Khoury, M., and Levenberg, S. (2009). A microfluidic droplet generator based on a piezoelectric actuator. Lab Chip *9*, 516–520.

Brouzes, E., Medkova, M., Savenelli, N., Marran, D., Twardowski, M., Hutchison, J.B., Rothberg, J.M., Link, D.R., Perrimon, N., and Samuels, M.L. (2009). Droplet microfluidic technology for single-cell high-throughput screening. Proc. Natl. Acad. Sci. USA *106*, 14195–14200.

Cai, L., Friedman, N., and Xie, X.S. (2006). Stochastic protein expression in individual cells at the single molecule level. Nature *440*, 358–362.

Campbell, K., and Groisman, A. (2007). Generation of complex concentration profiles in microchannels in a logarithmically small number of steps. Lab Chip *7*, 264–272.

Chabert, M., Dorfman, K.D., de Cremoux, P., Roeraade, J., and Viovy, J.L. (2006). Automated microdroplet platform for sample manipulation and polymerase chain reaction. Anal. Chem. *78*, 7722–7728.

Chalasani, S.H., Chronis, N., Tsunozaki, M., Gray, J.M., Ramot, D., Goodman, M.B., and Bargmann, C.I. (2007). Dissecting a circuit for olfactory behaviour in Caenorhabditis elegans. Nature *450*, 63–70.

Chen, D.L.L., and Ismagilov, R.F. (2006). Microfluidic cartridges preloaded with nanoliter plugs of reagents: an alternative to 96-well plates for screening. Curr. Opin. Chem. Biol. *10*, 226–231.

Chen, D., Du, W., Liu, Y., Liu, W., Kuznetsov, A., Mendez, F.E., Philipson, L.H., and Ismagilov, R.F. (2008). The chemistrode: a droplet-based microfluidic device for stimulation and recording with high temporal, spatial, and chemical resolution. Proc. Natl. Acad. Sci. USA *105*, 16843–16848.

Christopher, G.F., and Anna, S.L. (2007). Microfluidic methods for generating continuous droplet streams. J. Phys. D-Appl. Phys. *40*, R319–R336.

Chronis, N. (2010). Worm chips: Microtools for C. elegans biology. Lab Chip *10*, 432–437.

Clausell-Tormos, J., Lieber, D., Baret, J.C., El-Harrak, A., Miller, O.J., Frenz, L., Blouwolff, J., Humphry, K.J., Koster, S., Duan, H., et al. (2008). Droplet-based microfluidic platforms for the encapsulation and screening of Mammalian cells and multicellular organisms. Chem. Biol. *15*, 427–437.

Clausell-Tormos, J., Griffiths, A.D., and Merten, C.A. (2010). An automated two-phase microfluidic system for kinetic analyses and the screening of compound libraries. Lab Chip *10*, 1302–1307.

Cookson, S., Ostroff, N., Pang, W.L., Volfson, D., and Hasty, J. (2005). Monitoring dynamics of single-cell gene expression over multiple cell cycles. Mol. Syst. Biol. *1*, 2005.0024, 10.1038/msb4100032.

Cornejo, M., Nambi, D., Walheim, C., Somerville, M., Walker, J., Kim, L., Ollison, L., Diamante, G., Vyawahare, S., and de Bellard, M. (2010). Effect of NRG1, GDNF, EGF and NGF in the migration of a schwann cell precursor line. Neurochem. Res. *35*, 1643–1651.

Coupland, P. (2010). Microfluidics for the upstream pipeline of DNA sequencing—a worthy application? Lab Chip *10*, 544–547.

Courtois, F., Olguin, L.F., Whyte, G., Theberge, A.B., Huck, W.T., Hollfelder, F., and Abell, C. (2009). Controlling the retention of small molecules in emulsion microdroplets for use in cell-based assays. Anal. Chem. *81*, 3008–3016.

Delamarche, E., Juncker, D., and Schmid, H. (2005). Microfluidics for processing surfaces and miniaturizing biological assays. Adv. Mat. *17*, 2911–2933.

Dertinger, S.K.W., Chiu, D.T., Jeon, N.L., and Whitesides, G.M. (2001). Generation of gradients having complex shapes using microfluidic networks. Anal. Chem. *73*, 1240–1246.

Dorfman, K.D., Chabert, M., Codarbox, J.H., Rousseau, G., de Cremoux, P., and Viovy, J.L. (2005). Contamination free continuous flow microfluidic polymerase chain reaction for quantitative and clinical applications. Anal. Chem. *77*, 3700–3704.

