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## Micro Total Analysis Systems for Cell Biology and Biochemical Assays

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Novel applications of micro total analysis systems ( $\mu$ TAS) are addressing fundamental biological questions, creating new biomedical reagents, and developing innovative cell and biochemical assays. These efforts impact progress in all areas of  $\mu$ TAS from materials to fluidic handling as well as detection and external control systems. Three areas show the greatest current and potential impact on the biomedical sciences: improvements in device fabrication and operation, development of enabling technologies, and advancements at the interface with biology (Figure 1). The range of materials from which devices can be fabricated has expanded considerably and now includes paper, fabric and thread, and a multitude of polymers as well as more conventional materials. Thus device substrates and component materials suitable for nearly all biological applications are readily available. Devices are also becoming increasingly integrated with advancements in sample handling and preparation, key first steps in any biological analysis. Another growing area focuses on modular components that can be mixed and matched on-demand and applied to many different assays, so-called programmable microfluidics. This development should enhance the rate at which new bioassays are generated as well as customize existing experimental protocols. A second area of rapid advancement has been the development new technologies that enable assays that cannot be efficiently performed by any method except  $\mu$ TAS. Novel analyses of single cells are enabled due to effective manipulation of picoliter-scale volumes. Synthesis and screening of large-scale libraries has become increasingly feasible due to the fast processing speeds and combinatorial mixing of reagents provided by lab-on-chip systems. Increased automation within a completely contained system has now begun to provide some of the first true  $\mu$ TAS diagnostic devices for clinical medicine. The third area in which  $\mu$ TAS has begun to yield high dividends is the interfacing of living entities with microdevices to create biological communities, including tissues and organs on-chip. Control of cell placement in multiple dimensions has produced biological systems midway between the conventional tissue-culture dish and an intact animal. Thus the complexities of living constructs can be recreated in a controlled experimental environment permitting groundbreaking biological questions to be addressed. Application of  $\mu$ TAS in all of these areas continues to be highly interdisciplinary, utilizing techniques and strategies from almost every scientific field. This multidisciplinary focus insures continued relevance to the biological community as well as a bright future.

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Due to the rapid progress of  $\mu$ TAS or “lab-on-a-chip” systems, this review focuses on advances impacting cell biology and biochemistry and covers the time span from March 2010 through August 2011. The material for the review was compiled using several strategies: reviews of high impact journals such as *Analytical Chemistry*, *Lab on a Chip*, *Science*, *Nature*, and *PNAS*; extensive key word searches in databases such as PubMed, SciFinder, Web of Science, and Google Scholar; and screens of other recent topical reviews. Although several thousand papers were identified and over a thousand papers received a detailed examination, we focused on the most novel and exciting methods, devices, and applications in the areas of cell biology and biochemistry. We also endeavored to cover the most prominent work from a range of labs and countries. Ultimately we were limited by space constraints and our desire to craft a readable commentary on the state of the field. We apologize in advance for omitted papers and welcome feedback regarding any oversights on our part.

## 1. Fabrication and Operation

Although  $\mu$ TAS are rapidly maturing, ongoing efforts continue to yield improvements and innovation in fabrication technologies and modular subcomponents on-chip. In developing an analytical device, researchers begin by fabricating a prototype and testing unit operations, such as preparing, handling, and detecting small volumes of samples. This section reviews new device fabrication methods, addresses sample preparation and handling, and introduces innovative microfabricated detectors. Interestingly, these areas of continued progress represent the first and last steps in analysis. Microchip separations, which fall between sample preparation and detection, are now quite sophisticated, perhaps because many methods transfer readily from macroscale systems. Consequently, new separation methodologies on-chip are included in the section on integration and automation (Section 2). Here, we concern ourselves with the important and challenging processes that bookend any micro-analysis.

### 1.1 Fabrication

To choose a material for an application, several characteristics must be considered: cost, robustness, surface chemistry, optical and electrical properties, biocompatibility, ease of fabrication and integration, and feasibility of large-scale production. Depending on the application, devices may be fabricated from polymers, glass, silicon, paper, fabric thread, or a combination of these materials. Fabrication technologies are categorized here based on the type of material used.

**Polymers**—Among polymers, polydimethylsiloxane (PDMS) continues to be the most popular material for  $\mu$ TAS applications due to its easy fabrication, physical properties, and low cost. Among 255 references in this article, 144 studies use PDMS. Replica micromolding of PDMS against a master is well established and widely used, and a recent protocol, including a tutorial video, provided detailed instructions on making PDMS microfluidics.<sup>1</sup> On-going microfabrication efforts in PDMS focus on developing simple approaches to generate complex devices. A 3-dimensional (3D) microfluidic channel with a near-perfect circular cross section was obtained by a metal wire removal process.<sup>2</sup> Through-holes between layers in 3D microfluidics were generated simply and on-demand by high-throughput, localized tearing of a PDMS membrane.<sup>3</sup> The elastomeric property of PDMS is often exploited in building functional biomimetic devices. To reconstitute organ-level lung functions on a chip, a two-layer PDMS system contained two closely apposed microchannels separated by a thin, porous, flexible membrane (Figure 2a), and physiological breathing movements were mimicked by cyclic stretching of the flexible membrane using a vacuum applied to side microchannels.<sup>4</sup> Although academic laboratories favor PDMS for

rapid prototyping, researchers need to be aware of its limitations, such as rapid hydrophobic recovery after surface oxidation, adsorption and absorption of hydrophobic molecules, and swelling in common organic solvents.

Plastics, including poly(methyl methacrylate), polystyrene, polycarbonate and cyclic olefin copolymer, are increasingly common alternatives to PDMS. These materials can be processed by hot embossing or injection molding for high throughput and cost-effective mass production of microfluidic devices. In academic laboratories, hot embossing is more suitable than injection molding due to the relatively low cost of embossing equipment. For example, inexpensive and robust masters were recently fabricated photolithographically from SU-8 photoresist on copper substrates, then used for hot embossing of microfluidic reactors in a range of thermoplastic polymers including cycloolefin, polycarbonate, and UV-transparent acrylic polymers.<sup>5</sup> Polystyrene, the most commonly used material for *in vitro* cell-based research, was rapidly prototyped by embossing and bonding.<sup>6</sup> In addition to hot embossing and injection molding, other fabrication methods were used for plastic lab-on-a-chip devices, including microthermoforming,<sup>7</sup> roll-to-roll fabrication,<sup>8</sup> and casting.<sup>9</sup> This casting method generated prefabricated microfluidic blocks of epoxy SU-8 from flexible silicone molds. The blocks were quickly assembled into sophisticated microfluidic devices for a wide range of applications, potentially allowing laboratories to prototype new devices from pre-made blocks without investing in fabrication infrastructure (Figure 2b). Recent research also explored specialty polymers for microfluidic applications. Fluorinated thermoplastics, such as Teflon, were processed by a thermal embossing method using PDMS as master to yield Teflon microfluidic chips that exhibited extreme resistance to organic solvents (Figure 2c).<sup>10</sup> A photosensitive polymer formulation, SU-8 photoresist, was used for fast prototyping of monolithic 3D micro-systems by a mask-less micro-projection lithography platform.<sup>11</sup> Plastics overcome some limitations of PDMS, but their relatively complex fabrication still presents a bottleneck for widespread use for prototyping in academic laboratories. Relative to PDMS, plastics remain more suitable for industrial mass production of microfluidic devices rather than the rapid prototyping seen in most academic laboratories.

**Glass and silicon**—Compared to polymers, other materials are used for a relatively small proportion of published  $\mu$ TAS devices, most likely due to their more complex fabrication steps. Among inorganic materials, glass is most attractive due to its chemical inertness, optical transparency, and thermal stability. While fabrication of glass-based microfluidics generally involves etching and bonding, a new fabrication method used a spatiotemporally focused femtosecond laser beam to generate hollow microfluidic channels with a circular cross-section.<sup>12</sup> Unlike glass, silicon microfabrication is derived from the electronics industry. These mature fabrication methods enable the integration of various functionalities into a monolithic device. Complex 3D silicon microfluidic structures were produced by a single-mask, single-etch process by utilizing reactive ion etch lag, in which etching of a small trench lagged behind a large trench.<sup>13</sup> The channel was then sealed by deposition of dielectric films, avoiding the need for wafer to wafer bonding. While their fabrication can be complex, glass and silicon devices are particularly suitable for electrowetting-based microfluidics, highlighted in Section 1.3.

**Paper and thread**—Paper and thread have been exploited as the new substrates for low cost, disposable analytical devices. Inexpensive microfluidic platforms can be fabricated from paper by a number of methods; one notable strategy simply treated hydrophobic paper with a computer-controlled CO<sub>2</sub> laser.<sup>14</sup> Fabric thread was used to fabricate 3D and semiquantitative analytical devices by sewing it onto other materials, such as a polymer film.<sup>15</sup> Silk yarns were treated with the appropriate reagent solutions, dried and handloom-woven in one step into integrated fabric chips.<sup>16</sup> The wicking rate and the absorptive

capacity were conveniently adjusted by varying yarn parameters such as the twist frequency and weaving coverage. Cotton yarn and knots were used to build low cost passive microfluidic circuits which were capable of combining, mixing, splitting, and serially diluting streams of liquid.<sup>17</sup> Recent progress in fluid handling and detection on paper devices is highlighted in Section 2.1.

