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Integrated nanoliter systems

Jong Wook Hong & Stephen R Quake

Microfluidic chip platforms for manipulating liquid volumes in the nanoliter range are slowly inching their way into mainstream genomic and proteomic research. The principal challenge faced by these technologies is the need for high-throughput processing of increasingly smaller volumes, with ever higher degrees of parallelization. Significant advances have been made over the past few years in addressing these needs through electrokinetic manipulation, vesicle encapsulation and mechanical valve approaches. These strategies allow levels of integration density and platform complexity that promise to make them into serious alternatives to current robotic systems.

There have been many driving forces to exploit the potential benefits of micro-sized apparatus relative to systems of conventional size, including reduced consumption of samples and reagents, shorter analysis times, greater sensitivity, portability that allows *in situ* and real-time analysis, and disposability[1]. A unifying vision for the field has been the notion that, in the same way that integrated circuits used miniaturized transistors to automate computation, microfluidic chips could accomplish large-scale automation of biological processing using nanoliter volumes. Although the first microfabricated, miniaturized gas chromatograph was described in 1975, the introduction of miniaturized formats for analytical chemistry and biology using liquids in microfabricated chips did not begin in earnest until the early 1990s. The ensuing decade was a period of furious technological development, and many individual microfluidic components with the ability to perform biological manipulations on nanoliter volumes were demonstrated[2–5]. However, the actual impact of microfluidics on the life sciences and biotechnology thus far has been limited—very few academic biology laboratories use microfluidic devices on a routine basis, and when faced with a decision on how to automate, most companies still choose macroscopic robots. The disconnect in this area between technology developers and technology users is particularly dramatic, and has lasted longer than many observers expected.

One reason for this disconnect has been the difficulty of making the transition from simple microfluidic components to highly integrated systems. Individual microfluidic components, even if they are capable of analyzing nanoliters of material, are often of little use unless they can be integrated together in a functional system. An exception to this rule is when a microfluidic component takes advantage of novel fluid physics available only at the microscale. There have been several scientific demonstrations that take advantage of these effects[6] (see **Box 1**), and some of them may find their way to commercialization.

The first microfluidic systems with substantial complexity operated with microliter volumes of reagents and hence had limited scalability. Today, we are seeing the emergence of truly integrated microfluidic systems for biotechnology that operate on nanoliters of material, which can be termed 'nanofluidic systems'. In this review, we discuss recent results indicating that this technology may now be poised to realize its potential and have a markedly increased impact on the life sciences. We first describe technology platforms that allow scalable nanoliter manipulations, and go on to discuss system design strategies and specific biotechnology applications.

Department of Applied Physics, California Institute of Technology, MC 128-95, Pasadena, California 91125, USA. Correspondence should be addressed to S.R.Q. (quake@caltech.edu).

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Analogies with integrated electronic circuits

The main technology platforms for micro- and nanofluidic research are based on microfabrication techniques, such as photolithography, that were originally developed for the semiconductor industry[7]. The initial motivation was the idea that fabrication technologies used for manipulating electrons in ever-more-complicated ways could also be used to make devices that would manipulate fluids. Common substrates used in these devices are glass and silicon, but such hard materials are not ideally suited for implementing robust liquid control systems such as valves and pumps. Thus, alternative fabrication methods and materials such as soft lithography with silicone rubber became popular, resulting in the emergence of a wide variety of techniques and materials to make fluidic devices. The details of fabrication notwithstanding, there are many interesting analogies between integrated nanofluidic system design and the development of integrated circuits, and it remains instructive to make conceptual comparisons between the two fields.

One of the most significant achievements of the semiconductor electronics industry has been to automate computation to an extent never before imagined. Early automated computation in the World War II era was used for solving calculational problems and consisted of teams of people operating adding machines. This was followed by complete electronic automation with vacuum tubes, and computers such as ENIAC that filled entire rooms. However, it was not until the development of the silicon integrated circuit, and the tremendous complexity, economy of scale and computational power it enabled, that the true power of automated computation was realized: word processing, spreadsheets, databases, email and so forth. The scalability of integrated circuit technology was thus crucial to its success.