Dube, S., Qin, J., and Ramakrishnan, R. (2008). Mathematical Analysis of Copy Number Variation in a DNA Sample Using Digital PCR on a Nanofluidic Device. PLoS ONE *3*, e2876.

Fan, R., Vermesh, O., Srivastava, A., Yen, B.K.H., Qin, L., Ahmad, H., Kwong, G.A., Liu, C.-C., Gould, J., Hood, L., et al. (2008). Integrated barcode chips for rapid, multiplexed analysis of proteins in microliter quantities of blood. Nat Biotech *26*, 1373–1378.

Frenz, L., Blouwolff, J., Griffiths, A.D., and Baret, J.C. (2008). Microfluidic production of droplet pairs. Langmuir *24*, 12073–12076.

Gerdts, C.J., Tereshko, V., Yadav, M.K., Dementieva, I., Collart, F., Joachimiak, A., Stevens, R.C., Kuhn, P., Kossiakoff, A., and Ismagilov, R.F. (2006). Time-controlled microfluidic seeding in nL-volume droplets to separate nucleation and growth stages of protein crystallization. Angew. Chem. Int. Ed. Engl. *45*, 8156–8160.

Grover, W.H., Ivester, R.H.C., Jensen, E.C., and Mathies, R.A. (2006). Development and multiplexed control of latching pneumatic valves using microfluidic logical structures. Lab Chip *6*, 623–631.

Hansen, C.L., Skordalakes, E., Berger, J.M., and Quake, S.R. (2002). A robust and scalable microfluidic metering method that allows protein crystal growth by free interface diffusion. Proc. Natl. Acad. Sci. USA *99*, 16531–16536.

Hellmich, W., Pelargus, C., Leffhalm, K., Ros, A., and Anselmetti, D. (2005). Single cell manipulation, analytics, and label-free protein detection in microfluidic devices for systems nanobiology. Electrophoresis *26*, 3689–3696.

Herold, K.E., and Rasooly, A. (2009). Lab on a Chip Technology: Fabrication and Microfluidics, *Vol. 1* (Norwich, UK: Caister Academic Press).

Holt, R.A., and Jones, S.J.M. (2008). The new paradigm of flow cell sequencing. Genome Res. *18*, 839–846.

Holtze, C., Rowat, A.C., Agresti, J.J., Hutchison, J.B., Angile, F.E., Schmitz, C.H.J., Koster, S., Duan, H., Humphry, K.J., Scanga, R.A., et al. (2008). Biocompatible surfactants for water-in-fluorocarbon emulsions. Lab Chip *8*, 1632–1639.

Hua, Z.S., Xia, Y.M., Srivannavit, O., Rouillard, J.M., Zhou, X.C., Gao, X.L., and Gulari, E. (2006). A versatile microreactor platform featuring a chemical-resistant microvalve array for addressable multiplex syntheses and assays. J. Micromech. Microengin. *16*, 1433–1443.

Huang, B., Wu, H., Bhaya, D., Grossman, A., Granier, S., Kobilka, B.K., and Zare, R.N. (2007). Counting low-copy number proteins in a single cell. Science *315*, 81–84.

Hudlicky, M., and Pavlath, A.E. (1995). Chemistry of organic fluorine compounds II: a critical review (Washington, DC: American Chemical Society).

Huebner, A., Srisa-Art, M., Holt, D., Abell, C., Hollfelder, F., deMello, A.J., and Edel, J.B. (2007). Quantitative detection of protein expression in single cells using droplet microfluidics. Chem. Commun. (Camb.), 1218–1220.

Inglis, D.W., Davis, J.A., Zieziulewicz, T.J., Lawrence, D.A., Austin, R.H., and Sturm, J.C. (2008). Determining blood cell size using microfluidic hydrodynamics. J. Immunol. Methods *329*, 151–156.

Jeon, N.L., Dertinger, S.K.W., Chiu, D.T., Choi, I.S., Stroock, A.D., and Whitesides, G.M. (2000). Generation of solution and surface gradients using microfluidic systems. Langmuir *16*, 8311–8316.

Joensson, H.N., Samuels, M.L., Brouzes, E.R., Medkova, M., Uhlen, M., Link, D.R., and Andersson-Svahn, H. (2009). Detection and analysis of low-abundance cell-surface biomarkers using enzymatic amplification in microfluidic droplets. Angew. Chem. Int. Ed. Engl. *48*, 2518–2521.