**Multi-component fabrication**—While most devices are manufactured by the microfabrication technologies mentioned above, many devices require components made from other materials to be integrated into the device to offer mechanical, electrical, optical, acoustic or magnetic functionalities. These components can be co-fabricated with the device or integrated on-chip after primary fabrication is complete. Often multi-component fabrication involves embedding a secondary material in a channel; for example, monolithic 3D porous silica microstructures in an SU-8 microchannel resulted in a four-fold increase in mixing efficiency,<sup>18</sup> micropatterned carbon nanotube forests confined inside PDMS channels captured particles ranging over three orders of magnitude in size,<sup>19</sup> and free-standing lipid membranes formed in a microfluidic chamber array provided a unique platform for studying membrane transport.<sup>20</sup> To build a portable, inexpensive and low-power heater, silver-filled epoxy was injected into and solidified in a PDMS microfluidic chip.<sup>21</sup> Optical readout has become the predominant detection method in lab-on-a-chip, driving efforts to merge microfluidics with photonic elements. An integrated multiple internal reflection system was built on a PDMS microfluidic chip for cell screening.<sup>22</sup> This device included self-alignment of microchannels, microlenses, and air mirrors. PDMS was dyed with ink for integration of robust, low cost filters for optical sensing in disposable labs-on-a-chip.<sup>23</sup> For electrical control, PDMS was mixed with multi-walled carbon nanotubes and processed by standard soft lithography into flexible 3D electrodes.<sup>24</sup> As evidenced by these devices, a functional, integrated, multicomponent device requires both creative design and careful fabrication.

**Surface modification**—Microfluidic systems require precisely tailored surface properties for many applications, and a variety of surface modification methods are now available, including plasma treatment, formation of self-assembled monolayers, application of dynamic or semi-permanent coatings, or covalent grafting of synthetic and biological molecules. Surface modification was used to aid the irreversible bonding of various plastic substrates with PDMS by forming a chemically robust amine-epoxy bond at the interfaces.<sup>25</sup> Since one of the limitations of PDMS is its unstable surface properties after modification, surface modification methods that generate long-term stable surface properties are needed. Protein-reinforced supported bilayer membranes offered long term stability of chip performance, even when stored in a dehydrated state for up to one month.<sup>26</sup>

## 1.2 Sample preparation

Sample preparation is often the most challenging step for an integrated microdevice. Since raw biological samples contain a complex mixture of compounds, a preliminary purification and/or concentration step is often essential. Extensive sample preparation “off-chip” greatly reduces the utility of  $\mu$ TAS, so these processes should take place on-chip whenever possible. On-chip processing is particularly important for low-volume, rare or valuable samples and is critical to realizing true sample-to-answer  $\mu$ TAS. However, the diversity of sample preparation methods has required that each technique be optimized for on-chip use independently. Consequently, the miniaturization of conventional sample preparation has lagged behind the development of analysis and detection techniques. Nevertheless, recent progress in microscale sample preparation techniques is evident.

**Extraction and purification**—Raw biological samples (e.g. blood, sputum, tissue, soil and food) often consist of a variety of components, so the analytes (e.g. nucleic acid, proteins, plasma, cells) must be extracted or purified from the raw samples prior to analysis. On-chip extraction and purification lead to substantial reduction of workloads and sample volumes. Separation of plasma from whole blood is highly desirable for minimizing the noise associated with blood cells during analysis. On-chip plasma separation was realized by utilizing either capillary force through a disposable bead-packed microchannel,<sup>27</sup> or hydrodynamic force in a microfluidic network featuring a series of constrictions and bifurcations.<sup>28</sup> On-chip extraction and purification of rare cells from blood has become a promising diagnostic tool and will be covered in Section 3.2.

Deoxyribonucleic acid (DNA) extraction involves the removal of DNA from the cells or viruses so that further analysis can be performed on the genetic material. DNA was extracted from whole blood samples using a microchannel packed with a silica solid phase and a standard syringe pump as a single pressure source driving the extraction process in a total column volume of 1.2  $\mu\text{L}$ .<sup>29</sup> Continuous DNA extraction and purification from cell lysate was realized on a microfluidic chip based on phase-transfer magnetophoresis using superparamagnetic beads.<sup>30</sup> Following DNA extraction, polymerase chain reaction (PCR) was used to amplify DNA molecules to an appropriate concentration for analysis (see Section 2.4).

Disease biomarkers, an important class of diagnostic tools, are often present in serum, saliva, or tissue in minute concentrations. Thus detection of these early disease indicators frequently requires extraction and purification. Using a microfluidic purification chip, protein biomarkers such as cancer antigens were extracted from a 10- $\mu\text{L}$  sample of whole blood via surface binding with their cognate antibodies.<sup>31</sup> They were then released from the chip in concentrated form by photocleavage for label-free detection. Using a micromagnetic separation chip, low-femtomolar concentrations of target proteins were extracted from serum by trapping magnetic beads coated with capture antibody and aptamers.<sup>32</sup> After removal of the external magnetic field, the bead-bound target complexes were eluted from the chip for detection. This process can potentially be expanded for multiplexed detection of other protein biomarkers and biomolecular targets through appropriate design of the aptamers.

**Concentration and dilution**—After purification, the concentration of analytes can be adjusted on the chip by concentration, dilution, or gradient formation. Numerous on-chip methods for concentrating samples bring them within the limit of detection, allowing researchers to choose the best option from a wide variety of techniques. For example, analytes were preconcentrated on microfluidic devices to detectable levels using a highly ion-conductive charge-selective polymer structure,<sup>33</sup> a thermoswitchable poly(N-isopropylacrylamide) hydrogel plug,<sup>15</sup> microscale isoelectric fractionation ( $\mu\text{IF}$ ) membranes,<sup>34</sup> a porous polypropylene membrane,<sup>35</sup> or a massively parallel nanofluidic device.<sup>36</sup> On the other hand, analytes were diluted on-chip to generate a large number of universal stepwise monotonic concentrations with a wide range of logarithmic and linear scales, which will be useful for high throughput screening.<sup>37</sup> Gradient formation on microfluidic chips is particularly useful for controlling the cellular microenvironment, covered in Section 3.4.

**Eliminating sample preparation**—The inability to detect analytes in complex mixtures (e.g. blood, tissue biopsies, etc.) has been a major stumbling block in the development of  $\mu\text{TAS}$ . While many important efforts focus on on-chip purification and concentration to increase selectivity and sensitivity,  $\mu\text{TAS}$  capable of performing these operations can be complex to the point of impracticality. Additionally,  $\mu\text{TAS}$  devices that directly analyze

samples without extensive sample preparation will be useful in developing countries and other settings lacking extensive laboratory infrastructure. The most robust platforms may be those in which sample pretreatment is not necessary; either because the complex sample is examined in its entirety or because off-target substances do not significantly affect detection of the target molecule. A paper spray method offered a novel, inexpensive, rapid method of direct analysis of a complex mixture such as whole blood for mass spectrometry.<sup>38</sup> Analyte traveled through the porous paper medium by capillary action while blood cells were retained, so nearly no sample preparation was required prior to analysis. Chromatographic separations were also performed prior to paper spray, and this technique is likely to have a major impact on the paper microfluidics sector (Section 2.1). A functionalized gold-nanoparticles (GNPs) sensor was developed for detection of volatile organic compounds (VOCs) from exhaled breath.<sup>39</sup> The array may offer promise in differentiating between ‘healthy’ and ‘cancerous’ breath for some patients. Since only exhaled breath is collected for direct analysis, absolutely no sample preparation is needed for this technology.

### 1.3 Sampling handling

Sampling handling has always been a strength of microfluidic systems, which provide simple and precise control of small volumes of fluids. Impressive new approaches continue to be reported for precise positioning, mixing, and splitting of samples in  $\mu$ TAS applications.

**Droplet microfluidics**—Droplet microfluidic systems continue to advance sample containment and sample handling. While passive droplet generation and mixing are well-understood, on-demand capabilities and more complex manipulations are still emerging. Notable recent work in this area includes novel droplet generation schemes, as well as new sorting, reagent introduction, and sampling techniques. Passive droplet generation systems achieve high throughput and low volume variability at equilibrium, but droplets cannot be produced on-demand and volumes cannot be rapidly modulated. An innovative laser pulse-driven droplet generation mechanism, in which a cavitation bubble ejected droplets from an aqueous channel into an adjacent oil-filled channel, produced droplets ranging from 1 to 150 pL and at kHz rates (Figure 3a).<sup>40</sup> Alternatively, other strategies produced droplets at low Hz rates, taking as compensation greater control over droplet size and composition. As a culmination of earlier work, an automated microfluidic droplet-generating system called Droplab used a syringe pump to pull samples into the tapered end of a short capillary that was physically moved between samples via an automated sample presentation system.<sup>41</sup> Such precise control over droplet size and composition has not yet been demonstrated with alternative systems.