In the past decade, biology has very quickly advanced from a state of mostly manual labor to one of early automation. Current biological automation is roughly comparable to the vacuum tube era of electronics:

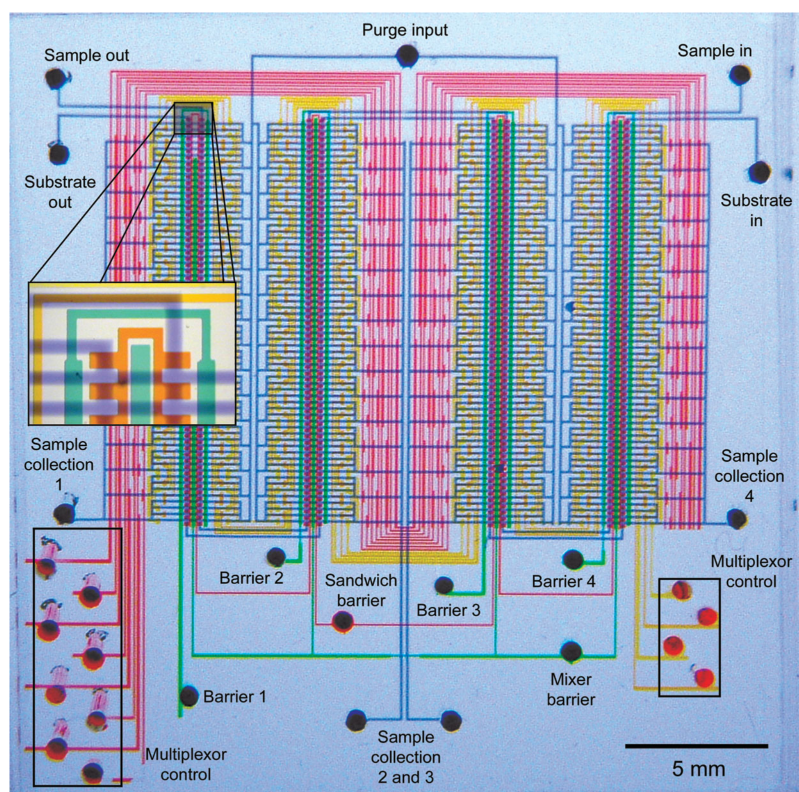

Figure 1 Optical micrograph of a nanofluidic system that can be used for parallelized high-throughput screening of fluorescence-based single-cell assays. The various inputs have been loaded with food dyes to show the channels and subelements of the fluidic logic. This chip has 2,056 valves, which are used to manipulate 256 compartments containing bacterial cells expressing an enzyme of interest (or a library of mutants of that enzyme) that are combined on a pairwise basis with 256 other compartments containing a fluorogenic substrate used to assay for a desired activity. Cells that display a particularly interesting activity can be selected and recovered from the chip using valve-based addressing of the compartments. (Adapted from ref. 18.)

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entire rooms are filled with robotic fluid-handling systems that shuttle plates between large machines. One goal of nanofluidic systems is to automate biology to an extent comparable to what semiconductor electronics achieved for computation, with the faith that similar, nontrivial, perhaps completely unanticipated new approaches to biology will emerge. However, integration of nanoliter systems with microfluidic chips has to this point lagged behind the possibilities offered by robots and conventional fluid-handling tools in both sophistication and parallelism. Furthermore, as there has been a significant investment into robotic systems and proven results with them, it is not good enough for chip-based approaches merely to equal the capabilities of such systems. It is only when the sophistication of fluidic chip integration surpasses robotic capabilities that one will see widespread adoption of the chips in industrial settings based merely on their utility in automation. This problem is comparable to the one faced by pioneers in the semiconductor industry (G. Moore, personal communication).