Kartalov, E.P., Walker, C., Taylor, C.R., Anderson, W.F., and Scherer, A. (2006a). Microfluidic vias enable nested bioarrays and autoregulatory devices in Newtonian fluids. Proc. Natl. Acad. Sci. *103*, 12280–12284.

Kartalov, E.P., Zhong, J.F., Scherer, A., Quake, S.R., Taylor, C.R., and Anderson, W.F. (2006b). High-throughput multi-antigen microfluidic fluorescence immunoassays. Biotechniques *40*, 85–90.

Keymer, J.E., Galajda, P., Muldoon, C., Park, S., and Austin, R.H. (2006). Bacterial metapopulations in nanofabricated landscapes. Proc. Natl. Acad. Sci. USA *103*, 17290–17295.

Kim, M.J., and Breuer, K.S. (2007). Controlled mixing in microfluidic systems using bacterial chemotaxis. Anal. Chem. *79*, 955–959.

Kiss, M.M., Ortoleva-Donnelly, L., Beer, N.R., Warner, J., Bailey, C.G., Colston, B.W., Rothberg, J.M., Link, D.R., and Leamon, J.H. (2008). High-throughput quantitative polymerase chain reaction in picoliter droplets. Anal. Chem. *80*, 8975–8981.

Knight, J.B., Vishwanath, A., Brody, J.P., and Austin, R.H. (1998). Hydrodynamic focusing on a silicon chip: mixing nanoliters in microseconds. Phys. Rev. Lett. *80*, 3863.

Koster, S., Angile, F.E., Duan, H., Agresti, J.J., Wintner, A., Schmitz, C., Rowat, A.C., Merten, C.A., Pisignano, D., Griffiths, A.D., et al. (2008). Drop-based microfluidic devices for encapsulation of single cells. Lab Chip *8*, 1110–1115.

Lanigan, P.M., Ninkovic, T., Chan, K., de Mello, A.J., Willison, K.R., Klug, D.R., Templer, R.H., Neil, M.A., and Ces, O. (2009). A microfluidic platform for probing single cell plasma membranes using optically trapped Smart Droplet Microtools (SDMs). Lab Chip *9*, 1096–1101.

Lee, C.H., and Lal, A. (2004). Single microdroplet ejection using an ultrasonic longitudinal mode with a PZT/tapered glass capillary. IEEE Trans. Ultrason. Ferroelectr. Freq. Control *51*, 1514–1522.

Lee, K., Kim, C., Kim, Y., Jung, K., Ahn, B., Kang, J., and Oh, K. (2010a). 2-layer based microfluidic concentration generator by hybrid serial and volumetric dilutions. Biomedical Microdevices *12*, 297–309.

Lee, W.G., Kim, Y.G., Chung, B.G., Demirci, U., and Khademhosseini, A. (2010b). Nano/microfluidics for diagnosis of infectious diseases in developing countries. Adv. Drug Del. Rev. *62*, 449–457.

Lemmo, A.V., Rose, D.J., and Tisone, T.C. (1998). Inkjet dispensing technology: applications in drug discovery. Curr. Opin. Biotechnol. *9*, 615–617.

Li, L., and Ismagilov, R.F. (2010). Protein crystallization using microfluidic technologies based on valves, droplets, and SlipChip. Annu. Rev. Biophys. *39*, 139–158.

Li, L., Mustafi, D., Fu, Q., Tereshko, V., Chen, D.L.L., Tice, J.D., and Ismagilov, R.F. (2006). Nanoliter microfluidic hybrid method for simultaneous screening and optimization validated with crystallization of membrane proteins. Proc. Natl. Acad. Sci. USA *103*, 19243–19248.

Li, L., Boedicker, J.Q., and Ismagilov, R.F. (2007). Using a multijunction microfluidic device to inject substrate into an array of preformed plugs without cross-contamination: comparing theory and experiments. Anal. Chem. *79*, 2756–2761.

Li Jeon, N., Baskaran, H., Dertinger, S.K.W., Whitesides, G.M., Van De Water, L., and Toner, M. (2002). Neutrophil chemotaxis in linear and complex gradients of interleukin-8 formed in a microfabricated device. Nat. Biotech. *20*, 826–830.

Lin, F., Saadi, W., Rhee, S.W., Wang, S.-J., Mittal, S., and Jeon, N.L. (2004). Generation of dynamic temporal and spatial concentration gradients using microfluidic devices. Lab Chip *4*, 164–167.