Other recent research focuses on controlling droplet transport. A novel approach to droplet sorting used piezoelectric membrane actuation to temporarily alter the hydrodynamic resistance of two daughter channels, thereby diverting a passing droplet into a side channel.<sup>42</sup> Alternatively, a permanent difference of hydrodynamic resistance between daughter channels was exploited as a reliable method of splitting sample plugs, providing a simple, passive mechanism of “sampling” from plugs in segmented flow.<sup>43</sup> Droplets were also manipulated using microfabricated depressions and canals within the walls of microfluidic channels; droplets squeezed into these structures were reversibly anchored in place or guided along a distinct path.<sup>44</sup>

A number of experiments that could be performed in droplet microfluidic systems require droplets to be modified post-production—modifications include introduction of additional reagents, diversion of some or all of a droplet toward detection equipment, and selective removal of particular analytes from the droplet. In recent work, reagents were introduced

into surfactant stabilized droplets by bringing them into close physical contact with a side channel containing a pressurized reagent. An electric field destabilized the droplet/carrier phase interface to allow transient fusion with the reagent channel contents (Figure 3b).<sup>45</sup> If the electric field was switched off, fusion did not occur, allowing selective reagent addition to specific droplets. Multiple, individually controlled injectors manufactured in series permitted much more complex reagent addition schemes than were previously possible. The reverse operation – sampling from droplet contents – can also be performed. Application of an electric field extracted samples from droplets into an aqueous buffer channel running underneath a main channel. This sampling methodology allowed traditional microchip capillary electrophoresis (CE) methods to be interfaced with droplet microfluidics.<sup>46</sup> In contrast to the previous method, in which an unmodified portion of a droplet was sent to a detector, other methods increase selectivity and sensitivity by sampling analytes in a chemically-specific fashion, for example following liquid-liquid extraction. An electrowetting-on-dielectric (EWOD) device was used to position, merge, mix, and separate aqueous and ionic liquid droplets for solute extraction.<sup>47</sup>

The nature of the carrier phase is of great interest to a biologist seeking to use droplet microfluidics. Aqueous droplets do not mix with a hydrophobic carrier phase oil to any appreciable extent—however, these aqueous droplets often contain substances which will readily partition into an exterior environment of hydrocarbon or silicone-based oil. Depletion of non-polar solutes from aqueous droplets can have very serious consequences, especially when working with media for intermediate or long-term cell culture. The use of fluorocarbon oils goes a long way toward remedying this problem, but a clever alternative exists in replacing the oil phase with an aqueous carrier phase. In this case the droplet compartment is isolated from the outside environment by a lipid bilayer. Aqueous droplets were sheathed in a thin layer of lipid from the oil phase by passing them by a “skim” before ejecting them into an aqueous channel (Figure 3c).<sup>48</sup> Although these synthetic vesicles were prepared with an eye toward performing reductionist biological studies (e.g. examining membrane protein biophysics in a simple system), this method also presents an innovative way of sequestering solutes in a digital microfluidic system.

**Mixing, actuating, and pumping**—Mixing, moving, and pumping liquids at the microscale constitute the basic unit operations of lab-on-a-chip devices, required for a variety of chemical and physical operations on-chip. Often, the rapid, controlled mixing afforded by microfluidics facilitates otherwise impossible observations of biochemical kinetics. In microfluidic devices, hydrodynamic flow is always laminar because of low Reynolds numbers; however, rapid and efficient mixing is possible using carefully designed geometrical constraints. For example, an ultrafast microfluidic mixer using 3D flow focusing achieved mixing on a 10- $\mu$ s scale.<sup>49</sup> This type of rapid mixing in microfluidic systems allowed intimate investigations of protein folding. For example, one microfluidic mixer examined intramolecular diffusion of an unfolded protein during folding.<sup>50</sup> Single-molecule fluorescence resonance energy transfer combined with microfluidic mixers permitted direct observation of individual biomolecule conformations on millisecond to second time scales.<sup>51</sup> Another single-molecule microfluidic mixer enabled detailed mapping of the binding-induced folding kinetics of proteins.<sup>52</sup> Integration of valves and pumps with microfluidic mixing extended these devices’ applicability; one integrated device automatically performed reagent titrations to screen multidimensional chemical space for conformational and enzymatic changes in biomolecules.<sup>53</sup> Combined with small angle X-ray scattering, another system provided a customized tool to study reaction kinetics of biomolecular assembly processes, such as intermediate filaments.<sup>54</sup>

Microactuators and pumps are also useful for manipulating small sample volumes, and recent research develops mechanical alternatives to electroosmotic flow. A miniature

peristaltic pump consisted of a helical bundle of microfluidic channels wrapped around a central camshaft and was operated by manually or mechanically rotating the non-cylindrical cam, which compressed the helical channels to induce peristaltic flow.<sup>55</sup> To sort mouse embryoid bodies, electrically-driven hydrogel actuators operated at low driving voltages (<1.2 V) and in cell culture media without damaging cells.<sup>56</sup> Biomimetic pumping methods were another promising approach for moving small sample volumes. For example, magnetically actuated artificial cilia were constructed from self-assembled chains of spherical superparamagnetic particles<sup>57</sup> or from a flexible magnetic nanoparticle-PDMS composite.<sup>58</sup> These artificial cilia mimicked the beating movement of airway cilia to generate fluid flow. Although neither type of cilia was integrated with microchannels to date, the suitability of these structures for microfluidic application was evident and supported by theoretical results.<sup>59</sup> Another example of bio-inspired pumping mimicked stomatal transpiration in plants and obtained a controllable flow rate of 0.13–3.74  $\mu\text{L}/\text{min}$  in microfluidic systems.<sup>60</sup> Free-standing micro- and nanomachines also offered new functionality, such as separation of drugs, cell sorting, and biosensing. A self-propelled catalytic Ti/Fe/Pt rolled-up microtube swam in a controllable manner within microfluidic channels.<sup>61</sup> It easily loaded multiple cargoes and transported them to desired locations in the microfluidic chip. When the micromachine was functionalized with targeting ligands, it selectively captured cancer cells, opening a new approach for capture and isolation of rare cells from biological fluids.<sup>62</sup>

**Programmable microfluidics**—Increasingly sophisticated sample handling techniques are leading to generic devices suitable for a wide range of analyses – so-called “programmable microfluidics.” In some cases, flexibility and programmability are achieved by valving. A digital microfluidic platform composed of a 2-dimensional array of microvalves automated quantitative, multi-step biomolecular assays.<sup>63</sup> A flexible microfluidic processor system with onboard pumps and valves performed metering, mixing, and reaction incubation in a series of molecular biology steps for messenger RNA (mRNA) amplifications.<sup>64</sup> At the extreme end of programmable microfluidics are devices in which channels are formed on-the-fly from the assay buffer and sample themselves. A hybrid integrated circuit/microfluidic chip simultaneously controlled thousands of living cells and pL volumes of fluid, enabling a variety of chemical and biological tasks.<sup>65</sup> A microfluidic device performed a variety of low- and high-level functions without hardware modifications; instead, each task was fully implemented by software programming.<sup>66</sup> Programmable electrowetting manipulated droplets by application of electrostatic forces on an array of electrodes. In one study, directional channel formation, as well as splitting and merging, resulted in virtual electrowetting channels formed by application of voltage to an array of polymer posts.<sup>67</sup> In another study, light triggered droplet transport on an open and featureless surface using a single-sided continuous optoelectrowetting mechanism.<sup>68</sup> Continuous transport, splitting, merging, and mixing of droplets were possible. The ability to perform multiple sample processing steps on demand also benefited these digital microfluidic systems. Porous polymer monoliths were formed *in situ* to carry out a digital microfluidic solid-phase extraction using microliter droplets of samples and reagents.<sup>69</sup> Surface acoustic waves (SAW) also provided a new route to program complex fluidic functions into a microchip. For example, a disposable phononic chip executed microcentrifugation for particle and cell concentration in microliter droplets.<sup>70</sup>

**Simple, rugged devices**—Devices with a multitude of chambers, valves, and connections are often impressive, but this complexity can lead to devices that are difficult and expensive to produce and more prone to failure. While some applications demand highly engineered devices, other assays can be adapted to simple, rugged devices with few moving parts and the potential for “hassle-free” usage. A self-powered, self-contained



microfluidic blood analysis system extracted plasma from whole-blood and performed multiple protein binding assays with high sensitivity (Figure 4a).<sup>71</sup> It did not require any external pumping, connections, tethers, or tubing to deliver and analyze whole blood. A similarly simple device self-digitized samples into a large array of discrete volumes; the user simply primed the chip with oil, introduced aqueous sample, which divided itself into an array of chambers, then followed with oil again (Figure 4b).<sup>72</sup> The digitized samples can be mixed with additional reagents and removed for downstream manipulation or analysis. A microfluidic pipette was developed to enable high-resolution spatial control of the chemical microenvironment of selected single cells (Figure 4c).<sup>73</sup> Because of its simplicity, it has potential to be a routine research tool in pharmacological and physiological studies of isolated biological cells. A common problem with complex microfluidic geometries is the probability of trapping air bubbles. A new microfluidic design, called a phaseguide, based on a step-wise advancement of the liquid–air interface using the meniscus pinning effect, gave complete control over filling and emptying of any type of microfluidic structure, independent of the chamber and channel geometry.<sup>74</sup> Advances like these are necessary to bring microfluidic technology to a wider audience. Currently, few life scientists use microfluidic technology in everyday laboratory practice, but rates of adoption will increase if simpler, more rugged devices are developed. A new direction in microfluidic design is needed to combine robust simplicity with functionality.