Technology platforms for scalable nanoliter systems

The earliest integrated microfluidic systems were used for sample preparation followed by polymerase chain reaction (PCR) or microarray analysis. They are good examples of how microfluidics can be applied to systems integration, but they consume large amounts (microliters) of reagents. Yuen *et al.*[8] reported a microchip module for sample preparation and PCR on a silicon-glass microchip. They used human blood samples, isolated white blood cells from them and amplified a human coagulation factor V gene (226 bp in length) from 8–9 µl of sample mixture. In another paper, Anderson *et al.*[9] developed a highly integrated monolithic device for a series of biochemical analyses, reverse transcription–PCR (RT-PCR), PCR, DNA fragmentation, dephosphorylation, terminal transferase labeling, dilution, hybridization, washing *et cetera*, on a device smaller than a credit card. Their device was integrated with a commercial DNA microarray, and both devices performed a serial set of reactions but without parallel processing.

Developing devices with integration densities, complexities and capabilities that rival current robotic systems will require technology platforms that are capable of manipulating smaller volumes while performing more complex tasks with higher degrees of parallelization and integration. Scalability is therefore essential. Three possible technology platforms may address these needs: electrokinetic manipulation, vesicle encapsulation and mechanical valving. A fourth platform, dubbed microfluidic tectonics, has recently been reviewed elsewhere and will not be discussed here[10].

Electrokinetic manipulation

Electrokinetic manipulation of fluids has been a popular research topic since the first report of capillary electrophoresis on a microfluidic chip. This method is ideally suited for separations applications and is amenable to parallelization; a recent example of parallelized capillary array electrophoresis (µCAE) was demonstrated by Emrich *et al.*[11]. They fabricated 384 capillary lanes on a 200-mm-diameter glass substrate sandwich and showed a 98.7% success rate in genotyping, with hereditary hemochromatosis as a model system. Although the system was loaded serially, electrophoresis and readout were carried out in a parallel manner.

Aside from simple separation applications, electrokinetics has also been used to manipulate a variety of different fluids, molecules and even cells on chips. Although electrokinetic manipulation has proved useful for loading and electrophoresis of samples, thus far it is unclear whether this platform will be able to scale in complexity to rival robotic systems. The challenges are similar to those faced by designers of analog computers: when every component of the system is coupled together, it is difficult to control, debug and design circuits. For these reasons, analog computers became useful for niche applications but never achieved the complexity possible with digital design. Electrokinetic systems face these and other obstacles; for example, the transport properties depend intimately on the charge of the molecules or particles being manipulated, and these specific dependencies make it difficult to design robust systems. Furthermore, molecules tend to 'leak' through intersections by diffusion, limiting the time scales over which fluids may be metered.

Recently, Kuo *et al.*[12] have attempted to address some of these challenges by building hybrid electrokinetic devices that use nanoporous membranes as gateable interconnects. They fabricated two different microfluidic channels with poly(dimethylsiloxane) (PDMS) and

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Box 1 Novel fluid physics

Aside from new capabilities emerging from the pure scale of systems integration, there are also interesting fluid physics inherent to microfluidic devices that can be exploited in the design and implementation of systems that can work at the nanoliter scale. There are several instances in which the low Reynolds number, or lack of turbulent phenomena, in microfluidic devices has been used for novel purposes—for example, in the generation of nontrivial chemical gradients to study chemotaxis[25] and the use of ultrafast mixing to study protein folding[26]. Microfluidic systems also have low Grashof numbers, or absence of density-driven convection. This property has been exploited to demonstrate that highly efficient protein crystallization kinetics can be achieved in microfluidic devices, outperforming conventional macroscopic methods of protein growth[23]. Finally, in some situations biochemical reactions progress more efficiently in small volumes because deleterious phenomena, such as competition with parasitic side reactions, are minimized (some evidence shows that this is the case with PCR; M.A. Unger, personal communication). The maximum potential of these ideas will be harnessed when the advantages provided by such novel physical properties are fully integrated within microfluidic systems.