Link, D.R., Anna, S.L., Weitz, D.A., and Stone, H.A. (2004). Geometrically mediated breakup of drops in microfluidic devices. Phys. Rev. Lett. *92*, 054503.

Link, D.R., Grasland-Mongrain, E., Duri, A., Sarrazin, F., Cheng, Z.D., Cristobal, G., Marquez, M., and Weitz, D.A. (2006). Electric control of droplets in microfluidic devices. Ange. Chem. Int. Ed. *45*, 2556–2560.

Lockery, S. (2007). Channeling the worm: microfluidic devices for nematode neurobiology. Nat Meth *4*, 691–692.

Maerkl, S.J., and Quake, S.R. (2007). A systems approach to measuring the binding energy landscapes of transcription factors. Science *315*, 233–237.

Manz, A., Graber, N., and Widmer, H.M. (1990). Miniaturized total chemical-analysis systems—a novel concept for chemical sensing. Sens. Actuators B Chem. *1*, 244–248.

Marcus, J.S., Anderson, W.F., and Quake, S.R. (2006). Microfluidic single-cell mRNA isolation and analysis. Anal. Chem. *78*, 3084–3089.

Marcy, Y., Ouverney, C., Bik, E.M., Lösekann, T., Ivanova, N., Martin, H.G., Szeto, E., Platt, D., Hugenholtz, P., Relman, D.A., et al. (2007). Dissecting biological "dark matter" with single-cell genetic analysis of rare and uncultivated TM7 microbes from the human mouth. Proc. Natl. Acad. Sci. USA *104*, 11889–11894.

Margulies, M., Egholm, M., Altman, W.E., Attiya, S., Bader, J.S., Bemben, L.A., Berka, J., Braverman, M.S., Chen, Y.J., Chen, Z.T., et al. (2005). Genome sequencing in microfabricated high-density picolitre reactors. Nature *437*, 376–380.

Martin, K., Henkel, T., Baier, V., Grodrian, A., Schön, T., Roth, M., Köhler, J., and Metze, J. (2003). Generation of larger numbers of separated microbial populations by cultivation in segmented-flow microdevices. Lab Chip *3*, 202–207.

Martinez, A.W., Phillips, S.T., and Whitesides, G.M. (2008). Three-dimensional microfluidic devices fabricated in layered paper and tape. Proc. Natl. Acad. Sci. USA *105*, 19606–19611.

Mazutis, L., and Griffiths, A.D. (2009). Preparation of monodisperse emulsions by hydrodynamic size fractionation. Appl. Phys. Lett. *95*, 204103-1–204103-3. 10.1063/1.3250432.

Mazutis, L., Baret, J.C., and Griffiths, A.D. (2009). A fast and efficient microfluidic system for highly selective one-to-one droplet fusion. Lab Chip *9*, 2665–2672.

Melin, J., and Quake, S.R. (2007). Microfluidic large-scale integration: the evolution of design rules for biological automation. Annu. Rev. Biophys. Biomol. Struct. *36*, 213–231.

Metzker, M.L. (2010). Applications of next-generation sequencing technologies—the next generation. Nat. Rev. Genet. *11*, 31–46.

Miller, E.M., and Wheeler, A.R. (2009). Digital bioanalysis. Anal. Bioanal. Chem. *393*, 419–426.

Morton, K.J., Loutherback, K., Inglis, D.W., Tsui, O.K., Sturm, J.C., Chou, S.Y., and Austin, R.H. (2008a). Crossing microfluidic streamlines to lyse, label and wash cells. Lab Chip *8*, 1448–1453.

Morton, K.J., Loutherback, K., Inglis, D.W., Tsui, O.K., Sturm, J.C., Chou, S.Y., and Austin, R.H. (2008b). Hydrodynamic metamaterials: microfabricated arrays to steer, refract, and focus streams of biomaterials. Proc. Natl. Acad. Sci. USA *105*, 7434–7438.

Nagrath, S., Sequist, L.V., Maheswaran, S., Bell, D.W., Irimia, D., Ulkus, L., Smith, M.R., Kwak, E.L., Digumarthy, S., Muzikansky, A., et al. (2007). Isolation of rare circulating tumour cells in cancer patients by microchip technology. Nature *450*, 1235–1239.

Nam-Trung, N., and Zhigang, W. (2005). Micromixers—a review. J. Micromech. Microeng. *15*, R1.

Niu, X., Gulati, S., Edel, J.B., and deMello, A.J. (2008). Pillar-induced droplet merging in microfluidic circuits. Lab Chip *8*, 1837–1841.