#### 1.4 Microfabricated Detectors

Microfabrication lends itself to the improvement of established detection methods and development of new strategies. A photothermal detector, previously demonstrated with capillaries, was recently applied to microfluidics with great success.<sup>75</sup> The detector used laser illumination to heat the sample by nonradiative relaxation. The resulting temperature change produced a corresponding change in viscosity and therefore conductivity. Prior implementation with capillaries used contactless conductivity measurements, but the ease with which electrodes can be interfaced with microchannels permitted contact measurements in the microfluidic system, dropping the detection limit for this label-free measurement to 5 nM. Microfabrication also lends itself to mechanical detectors, including cantilevers. Microfabricated cantilevers in PDMS channels were transiently deflected by non-specific binding of bovine serum albumin (BSA), and in future iterations, cantilever arrays could be individually coated with binding agents for analyte-specific detection.<sup>76</sup> Microfabricated cantilevers were also incorporated in a DVD platform for high-throughput bio-molecular sensing, in which optics and mechanics from a DVD player were used to handle liquid samples and to read-out cantilever deflection and resonant frequency.<sup>77</sup> Microfabricated detectors were particularly useful for detection of a single cell, single particle, or single molecule. A microfabricated cantilever containing a microchannel responded to the buoyant mass of particles with femtogram resolution as they flowed through it. Repeated measurements of individual bacterial and mammalian cells provided single cell measurements of instantaneous growth rates,<sup>78</sup> and a recent advance allowed the detector response to be readout piezoelectrically, rather than optically.<sup>79</sup> Another microfabricated detector rapidly counted and characterized nanoparticles passing through a nanoconstriction at rates up to 500,000 nanoparticles per second.<sup>80</sup>

## 2. Enabling technologies: Cheaper, faster, smaller volume analyses

In addition to improving fabrication and unit operations, many researchers are making substantive contributions to overall device designs. The results are enabling technologies that permit lower cost, faster, and/or highly automated analyses relative to prior state-of-the-art. We highlight paper microfluidics as an emerging paradigm in low cost microscale analysis. In high speed analysis, we recognize major contributions to binding assays and high throughput screening. Increased integration and automation also decrease analysis

times and make devices compatible with operation in the field and for point-of-care. Finally, we examine recent progress in nucleic acid analysis as a case study representative of the future of this field.

## 2.1 Paper microfluidics

The past eighteen months have seen an explosion of research into paper-based microfluidic devices. These devices are similar to the dipsticks and lateral flow tests commonly used as inexpensive diagnostic tools; however, paper microfluidics typically integrate additional steps and more sophisticated fluid handling. Current research is focused on recapitulating conventional microfluidic operations on these lower cost platforms, and major areas of progress include controlling fluid flow and integrating detection systems.

**Fluid flow in paper-based devices**—Fluid flow in paper microfluidics can be controlled by adjusting channel length<sup>81</sup> or width<sup>82</sup> or by including a soluble<sup>82</sup> or insoluble barrier.<sup>83</sup> In two-dimensional paper devices, hydrodynamic focusing and an H-filter were achieved by varying channel lengths to adjust flow rates (Figure 5a). Similarly, varying channel lengths contributed stable flow control for a Y-mixer and a dilution system in a multi-layer microfluidic device. Indeed, the capillary-driven flow in paper microfluidics tends to be more stable than mechanically-pumped pressure-driven flow in traditional microchannels, improving the performance of these filters and mixers.<sup>81</sup> Three-dimensional paper microfluidics permit more complex fluid control. “Metering” layers spotted with paraffin wax controlled the time required for a fluid to penetrate to the next device layer. Varying the mass of the wax deposited resulted in time delays from 30 s to 2 h.<sup>83</sup> The meters were also interfaced with a timing indicator that changed color when an assay was complete or ready for readout. The timing unit allowed the device to self-correct for humidity, a common source of variation in paper-based devices.<sup>84</sup> Multi-layer devices fabricated with paper and tape can also contain single-use “on” buttons. Pressing down on these buttons permitted fluid to flow through the newly connected conduit, effectively creating a new fluid path on the device (Figure 5b).<sup>85</sup>

**Detection methods for paper devices**—While most paper-based assays to date rely on colorimetric detection, alternatives are emerging. Chemiluminescence<sup>86</sup> and electrochemiluminescence<sup>87</sup> were demonstrated with appropriate reagents and analytes. Additionally, commercially available glucose meters, typically used with test strips, have been adapted to electrochemical detection of glucose, cholesterol, lactate, and ethanol on paper microfluidics.<sup>88</sup> These glucose meters represent a sensitive and portable detection system that is widely available at low cost. In contrast, paper microfluidics have also been interfaced with mass spectrometry, a detection system that is substantially more expensive but provides mass information. Using a recently developed ambient ionization method, samples were directly sprayed from a macroscopically sharp paper tip at high voltage (Figure 5c).<sup>38</sup> This direct paper spray was effective for a wide range of analytes, including small molecules, lipids (phosphatidylcholine), peptides (angiotensin I), and small proteins (cytochrome C). Paper spray has also been implemented with surface acoustic wave-assisted sample delivery, which facilitated atomization and ionization of samples.<sup>89</sup> These new detection methods extend paper microfluidics to new assays and applications.

**Outlook**—While paper devices are progressing rapidly, the field remains immature. Recently published applications demonstrate this fact: most papers describe simple assays, including blood typing<sup>90</sup> and standard urine and saliva tests,<sup>91</sup> for which mature diagnostic tests based on dipsticks or lateral flow methods already exist. Paper devices also have inherent limitations, including optical opacity and limited options for surface modification compared to glass. However, the exceedingly low cost of these devices, often estimated at

pennies apiece,<sup>88</sup> makes them competitive for certain applications. Third world diagnostic systems must be as low cost, simple, and rugged as possible, so paper devices are well-suited to these applications. Additionally, paper microfluidics are ideal for regular, frequent monitoring of specific analytes. Just as blood glucose meters allow diabetics to monitor their glucose outside of clinical settings, paper devices will find similar applications for home use, particularly as the field of personalized medicine matures.

## 2.2 High speed analysis

While paper microfluidics research is motivated primarily by decreasing cost of analyses, more traditional microfluidic systems are becoming faster as well as cheaper. As noted above, microfluidics provide precise, automated sample handling, and recent research harnesses this advantage to decrease analysis times and reagent consumption, particularly in binding assays and high-throughput screening of new reagents or assay conditions.

**Microscale binding assays**—Immunoassays are a widely used tool in biochemistry due to their specificity and sensitivity; however, these assays tend to be time-consuming due to their laborious, multistep protocols and expensive because of the consumption of antibodies. Recent microfluidics-based techniques decrease both analysis time and reagent consumption of commonly used immunoassay formats. Two platforms for microfluidic western blotting were demonstrated in the past 18 months. In the first, electrophoretic separation, sample transfer, and blotting were integrated on a single device (Figure 6a).<sup>92</sup> This high level of integration, combined with a photopatterned discontinuous gel,<sup>93</sup> facilitated extremely rapid assays of a protein of interest; in this case, prostate specific antigen (PSA) concentration was determined in just 5 minutes.<sup>92</sup> Multiple proteins can be detected simultaneously using additional antibody-labeled blotting regions.<sup>94</sup> The second platform, while less integrated and slower, also decreased antibody consumption and measured several proteins simultaneously, permitting controls and internal molecular weight markers to be assessed along with samples.<sup>95</sup> Immunosubtraction assays also benefitted from microfluidic implementation with a decrease in analysis time from hours to minutes combined with an increase in sensitivity.<sup>96</sup> Indeed, automated on-chip immunoassays commonly yield lower detection limits than manual methods. A recent amperometric immunoassay gave 2- to 30-fold lower detection limits for cancer biomarkers compared to manual analysis.<sup>97</sup> Similarly, a microfluidic bead-based immunoassay lowered detection limits (compared to surface plasmon resonance), allowing kinetic measurements to be made on antibodies secreted from individual cells.<sup>98</sup> Finally, microfluidic immunoassays also decrease the required sample size. Directly interfacing microchannels with a tissue sample allowed assays to be performed ten times faster, with ten times less antibody and on scarce samples, such as needle biopsies of breast cancer tissue.<sup>99</sup>