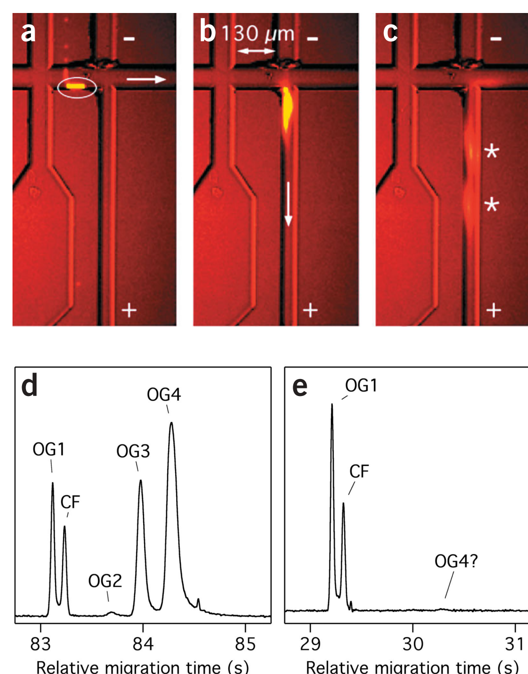

Figure 2 Automated microfluidic single-cell analysis device. (**a**) A Jurkat cell loaded with calcein AM (shown in white oval) is hydrodynamically transported to the lysis intersection (right-hand intersection). The cell image is distorted because the integration time of the CCD camera is relatively long compared to the cell velocity. The arrow depicts the direction of cell transport in the horiz-ontal channel. (**b**) The cell encounters the electric field in the right vertical channel and is lysed. The fluorescently labeled contents are injected into the separation channel and migrate toward the anode. Arrow, direction of lysate migration in the separation channel. (**c**) Two fluorescent components (marked by asterisks) in the separation channel are electrophoretically separated. (**d**) An electropherogram from a normal single Jurkat cell loaded with Oregon Green and carboxyfluorescein (CF). The peaks correspond to completely hydrolyzed Oregon green diacetate (OG1), Oregon green diacetate metabolites (OG2, OG3 and OG4), and CF. (**e**) Electropherogram from an anomalous Jurkat cell similarly loaded. This separation lacked several Oregon green metabolites seen in **d**. Approximately 10% of the Jurkat cells examined exhibited anomalous metabolite patterns. Single cell analysis rates are ~10/min, or more than 100 times faster than conventional approaches. (See ref. 22 for details.) (Figure courtesy of Michael Ramsey, Oak Ridge National Laboratory, Oak Ridge, Tennessee, USA.)

inserted 200-nm polycarbonate nuclear track–etched (PCTE) membrane having a disperse distribution of pore diameters between the two fluidic layer, enabling control of net fluid flow based different physical characteristics of the sample by electroosmotic control of the fluid. This provides an extra degree of control for electrokinetic manipulation, in that the effect of the bias voltage depends on the pore diameters.

Vesicle encapsulation

Another basic platform concept is to encapsulate the reagents of interest in a droplet, vesicle or micelle, which can then be manipulated in channels. This greatly reduces such problems as diffusive leaking of reagents and allows simple pressure-based control. Manipulating droplets of water separated by air bubbles has led to a number of demonstrations of small-scale reactions with limited complexity; one of the most sophisticated examples was described by Burns *et al.*[13]. However, the compressibility of air and differential resistance of different fluids makes it challenging to scale this process up to more complex geometries. Thus, effort has focused on using two-phase fluid systems, such as water and oil, to generate vesicles that could be used as small but controlled chemical reactors[14,15]. The most recent example of this is work by Song *et al.*[16], who have constructed a fluidic system that simultaneously transports and mixes solutions in aqueous droplets several hundred picoliters in volume. The fluid forces acting on the droplets generate chaotic flows in the interior of these tiny volumes, leading to efficient and rapid mixing without dispersing the volumes in the droplets. The authors showed that a flow system could be used to measure reaction rates of calcium binding to a fluorescent dye on the millisecond timescale[16]. Other applications of this device include the design of control networks for many biological or chemical reactions.