Oh, K.W., and Ahn, C.H. (2006). A review of microvalves. J. Micromech. Microeng. *16*, R13–R39.

Ohno, K., Tachikawa, K., and Manz, A. (2008). Microfluidics: applications for analytical purposes in chemistry and biochemistry. Electrophoresis *29*, 4443–4453.

Ottesen, E.A., Hong, J.W., Quake, S.R., and Leadbetter, J.R. (2006). Microfluidic digital PCR enables multigene analysis of individual environmental bacteria. Science *314*, 1464–1467.

Paliwal, S., Iglesias, P.A., Campbell, K., Hilioti, Z., Groisman, A., and Levchenko, A. (2007). MAPK-mediated bimodal gene expression and adaptive gradient sensing in yeast. Nature *446*, 46–51.

Park, J.W., Vahidi, B., Taylor, A.M., Rhee, S.W., and Jeon, N.L. (2006). Microfluidic culture platform for neuroscience research. Nat. Protoc. *1*, 2128–2136.

Pushkarev, D., Neff, N.F., and Quake, S.R. (2009). Single-molecule sequencing of an individual human genome. Nat. Biotech. *27*, 847–850.

Qin, D., Xia, Y., and Whitesides, G.M. (1996). Rapid prototyping of complex structures with feature sizes larger than 20 mum. Adv. Mat. *8*, 917–919.

Rowat, A.C., Bird, J.C., Agresti, J.J., Rando, O.J., and Weitz, D.A. (2009). Tracking lineages of single cells in lines using a microfluidic device. Proc. Natl. Acad. Sci. USA *106*, 18149–18154.

Saadi, W., Wang, S.-J., Lin, F., and Jeon, N. (2006). A parallel-gradient microfluidic chamber for quantitative analysis of breast cancer cell chemotaxis. Biomed. Microdev. *8*, 109–118.

Schaerli, Y., Wootton, R.C., Robinson, T., Stein, V., Dunsby, C., Neil, M.A.A., French, P.M.W., deMello, A.J., Abell, C., and Hollfelder, F. (2009). Continuous-flow polymerase chain reaction of single-copy DNA in microfluidic microdroplets. Anal. Chem. *81*, 302–306.

Shendure, J., and Ji, H. (2008). Next-generation DNA sequencing. Nat. Biotech. *26*, 1135–1145.

Song, H., and Ismagilov, R.F. (2003). Millisecond kinetics on a microfluidic chip using nanoliters of reagents. J. Am. Chem. Soc. *125*, 14613–14619.

Song, H., Tice, J.D., and Ismagilov, R.F. (2003). A microfluidic system for controlling reaction networks in time. Angew. Chem. Int. Ed. Engl. *42*, 768–772.

Song, H., Li, H.W., Munson, M.S., Van Ha, T.G., and Ismagilov, R.F. (2006). On-chip titration of an anticoagulant argatroban and determination of the clotting

time within whole blood or plasma using a plug-based microfluidic system. Anal. Chem. *78*, 4839–4849.

Squires, T.M., and Quake, S.R. (2005). Microfluidics: Fluid physics at the nanoliter scale. Rev. Mod. Phys. *77*, 977.

Srinivasan, V., Pamula, V.K., and Fair, R.B. (2004). An integrated digital microfluidic lab-on-a-chip for clinical diagnostics on human physiological fluids. Lab Chip *4*, 310–315.

Stone, H.A., Stroock, A.D., and Ajdari, A. (2004). Engineering flows in small devices: Microfluidics toward a lab-on-a-chip. Annu. Rev. Fluid Mech. *36*, 381–411.

Stroock, A.D., Dertinger, S.K.W., Ajdari, A., Mezic, I., Stone, H.A., and Whitesides, G.M. (2002). Chaotic mixer for microchannels. Science *295*, 647–651.

Tam, J.M., Song, L., and Walt, D.R. (2009). DNA detection on ultrahigh-density optical fiber-based nanoarrays. Biosens. Bioelectron. *24*, 2488–2493.

Taylor, G. (1953). Dispersion of soluble matter in solvent flowing slowly through a tube. Proc. R. Soc. Lond. A Math. Phys. Sci. *219*, 186–203.

Taylor, A.M., Rhee, S.W., and Jeon, N.L. (2006). Microfluidic chambers for cell migration and neuroscience research. Methods Mol. Biol. *321*, 167–177.