Precise microfluidic handling also benefits other binding assays. Sub-micron deep channels spatially confine molecules, allowing single molecule detection at physiologically relevant concentrations. A recent application exploited this effect for single molecule studies of protein-protein interactions.<sup>100</sup> Quantum dot-labeled proteins interacted with one another, and the resultant protein complexes were detected individually based on the presence of two-color signals – one from each protein. This method interrogated individual complexes and took less than one hour to complete. Elaborate fluid handling systems in larger channels increase throughput by multiplexing. A microfluidic array multiplexed both ligands and analyte concentrations for surface plasmon resonance imaging of up to 264 separate combinations.<sup>101</sup> Another arrayed device assayed transcription factor binding. In this case, over 4,000 interactions were tested using a microfluidic network controlled by over 12,000 valves (Figure 6b).<sup>102</sup>

**High-throughput screening**—These dramatic multiplexing capabilities are also advantageous in screening large numbers of samples or assay conditions. Droplet-based microfluidics are ideal for high throughput screening; for example, a droplet screen for directed evolution of horse radish peroxidase performed  $10^8$  reactions in  $< 10$  h!<sup>103</sup> In addition to speed, droplets provide a controlled environment for each sample, an advantage in phage amplification. Phage libraries are a simple means of selecting and amplifying peptides; however, traditional methods discriminate against slow-growing phage. Encapsulating each phage in its own droplet made amplification independent of growth characteristics.<sup>104</sup> While these droplet-based systems showcase sophisticated fluid handling, simple devices also have advantages. For example, a straight microfluidic channel containing adherent cells improved screening of surface marker-binding peptides compared to biopanning.<sup>105</sup> Similarly, a simple microfluidic SELEX device combined with high throughput sequencing yielded aptamers with higher affinity and higher specificity than those obtained by conventional methods.<sup>106</sup> Microfluidic devices are also increasing throughput for single cell assays, as discussed in Section 3.1.

### 2.3 Integration and automation

The advances discussed above are rapidly being combined into highly integrated and automated devices. Integration of multiple steps on a single device prevents sample loss, decreases analysis time, and makes microfluidic analyses amenable to new detection methods and accessible to new users, including non-specialists, and in point-of-care situations. Major progress focuses on the sample handling demands of complex devices and integration with mass spectrometry and optical detection systems. Along with these advances come a growing number of true sample-to-answer  $\mu$ TAS and practical point-of-care systems.

**Sample handling and automation**—Improved sample handling makes integration of multiple steps on-chip easier and more efficient. In some cases, simple innovations have provided disproportionate advantages. One innovative method pre-printed channels with reagents, which were allowed to dry. When the assay began, these reagents were reconstituted at the appropriate concentration by sample flow, combining the convenience of a lateral flow assay with the sophisticated fluid manipulations possible in microfluidics.<sup>107</sup> A so-called “self-powered” device used subambient pressure, developed by depleting the PDMS channels of air prior to analysis, to draw sample through the device for the assay.<sup>71</sup> Similarly, a pumping mechanism based on the capillary action of filter paper drew reagents sequentially through the detection region of a valve-less, pump-less microfluidic device for protein screening in whole blood.<sup>108</sup> In more complex assays, however, valving has played a central role in integration. High pressure valves facilitated coupling of isoelectric focusing (IEF) with pressure-driven reverse-phase liquid chromatography (RPLC) of peptides.<sup>109</sup> In another device, a two-dimensional array of valves controlled mixing, rinsing, diluting, and sample labeling functions on a chip that automatically generated a standard curve during biochemical assays (Figure 6c).<sup>63</sup>

**Integration with mass spectrometry (MS)**—While most integration focuses on transferring analytical steps to a microfluidic device,  $\mu$ TAS systems with mass spectrometric detection couple on-chip sample preparation and separations to (for now) off-chip MS detection. These devices combine the sample handling capabilities of microfluidics with structural information from mass spectrometry, and benefits often include faster processing and higher sensitivity compared to experiments completed off-chip. For proteomics, on-chip sample preparation, including extraction, reduction and alkylation, and digestion, prior to electrospray ionization have increased sequence coverage for model proteins<sup>110</sup> and dramatically decreased processing time.<sup>111</sup> Microchips have also performed two-

dimensional separations (LC-CE) prior to MS,<sup>112</sup> and in the near future it should be possible to conduct an entire bottom-up proteomics experiment using an integrated microchip and a mass spectrometer. In a metabolomics example, one device controlled cell culture and stimulation, while a second device performed sampling, desalting, preconcentration, and electrospray.<sup>113</sup> Digital microfluidics based on electrowetting have also been interfaced with mass spectrometry via matrix-assisted or surface-assisted laser desorption ionization (MALDI or SALDI). Localized heating combined with electrowetting sped up both trypsin digestion and matrix crystallization for proteomic experiments with MALDI detection.<sup>114</sup> Alternatively, hydrophobic silicon nanowires facilitated SALDI, improving de-wetting and detection of low molecular weight analytes without matrix interference.<sup>115</sup> Whether in enclosed channels or droplets, each of these devices contributes to mass spectrometric analysis of complex mixtures, automating sample preparation and applying MS detection to small sample volumes.

**Integration of optical detection units**—Other recent developments focus on integration of optical components with additional microfluidic functionality. A highly integrated microfluidic fluorescence-activated cell sorter included both piezo activated sorting and an optofluidic waveguide. As a cell sample flowed through the waveguide, slits transformed the spatial signal into a temporal signal, permitting multipoint detection at a single photomultiplier tube (PMT).<sup>116</sup> Similarly, frequency-modulated fluorescence excitation allowed multi-color detection of electrophoretically-separated DNA at a single PMT.<sup>117</sup> Capturing multiple signals at a single detector may eventually decrease costs while increasing information content. Alternative strategies used planar and image-based detectors, often taking advantage of 96-well plate readers. One recent device combined microfluidics with the high throughput planar readout of a 96-well plate to measure nitric oxide release from red blood cells.<sup>118</sup> Another 96-well device for enzyme linked immunosorbent assays (ELISA) used six device layers to deliver reagents, incubate sample with antibody-labeled carbon nanotubes, and record chemiluminescence at a modular CCD-based detector.<sup>119</sup> Similarly, a recent radioassay for kinase activity in cancer biopsies used an integrated  $\beta$ -camera with microfluidics to minimize sample consumption.<sup>120</sup>

**True  $\mu$ TAS**—While the term “micro total analysis system” has been in use for some time, integration of all analytical steps onto complete “sample-to-answer” systems remains rare. In the past eighteen months, however, several true  $\mu$ TAS devices have been demonstrated. Major developments in this area include elimination of laboratory infrastructure for device operation, demonstration of autonomous operation, and integration of portable power supplies and data displays. Devices are increasingly reducing or eliminating the need for external equipment to complement their operation. As noted above, optical detection systems often add bulk to microscale analyses. This bulk was eliminated in a fluorescence detector for isotachopheresis using a photodiode and minimal optics, consisting of a miniature lens and an interference filter, fixed below the microfluidic chip and housed in a barrel.<sup>121</sup> Numerous devices rely on pneumatic valving to increase functionality, but they typically require bulky laboratory infrastructure, such as computers and gas tanks. A recent electrochemical system implemented a more compact version of pneumatic valving.<sup>122</sup> Another system of PDMS elastomeric microvalves combined with solenoids avoided gas-driven valving entirely. Solenoids with cylindrical plungers drove the hydraulics and were modified with a spring between the plunger and solenoid body to provide a return mechanism.<sup>123</sup>

In addition to requiring less space and equipment, true  $\mu$ TAS minimize the need for human intervention, a useful or even necessary advantage in certain applications. New  $\mu$ TAS for deployment in outer space demand particularly high levels of integration and automation since cargo load on spacecraft is limited and human intervention after launch may be

impossible. A prototype for microchip electrophoresis and spectral characterization analyzed samples, blanks, and standards for amine-containing molecules and polyaromatic hydrocarbons. Importantly, this system included substantial redundancy to compensate for potential failures, and further developments will automate sample preparation and self-cleaning to complete flight-ready instrumentation.<sup>124</sup> Another fully integrated microchip system monitored ammonia gas leaks in clean rooms. This device integrated gas sampling along with NH<sub>3</sub> extraction, concentration, and detection and operated stably and independently for at least three weeks (Figure 7a).<sup>125</sup> Equally important, this device targets a sector experienced with microfluidic systems, making it more likely to be widely adopted.