An unusual technique for fabricating micellar nanofluidic networks has recently been reported by Karlsson *et al.*[17], who eschewed conventional fabrication techniques in favor of pipettes. Their method is based on using surfactant membrane technology to build lipid nanotube-micelle networks, which then allow controlled transport of fluorescent nanoparticles. To construct the lipid nanotube system, they used several simple components: 5-μm-diameter carbon fibers and pipettes for positioning the vesicles, whose sizes ranged from 5 to 30 μm. Small nanotubes could be created from a micelle by patching onto the surface and pulling away, and they were able to measure fluid velocities as high as 60 μm/s inside the nanotubes.

Mechanical valves

The final platform that we will consider is the use of mechanical valves. This might represent, in some sense, the most robust solution to the challenges described above, because mechanical valves can be individually addressed and operate independently of the physical and chemical properties of the working fluid. The problem, historically, has been to devise a practical method of fabricating very small mechanical valves. There are several examples of integrated mechanical valves fabricated with conventional microelectrical mechanical systems (MEMS) technology; however, because of the stiffness and processing requirements of the materials involved, these tend to be large (millimeter scale) and difficult to fabricate, and thus the operational complexity of such devices has been severely limited.

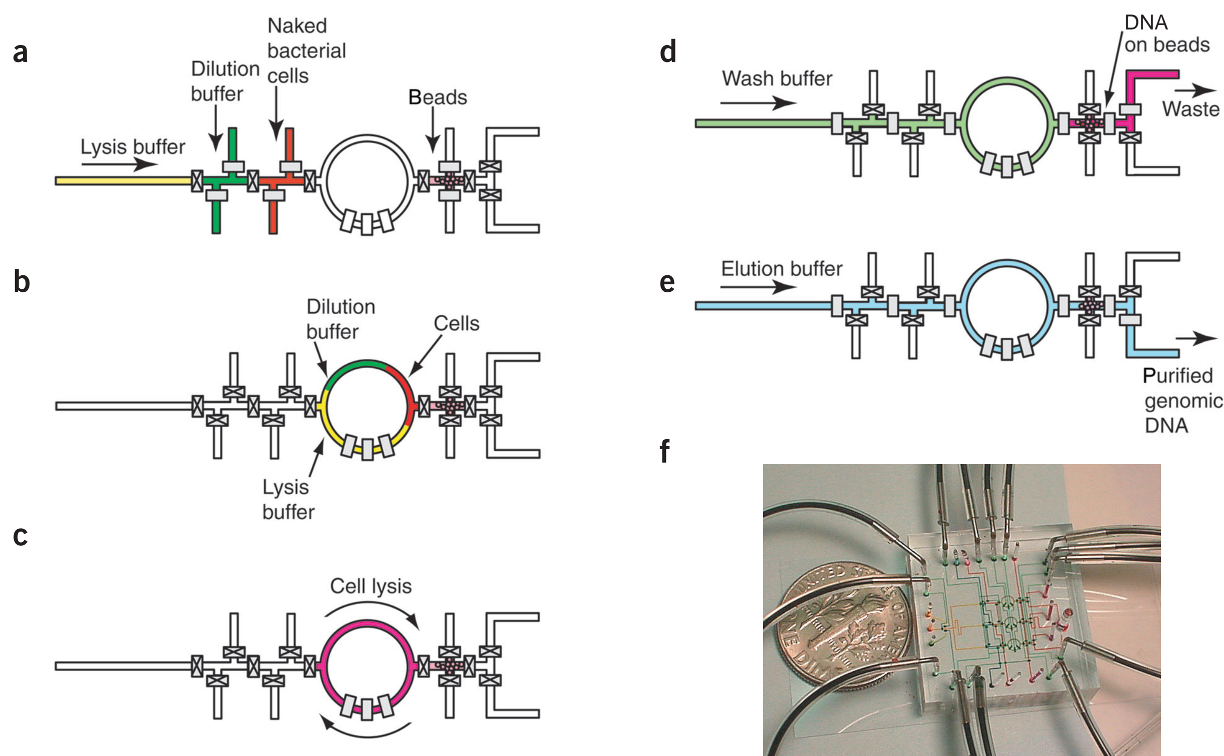

Figure 3 Parallel processing with nanoliters. (**a**–**e**) Process flow showing how an integrated processor for DNA purification can be implemented in a nanofluidic system. [☐], open valve; [×], closed valve. (**a**) Bacterial cell culture (indicated in red) is introduced into the chip through the 'cell in' port located in the uppermost part of the chip. Buffer (in green) for dilution of the cell sample is introduced through the 'buffer in' port located next to the 'cell in' port. The amount of dilution is determined by the ratio of channel lengths for cells and buffer; in this case it is 1:1. Lysis buffer (yellow) is introduced from the left side of the chip. (**b**) The cell sample, dilution buffer and lysis buffer slugs are introduced into the rotary mixer, which has a total volume of 5 nl. (**c**) The three liquids are circulated inside the reactor, resulting in efficient mixing and consequent lysis of the bacterial cells. (**d**) The lysate is flushed over a DNA affinity column and drained to the 'waste port'. (**e**) Purified DNA is recovered from the chip by introducing elution buffer from the left side of the chip. The recovered DNA can either be recovered from the chip or sent to another part of the chip for further analysis or manipulation. (**f**) Photograph of an actual nanofluidic system that implements three simultaneous parallel processes of the DNA recovery scheme illustrated above. The three parallel processes use distinct sample volumes of 1.6 nl, 1.0 nl and 0.4 nl, respectively. The parallel architecture allows complex processes to be implemented in parallel without increasing the control complexity of the system.