Taylor, R.J., Falconnet, D., Niemistö, A., Ramsey, S.A., Prinz, S., Shmulevich, I., Galitski, T., and Hansen, C.L. (2009). Dynamic analysis of MAPK signaling using a high-throughput microfluidic single-cell imaging platform. Proc. Natl. Acad. Sci. USA *106*, 3758–3763.

Terry, S.C., Jerman, J.H., and Angell, J.B. (1979). A gas chromatographic air analyzer fabricated on a silicon wafer. IEEE Trans. Electron Devices *26*, 1880–1886.

Tewhey, R., Warner, J.B., Nakano, M., Libby, B., Medkova, M., David, P.H., Kotsopoulos, S.K., Samuels, M.L., Hutchison, J.B., Larson, J.W., et al. (2009). Microdroplet-based PCR enrichment for large-scale targeted sequencing. Nat. Biotechnol. *27*, 1025–1094.

Thorsen, T., Roberts, R.W., Arnold, F.H., and Quake, S.R. (2001). Dynamic pattern formation in a vesicle-generating microfluidic device. Phys. Rev. Lett. *86*, 4163–4166.

Thorsen, T., Maerkl, S.J., and Quake, S.R. (2002). Microfluidic large-scale integration. Science *298*, 580–584.

Toriello, N.M., Douglas, E.S., Thaitrong, N., Hsiao, S.C., Francis, M.B., Bertozzi, C.R., and Mathies, R.A. (2008). Integrated microfluidic bioprocessor for single-cell gene expression analysis. Proc. Natl. Acad. Sci. USA *105*, 20173–20178.

Trimmer, W.S.N. (1989). Microrobots and micromechanical systems. Sens. Actuators *19*, 267–287.

Umbanhowar, P.B., Prasad, V., and Weitz, D.A. (2000). Monodisperse emulsion generation via drop break off in a coflowing stream. Langmuir *16*, 347–351.

Unger, M.A., Chou, H.P., Thorsen, T., Scherer, A., and Quake, S.R. (2000). Monolithic microfabricated valves and pumps by multilayer soft lithography. Science *288*, 113–116.

Urbanski, J.P., Thies, W., Rhodes, C., Amarasinghe, S., and Thorsen, T. (2006). Digital microfluidics using soft lithography. Lab Chip *6*, 96–104.

Vogelstein, B., and Kinzler, K.W. (1999). Digital PCR. Proc. Natl. Acad. Sci. USA *96*, 9236–9241.

Vyawahare, S., Sitaula, S., Martin, S., Adalian, D., and Scherer, A. (2008). Electronic control of elastomeric microfluidic circuits with shape memory actuators. Lab Chip *8*, 1530–1535.

Weaver, J.A., Melin, J., Stark, D., Quake, S.R., and Horowitz, M.A. (2010). Static control logic for microfluidic devices using pressure-gain valves. Nat Physiol. *6*, 218–223.

Weigl, B., Domingo, G., LaBarre, P., and Gerlach, J. (2008). Towards non- and minimally instrumented, microfluidics-based diagnostic devices. Lab Chip *8*, 1999–2014.

Wheeler, A.R., Throndset, W.R., Whelan, R.J., Leach, A.M., Zare, R.N., Liao, Y.H., Farrell, K., Manger, I.D., and Daridon, A. (2003). Microfluidic device for single-cell analysis. Anal. Chem. *75*, 3581–3586.

Whitesides, G.M. (2006). The origins and the future of microfluidics. Nature *442*, 368–373.

Williams, M.S., Longmuir, K.J., and Yager, P. (2008). A practical guide to the staggered herringbone mixer. Lab Chip *8*, 1121–1129.

Wu, H., Odom, T.W., Chiu, D.T., and Whitesides, G.M. (2002). Fabrication of complex three-dimensional microchannel systems in PDMS. J. Am. Chem. Soc. *125*, 554–559.

Xie, Y., Wang, Y., Chen, L., and Mastrangelo, C.H. (2008). Fourier microfluidics. Lab Chip *8*, 779–785.

Zare, R.N., and Kim, S. (2010). Microfluidic platforms for single-cell analysis. Annu. Rev. Biomed. Eng. *12*, 187–201.

Zhang, C., Xu, J., Ma, W., and Zheng, W. (2006). PCR microfluidic devices for DNA amplification. Biotechnol. Adv. *24*, 243–284.

Zhong, J.F., Chen, Y., Marcus, J.S., Scherer, A., Quake, S.R., Taylor, C.R., and Weiner, L.P. (2008). A microfluidic processor for gene expression profiling of single human embryonic stem cells. Lab Chip *8*, 68–74.