Eliminating the need for bulky external power supplies is another step toward fully integrated devices, particularly those for use in field work or in developing countries. Several recent devices run off batteries,<sup>123,126,127</sup> USB power,<sup>121</sup> or wireless power using RF circuits.<sup>128</sup> These portable options were sufficient to power many important analytical steps, including valving,<sup>123</sup> dielectrophoretic manipulations,<sup>128</sup> electrokinetic separations,<sup>121,126</sup> and electrochemical<sup>126</sup> or LIF detection.<sup>121</sup> Finally, while most  $\mu$ TAS are interfaced with a laptop for electronic control, data collection, and data display, some highly portable devices have included LED readout,<sup>127</sup> an integrated display,<sup>129</sup> preprogrammed microcontroller,<sup>123</sup> or smartphone-based operation.<sup>130</sup>

**Point-of-care analyses**—While many microfluidic systems purport to be point-of-care devices, few actually succeed in combining portability, speed, and user-friendliness with an important clinical application; however, there are a few notable recent exceptions. Lithium levels in blood were assayed by microchip CE with conductivity detection using prefilled cartridges. The user only needed to deposit a blood drop and insert the cartridge into the handheld reader. Results obtained by untrained users were indistinguishable from those obtained by trained scientists, suggesting that this system could be used by patients to monitor their lithium levels at home (Figure 7b).<sup>129</sup> Another handheld analyzer performed immunoassays with magnetic nanotags and detection with giant magnetoresistive sensors; this system was built around disposable sticks, required no wash steps, and was more sensitive than an ELISA.<sup>127</sup> Centrifugal microfluidic devices combined with portable detection systems are another growing point-of-care strategy. One cartridge accepted whole blood and output levels for cholesterol, triglycerides, glucose, aminotransferases, and creatinine kinase.<sup>131</sup> Another centrifugal device provided point-of-care data on dairy cows for farm applications. A briefcase-size sedimentation cytometer centrifuged up to 12 milk samples and automatically detected the size of the cell pellet for mastitis diagnosis and the thickness of the cream layer as an indicator of milk value.<sup>132</sup> Importantly, each of these devices was fully operable at the point-of-care without additional equipment and provided sample-to-answer functionality with readout in minutes. A third device combined micro-NMR with magnetic nanoparticle labeling of proteins for cancer diagnosis. Although this device required off-chip sample preparation to label the 4 protein panel, it was compatible with small sample sizes (fine needle biopsies), included smart phone control, and provided more accurate diagnoses than immunohistochemistry in less than one hour, making it an extremely promising point-of-care device.<sup>130</sup>

## 2.4 Nucleic acid analysis: a case study

Nucleic acid analysis is a relatively mature application of  $\mu$ TAS. As such, nucleic acid analysis on-chip serves as a case study in the progress and future of microfluidic systems generally. This section details advances in multiplexing, integration, and innovation in nucleic acid analyses in microfluidic devices that range from strikingly simple to astonishingly complex.

**Single-Cell PCR**—Many devices for multiplexed PCR harness microscale fluid handling to compartmentalize samples in droplets or nanoliter-scale microfabricated compartments. Single-cell genetic analysis is possible by encapsulation of individual cells in reagent-containing droplets. Microfluidic droplet-based sample preparation for emulsion PCR was previously demonstrated with a single channel; recently, it was scaled up to a 96 channel format, dramatically increasing throughput.<sup>133</sup> These advances in single-cell PCR are finding important biological applications: profiling viral diversity in individual infected bacteria,<sup>134</sup> sequencing environmentally important archaea,<sup>135</sup> and identifying rare mutations in individual cells.<sup>136</sup> The importance of microfluidic PCR to single-cell analysis will be further discussed in Section 3.1.

**Digital PCR**—Microscale fluid handling also benefits digital PCR, in which a single DNA strand is amplified. A recent electrowetting device divided an initial sample droplet into multiple subsamples and mixed each with a separate set of PCR reagents. The individual droplets traveled in a loop through regions of variable temperature for amplification. Each loop held multiple droplets, and printed circuit board fabrication resulted in easy and affordable fabrication of multiple loops, further increasing the potential for multiplexing.<sup>137</sup> Large numbers of PCR samples can also be multiplexed by dividing them into microfabricated compartments. Recent developments in this area focused on simplifying sample handling. For example, SlipChips have been used to mix and compartmentalize samples by sliding together micropatterned top and bottom plates, mating wells and ducts in the two layers without pumps or valves.<sup>138,139</sup> Another valveless design used centrifugal force to dispense samples into 1,000 microfabricated wells on a spinning disk prior to digital PCR.<sup>140</sup> While these are far from the first examples of multiplexed PCR on-chip, these recent publications demonstrate the drive to make microfluidic PCR more user friendly and accessible. Eliminating valves can also increase the density with which micro-compartments are fabricated. A recent device reached a density of 440,000 chambers per cm<sup>2</sup> by isolating microfabricated PCR compartments with an oil phase wall.<sup>141</sup>

**Integrated PCR systems**—Highly integrated and automated devices are making point-of-care, forensic and biosafety applications of microchip PCR practical. For example, a recent device lysed cells, extracted and amplified their mitochondrial DNA, and executed a CE-LIF analysis to identify disease-linked deletions in just 150 min.<sup>142</sup> Such highly integrated devices increase accessibility, and automation improves reliability and decreases analysis time. These advantages are especially important in forensics, which benefits from rapid analyses that can be performed on-site with minimal user input. Most forensic DNA analysis examines short tandem repeats (STR) to produce a so-called genetic fingerprint, and several highly integrated devices target STR analysis. One chip extracted and amplified DNA from whole blood and semen,<sup>29</sup> and more complex devices have integrated extraction and sample preparation, amplification, CE separation, and LIF detection for analysis of lysed cells from cheek swabs (Figure 7c).<sup>143,144</sup> Importantly, these devices provided results within just 3–4 h of sampling and required negligible user input after sample loading. Consequently, they are promising for use by non-scientists in the field.

Microfluidic analyses are also being developed to identify microbes. High levels of integration in these devices reduce the need for extensive user training and allow implementation both at point-of-care and in the field. A microfluidic cassette for pathogen analysis included preloaded reagents and combined cell lysis, nucleic acid extraction, reverse transcription, PCR, amplicon labeling, and detection at a lateral flow strip.<sup>145</sup> Applications included detection of the food borne pathogen *Bacillus cereus* and HIV. An alternative method, multi locus sequence typing (MLST), identifies bacterial species by amplifying a handful of housekeeping genes using highly conserved primers. A recently-demonstrated microdevice for MLST incorporated multiplexed PCR, Sanger sequencing,

ultrafiltration, electrophoretic separation, and LIF detection of sequenced fragments on-chip.<sup>146</sup> The entire process required only 1.5 h, making this device suitable for biomedical, forensic, and biosecurity applications that require rapid genotyping of unknown microbes. Recent fears related to pandemic influenza have made automated detectors for respiratory viruses an attractive target. A portable device with a power requirement of 3 W executed reverse transcription polymerase chain reaction (RT-PCR) and melting curve analysis of the products; integrated LED-based fluorescence detection of H5N1 viral RNA took just 35 minutes.<sup>147</sup> Another device used magnetic capture agents prior to PCR and electrochemical readout after amplification.<sup>148</sup> Other highly integrated systems have incorporated sample preparation on-chip, allowing nasopharyngeal swabs to be analyzed without off-chip extraction of viral RNA.<sup>149,150</sup> Isothermal amplification of influenza genes has also been demonstrated on-chip with a custom-made device reader.<sup>151</sup> The combination of portability, speed, and automation provided by  $\mu$ TAS makes these devices ideal for influenza detection in airports, clinics, and other non-laboratory settings. While these influenza systems have not yet been tested in the field, recent research demonstrates the possibility of using commercial microfluidic cartridges for PCR-based detection of tuberculosis and identification of rifampin resistant strains in resource-poor clinics around the world.<sup>152</sup> Next steps in this area include demonstration of further clinical applications and reduction of instrument and per-cartridge costs.

**PCR alternatives**—Microfluidic alternatives to PCR are also being developed. For DNA, a number of isothermal amplification methods, including recombinase polymerase amplification (RPA),<sup>153</sup> helicase dependent amplification (HDA),<sup>154</sup> and loop-mediated isothermal amplification (LAMP)<sup>155</sup> have been demonstrated on-chip and used to obtain important biological results. Multiple displacement amplification (MDA) combined on-chip with pyrosequencing provided single-cell genome information.<sup>135</sup> Isothermal rolling circle amplification (RCA) has been extended to protein detection by coupling appropriate primers to antibodies for proteins of interest.<sup>156</sup> For whole genome analysis, a sophisticated device isolated individual chromosomes from single cells into separate fluidic compartments for multiple strand displacement amplification, an isothermal amplification method suitable for whole genomes. After amplification, the products were removed from the fluidic device for haplotype analysis using genotyping arrays.<sup>157</sup> More recently, the same device was used to draft the genome of the CHO-K1 ancestral cell line.<sup>158</sup>

Some microfluidic methods for nucleic acid analysis eliminate the need for amplification altogether. Molecular beacons are becoming popular for ssDNA detection on microchips. These molecules undergo a change in fluorescence (by FRET or by quenching) that depends on the presence of target DNA.<sup>159–161</sup> Single-molecule methods based on labeled, linearized DNA also provide alternatives to PCR. Direct linear analysis of DNA fragments has been executed on-chip with DNA-based detection of toxins.<sup>162</sup> Long range analysis of genomic information from single cells was obtained using a new single molecule denaturation technique. YOYO-1 stained DNA was stretched into nanochannels and denatured with formamide and local heating, resulting in maps of fluorescence maxima and minima that are sequence dependent and predictable.<sup>163</sup> Another single molecule technique detected fluorescently labeled circulating nucleic acids in serum. As individual circulating DNA fragments flowed through a microfluidic constriction, fluorescence bursts from the stained fragments were used to count and size the DNA.<sup>164</sup> This technique discriminated between Stage I and Stage IV lung cancer samples based on the size distribution of circulating DNA in patient serum.