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One alternative that is gaining popularity is to use soft lithography to make monolithic mechanical valves out of rubber; this technology is highly scalable because of the ease of processing and low Young's modulus of rubber. We have built microfluidic memory arrays with 3,574 valves on a 1-inch-square chip using a multiplexing scheme that allows all the valves to be controlled through a handful of interconnects to the chip[18]. As a demonstration, we built a 1,000-chamber memory chip—a 25 × 40 grid addressed by 20 lines—that represents the highest degree of integration demonstrated in any nanofluidic platform to date. We have also shown that mechanical valves could be used for highly parallel high-throughput screening. A second chip integrated 2,056 valves with 256 compartments containing bacterial cells expressing an enzyme of interest (or a library of mutants of that enzyme) that could be combined on a pairwise basis with 256 other compartments containing a fluorogenic substrate used to assay for a desired activity[18] (**Fig. 1**).

The emergence of integrated nanoliter systems

To what extent can these technology platforms be used to make highly integrated, practical devices? Some of the earliest integrated nanofluidic devices used droplet-based and electrokinetic manipulation to show enzymatic reaction followed by electrophoretic analysis[13,19]. Electrokinetic manipulation is now being extended for use in automating serial processes for sample preparation. Recently, Broyles *et al.*[20] have demonstrated an example of integration of sample filtering, solid-phase extraction and open-channel electrochromatography on a quartz microchip. To accomplish this goal, they used a seven-channel array, 1 µm deep, for sample concentration and showed signal enhancement by using the chip. Although their target molecules were not biological samples, their scheme could be applied to the purification and separation of biomolecules, such as proteins or peptides.

Tang *et al.*[21] have provided another example of an integrated electrokinetic system that performs mixing, reaction and separation of reagents products for cycling probe technology (CPT), an isothermal signal amplification technique for specific DNA sequences that may provide an alternative to PCR. Their system—a glass chip (10.16 × 10.16 mm) containing a CPT reactor (with a volume of 160 nl) integrated with an electrophoretic separation system—was able to detect target DNA from the methicillin-resistant bacteria *Staphylococcus aureus*. Unlike PCR, which requires two or three different temperature zones for the reaction to take place, the CPT process only requires isothermal conditions for the reaction, usually 50–60 °C; this allows easy integration of genetic amplification and electrophoretic separation. Though the on-chip sensitivity was lower than that for off-chip experiments, with a sensitivity of 250 fM (25,000 target molecules), further optimization may improve