**RNA Assays**—Technologies to quantitate RNA on-chip with or without an amplification step are also increasingly important. Just as droplets and microfabricated compartments advance single-cell PCR, the same technologies are applicable to single-cell measurements



of mRNA. RT-PCR has been performed on single cells isolated in droplets<sup>165</sup> and microfabricated chambers,<sup>166</sup> permitting rapid characterization of gene expression in hundreds to thousands of cells. MicroRNAs (miRNA) are 18–24 nucleotide long RNA strands that regulate transcription by binding mRNA; this regulatory role makes them important players in many disease states. The small size of these molecules, as well as the high reactivity of RNA, makes miRNA analysis challenging. A new isotachopheresis method used a multistage sieving matrix to rapidly preconcentrate RNA, selectively focus miRNA while excluding longer RNA strands, and sensitively detect the miRNA in a low denaturant region. This technique was recently used to profile miRNA differences between confluent and subconfluent HeLa cells.<sup>167</sup>

### 3. Interfacing biology and $\mu$ TAS

Just as progress in integration and automation have brought  $\mu$ TAS closer to clinical applications, other advances in microfluidic technology are providing new tools for biological and biomedical research laboratories. This progress spans the broad scale over which life occurs: from single cells, to complex cellular communities and networks, to organs and organisms.

#### 3.1 Single-cell analysis

Recent investigations revealed a large degree of cell heterogeneity present within populations of a particular cell type. Analysis of DNA, RNA, proteins and other biomolecules from single cells is important for understanding cell-cell variation. Current methods utilizing microfluidics for measuring the chemical contents of single cells include fluorescence microscopy, flow cytometry, and chemical cytometry. Recent  $\mu$ TAS developments have investigated techniques for generating a large amount of data from single cells with high throughput and at low cost.

**Direct analysis of intact cells**—Imaging cytometry is a common strategy for analyzing whole cells and obtaining spatial information. By labeling specific cellular components with fluorescent markers, the location and quantity of cellular analytes may be observed. Microfluidics provides the high-throughput sample preparation required for imaging statistically relevant numbers of single cells. For example, 96 parallel microfluidic chambers were used to access the generation of a chromosomal YFP (yellow fluorescent protein) fusion library for single molecule quantitation of proteins and mRNAs in *Escherichia coli* (*E. coli*) (Figure 8a).<sup>168</sup> Quantifying copy numbers of specific proteins and mRNAs in individual *E. coli* cells provided evidence of cellular heterogeneity within a clonal population while demonstrating that protein copy number is uncorrelated with mRNA expression at the single cell level. In another study, microfluidics facilitated parallel assays for simultaneous imaging of four signaling proteins in a small number of individual cells (1,000–2,800 cells per chamber).<sup>169</sup> This simple device used multi-parameter immunocytochemistry measurements to investigate the heterogeneity of glioblastoma from small quantities of clinical tissue samples.  $\mu$ TAS devices have also been developed to provide automated media and reagent additions for real-time imaging of biological samples under various culture conditions. For example, a recent study applied a commercial microfluidic device (CellASIC ONIX) to investigate the effects of varying nutrient levels on the yeast metabolic cycle.<sup>170</sup> This research showed that single yeast cells loaded with specific strains of fluorescent reporters exhibited enhanced expression during specific metabolic periods.

Managing small volumes by microfluidics also allows interrogation of cellular transport processes as they occur in single cells. Recent studies in this area have included measurements of antibody secretion and transcription factor binding. Antibodies are

continuously secreted from certain cell populations and used for numerous biological applications. The ability to quantify antigen-antibody binding kinetics at a single-cell level was provided by a microfluidic sandwich assay that combined single bead-immobilized antigens and secreted antibodies.<sup>98</sup> Another device utilized an immunosandwich assay to measure the heterogeneity in the expression of up to 13 secreted proteins from single tumor cells using multiplexed bar-coding within discrete microfluidic chambers.<sup>171</sup> Within cells, the transcription factor NF- $\kappa$ B is an important molecule in cell dynamics. A commercial microfluidic device (Fluidigm) was utilized to examine the response of NF- $\kappa$ B with the addition of TNF- $\alpha$  at the single cell level.<sup>172</sup> A digital response in the expression of NF- $\kappa$ B with TNF- $\alpha$  addition was observed. The response of NF- $\kappa$ B was also examined as single cells were stimulated by IL-1 $\beta$ .<sup>173</sup> A microchip flow cytometer paired with an electroporation region allowed for monitoring of nucleocytoplasmic transport of NF- $\kappa$ B following addition of IL-1 $\beta$ . These devices show the strength of microfluidics for discrete and controlled fluid manipulations to analyze single cells. Beyond their utility in uncovering the heterogeneity of cell populations, these devices allow for analysis of samples containing a low abundance of target cells commonly encountered when dealing with small sample quantities or rare cells.

**Analysis of intracellular contents**—Image- and flow-based studies of intact cells are complemented by chemical characterization of cell lysates. Single-cell lysates are typically analyzed through PCR<sup>133,174</sup> and/or CE,<sup>98,175</sup> depending on the analyte-of-interest. PCR is a powerful technique for analyzing minute quantities of genetic material, due to the high degree of nucleic acid amplification. As noted in Section 2.4, microfluidic systems are contributing to single-cell PCR analysis<sup>133, 166</sup> and single-cell haplotyping.<sup>157,158</sup> These studies show how the high throughput and low reagent costs of microfluidic analyses make large-scale genetic analysis of single cells feasible (Figure 8b). An outstanding application of these benefits is the use of microfluidic arrays to generate global miRNA profiles.<sup>174</sup> Profiling the expression of 288 different miRNAs in single cells representing 27 hematopoietic subpopulations revealed that, while not specific to individual cell types, miRNA expression may be used in cell categorization. As the importance of these cell-cell variations is recognized, new methods for analyzing cell populations on a cell-by-cell basis will become more common in biological procedures. The simplicity, robustness and high-throughput nature of microfluidic devices to facilitate fluorescence microscopy and PCR assays will lead to increased utilization of these technologies over their macroscopic counterparts for performing common biological protocols.

Other cell contents, such as proteins and small-molecule metabolites, cannot be amplified by means such as PCR, so these analytes require efficient separations and sensitive detection for identification and analysis from single cells. To accomplish this, several approaches integrate sample preparation, analyte separation and subsequent identification on-chip. Of the various microfluidic approaches, microchip CE has shown the greatest promise for separating the contents of a lysed cell on-chip. Recent advances in this technology improve the usability of microchip CE through the development of PDMS/glass hybrid chips<sup>175</sup> and detection of amino acids through chemiluminescence.<sup>176</sup> The separation and analysis of intracellular components on-chip is still a field dominated by technology development and method validation using model cell lines. Microfluidic approaches for chemical cytometry have not been widely accepted by the biological community or applied to primary cells, likely due to the complexity and poor stability of many systems. These areas must improve in order to translate this research into the biological laboratory and make chemical cytometry as useful and accessible as other pivotal single-cell technologies, such as fluorescence-activated cell sorting (FACS).

### 3.2 Cell sorting

As emphasized above, cells exist as highly heterogeneous populations. Generating useful information about specific subpopulations often requires sorting and isolation of cells-of-interest prior to analysis. Common macroscale techniques for isolating cells include limiting dilution, magnetic-activated cell sorting (MACS) and FACS. For many applications, these methods can have drawbacks such as poor purity, low sorting yield and low throughput. For certain applications, microsystems resolve the limitations of their macroscale counterparts.