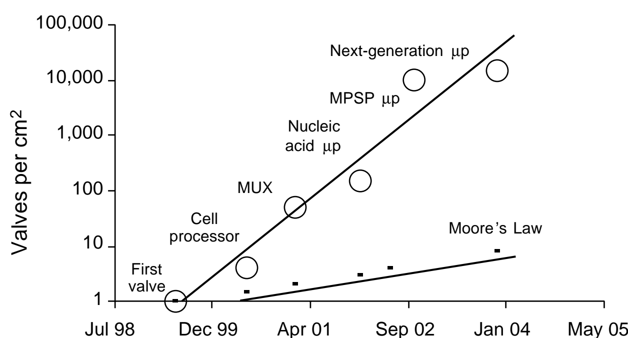

Figure 4 The analog of Moore's Law for nanofluidic systems. Valve densities in fluidic chips fabricated with soft lithography have increased exponentially with time. The current rate of growth is four times faster than the rate of growth of transistor densities in the semiconductor industry—it remains to be seen how long this can be maintained! MPSP, massively partitioning sniper processor; µp, microprocessor; MUX, multiplexer. (Figure courtesy of Fluidigm, San Francisco, CA, USA.)

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performance. Ramsey and collaborators have recently shown how sophisticated single-cell assays can be performed using electrokinetic methods[22] (**Fig. 2**).

Mechanical valve-based systems have also been used for automation of serial and parallel processes. Using extensive parallelization and valve-based fluid control schemes, Hansen *et al.*[23] and Liu *et al.*[24] have obtained significant economies of scale in both reagent consumption and pipetting steps for protein crystallization assays and PCR, respectively. Work in our group has shown how an integrated system could be used for trapping mammalian cells, lysis and mRNA purification from the cells (V. Studer, G. Hang, W.F. Anderson and S.R. Quake, unpublished data). The system has been used to purify measurable amounts of mRNA from as little as a single cell. This work has been further extended by developing an architecture in which such processes can be parallelized without increasing the control complexity of the chip (**Fig. 3**). The design has been applied to the development of a chip for parallel bioprocessing, showing DNA purification from small numbers of bacterial cells and demonstrating that the complete process, including cell isolation, cell lysis, DNA purification and DNA recovery, could be carried out on a single microfluidic chip in nanoliter volumes without any pre- or postsample treatment (J.W. Hong, W.F. Anderson and S.R. Quake, unpublished data). The chip is capable of simultaneously processing three different samples, thus illustrating how highly parallel architectures can be constructed to perform integrated batch processing functionalities with nanoliter sample volumes for other general biological and medical applications.

The future

What is the ultimate extent of systems integration possible with fluidic chips? Moore's Law is the observation that quantities of interest in the semiconductor industry, such as the number of transistors per square inch, grow exponentially with a doubling time of 18 months. If an analog to Moore's Law were applicable to microfluidics, it would allow us to predict future technological developments and anticipate new biological or medical applications that would be enabled by the ability to process samples at the nanoliter and picoliter scales. For one of the technology platforms discussed here, a similar law holds. The number of mechanical valves per square inch has grown exponentially, and with a doubling time that is four times faster than that reflected by Moore's Law (**Fig. 4**). Thus, it becomes possible to anticipate the future capabilities of nanofluidic systems, to invest research effort to remain on the exponential curve, and to begin to argue about where the basic physics will cause the exponential growth curve to become saturated.

Several scalable technology platforms are available for nanoliter fluid manipulation, and for each platform there has been some success in making integrated systems for biological analysis. Most component steps for molecular biology and biochemistry have now been implemented in these formats, and the ability to build scalable nanoliter systems out of such components presents a new challenge to the field: how can these pieces be put together to invent new applications for biological automation? In particular, can nanofluidic systems eclipse robots in performance and play the role for automation in biology that integrated circuits did for the automation of computation? In finding the answers to these questions, we will discover whether fluidic devices are ready to live up to their potential.

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