**Flow cytometry**—On-going progress in the field of flow cytometry on-chip has made these miniaturized systems more competitive with the performance of larger scale instrumentation. The complexity of sheath-based cell focusing techniques in microfluidic flow cytometry has led researchers to explore inertial techniques (inertial migration and Dean flow) to direct cells in microchannels. Dean flow is a phenomenon in which faster-moving fluid near the center of a curved microchannel tends to move in a direction tangential to the curve, due to its inertia. This creates vortices which focus particles such as cells. Integration of Dean-coupled inertial focusing with microfluidic flow cytometers<sup>177</sup> is a key advance in the attempt to move microfluidics into biomedical research labs to replace benchtop systems. The throughput of cell analysis on-chip may be increased through the use of hundreds of parallel channels simultaneously on-chip. A recent advance improved the data-acquisition rate for these parallel devices by utilizing one-dimensional imaging.<sup>178</sup> While this method was effective for rudimentary analysis, more sophisticated image processing techniques are required to generate the higher information content supplied by two-dimensional imaging. Integration of cell sorting capabilities into these parallel channel designs is complex and has yet been developed. Alternative non-optical detection methods, such as impedance measurements, represent a low-cost alternative to fluorescence-based cell detection. While this technology cannot readily distinguish between unlabeled cell subpopulations, antibody-based labeling with polystyrene beads generated quantifiable variation in the impedance response for specific cell subpopulations.<sup>179</sup> Advancements have also been made to integrate the preparation of biological samples on-chip prior to flow cytometry. One such example integrated an on-chip hybridization region for performing fluorescence *in situ* hybridization (FISH) on small numbers of bacteria with downstream analysis by flow cytometry.<sup>180</sup>

**Cell sorters**—A number of recent technologies aimed to isolate a specific cell-of-interest from a heterogeneous population of cells. FACS pairs fluorescence detection of cells with downstream sorting capabilities to isolate cells. Bench-top models possess sorting rates of 10,000 cells  $s^{-1}$  while their microchip counterparts remain substantially slower with typical rates of 100 cells  $s^{-1}$ . One recent strategy for advancing the throughput of microfluidic FACS systems utilized a piezoelectric actuator to improve the effectiveness of cell sorting.<sup>116</sup> This sorting strategy improved reproducibility and increased throughput (1,500 cells  $s^{-1}$ ). Despite these advances, further improvement in throughput, arising from faster detection and better fluid manipulation strategies, is necessary to compete with macroscale flow cytometers. Several microfluidic approaches other than FACS have been utilized to separate specific cells of interest. A microchannel with self-assembled antibody-functionalized magnetic beads efficiently captured cells with corresponding surface markers.<sup>181</sup> These functionalized magnetic rods were similar in function to the antibody-coated micropillars common in the collection of circulating tumor cells (CTCs) described in the next section. Another antibody-coated microchannel captured neutrophils on-chip for further analysis of their mRNA and proteins.<sup>182</sup>

**Sorting rare cells**—The throughput of both benchtop and microfluidic FACS is currently too low to isolate extremely rare cells from a large number of non-targeted cells, for

example, CTCs and some stem cell populations. CTCs are typically present at one cell in a background of  $10^6$ – $10^9$  other cells, requiring the processing of large fluid volumes to retrieve even small numbers of CTCs. It is generally impractical to analyze these extremely rare events by serial analysis techniques. These technological challenges have spurred the recent development of microfluidic devices capable of high throughput enrichment and analysis of low-abundance cells. To date most microfluidic-based techniques for isolating CTCs employ either affinity-based capture or collection based on native cell properties.

Affinity-based capture methods for CTCs typically utilize antibodies against specific surface markers (e.g. EpCAM) present on CTCs but not on red or white blood cells. Indeed the only FDA-approved method for CTC detection is a macroscale technology (Veridex) employing immunomagnetic labeling of CTCs for capture from whole blood. While it may seem counterintuitive to use microfluidics to process large fluid volumes, rare cell isolation can be achieved by taking advantage of the high surface area-to-volume ratio and massively parallel analysis offered by microscale devices. The collection and detection of magnetically-labeled CTCs was automated on a microdisk format.<sup>183</sup> Microfluidic channels coated with antibodies have also been used to capture CTCs. Recent methods increased the quantity of cell-substrate contact events by introducing chaotic mixing and increasing the effective surface area through the incorporation of a herringbone structure (Figure 8c)<sup>184</sup> or silicon nanopillars.<sup>185</sup> Another strategy improved the quality of these interactions by using antibodies other than anti-EpCAM.<sup>186</sup> Following collection, analysis of these CTCs benefited from integration of an automated imaging platform<sup>187</sup> or through cell release for downstream PCR, ligase detection reaction (LDR), and capillary electrophoresis assays on-chip.<sup>188</sup>

Immunoaffinity capture is an effective technique for collecting CTCs when the surface proteins on the cells are known and distinct from those of normal cells. However, antibodies specific to CTCs are not always available. Additionally, release of captured CTCs can be difficult due to the high-affinity antigen-antibody bond, and most release methods to date do not yield viable CTCs. Antibody-based methods can also be plagued by non-specific binding of white blood cells, which then require subsequent analyses for discrimination from CTCs. Many CTCs are larger and more rigid than red blood cells (6  $\mu\text{m}$  diameter) or white blood cells (8–20  $\mu\text{m}$ ). These physical differences permit larger CTCs to be captured on size-selective arrays while smaller cells flow through the device. Filtering whole blood through an array of 8–11  $\mu\text{m}$  diameter microcavities<sup>189</sup> or 5–7  $\mu\text{m}$  wide slots<sup>190</sup> selectively captured most cultured tumor cells intermixed into blood. While the slots are comparable in size to red blood cells, blood cells are highly deformable and pass through the pores. Variations in the flow profiles of cells of different sizes based on their inertial migration have also been exploited to separate larger CTCs from blood cells.<sup>191</sup> Another label-free sorting method takes advantage of differences in the dielectrophoretic forces experienced by different cell types. Integration of a hydrodynamic sorter followed by sorting with dielectrophoresis (DEP) improved enrichment of cells while operating at high flow rates.<sup>192</sup> When viable CTCs are required, these size- and DEP-based techniques are superior to those reliant on immunoaffinity binding. Additionally, because these methods do not require prior knowledge of cell surface markers, these can collect CTCs missing the targeted antigens. However the size-based techniques fail to collect small CTCs, and DEP methods are still being developed.

### 3.3 Integrated microfluidic cell culture

**Improving cell culture microenvironment**—While cell culture within microfluidic devices is well-established, recent advances increase the functionality of cell culture on-chip by improving fluid control with the end goal of automating a rather tedious operation. For

example, a cell culture chip with integrated environmental sensors and automated valves continuously cultured *E. coli* for up to 3 weeks.<sup>193</sup> Similarly, a digital array platform of 2.2 × 2.2 mm electrodes was able to culture hundreds of adherent cells within discrete 150-nL droplets and automate media exchange and cell splitting (Figure 9a).<sup>194</sup> Array platforms for cell culture have also enhanced investigations into the effects of culture conditions on cellular physiology. For example, a device featuring 128 independently-perfused culture chambers rapidly assessed the influence of the culture conditions on MAP kinase signaling pathways in wild-type yeast and seven mutants.<sup>195</sup> Another high-throughput cell culture device consisted of an array of 1,600 culture chambers loaded with individual nonadherent cells. Programmable media exchanges were then utilized to extend cell survival and evaluate effects of various environmental conditions on hematopoietic stem cells. In other examples, the addition of simple components led to substantial improvements in on-chip cell culture. For example, cell culture and assay steps were controlled simply through the addition of a hydrodynamic shear stress generator<sup>196</sup> or “timer channel”. Adjusting the dimensions of the “timer channel” yielded accurate sequential application of reagents for cell staining.<sup>197</sup> In another device, adherent cells cultured below a vacuum-actuated diaphragm could be controllably removed from their growth surface as the diaphragm was actuated providing a hydrodynamic shear stress to release cells.<sup>196</sup> These devices demonstrated that simple microfluidic devices can perform cell culture tasks in an automated fashion. In addition, advanced microfluidic platforms also improve culture of demanding cell types by precisely controlling the local environment and reagent flow profile to extend cell survival. This was accomplished using cell-free “feeding channels”<sup>198</sup> or controlled-source conditioned media infusions.<sup>199</sup>

**Directed cell growth**—Strategies to confine cells in channels or pattern cells on surfaces have been a robust area of investigation in the microfluidic field for many years. While numerous techniques exist for patterning cells on-chip, most are not sufficiently simple to enable widespread adoption by biologists. Thus a device utilizing capillary action accompanied by laminar flow to pattern cells in precise, non-overlapping patterns may enable biomedical researchers to pattern cells without having an extensive knowledge of microfluidics.<sup>200</sup> Easily adoptable cell-patterning methods have led to new biological studies of cell physiology and behavior. For example, the ability to pattern cells aided investigation of cell growth as a function of time, as evidenced by a recent study of aging in *E. coli*.<sup>201</sup> By seeding deep microfluidic wells with individual “mother cells,” investigators followed the daughter cells of up to 10<sup>5</sup> individual *E. coli* lineages (Figure 9b). Microfabricated channels were also used to guide the formation of cell-cell contacts, such as axonal connections between whole slices of rat hippocampus and cortex.<sup>202</sup> This directed formation of electrical contacts led to synchronization of the slices for use in drug testing. Patterning of channel surface chemistry prior to cell culture also directs cell growth, and precise control of a cell’s spatial environment makes new findings possible. For example, patterned N-cadherin, an extracellular matrix protein, guided axon growth from neurons within groups of cultured embryonic stem cells.<sup>203</sup> A two-compartment microfluidic device was used to fluidically isolate neuron cell dendrites.<sup>204</sup> Research performed with this device have suggested a mechanism for dendrite-to-nucleus cell signaling, and hold promise for future studies that require biochemical study of specific regions of the neuron.

The devices highlighted above aimed to automate common procedures and/or provide enhanced platforms for cell growth and analysis, directly expanding our biological capabilities. However, these devices often remain in the research laboratories of chemists and engineers. Acceptance of these and future devices by the biology community requires simplicity, robustness and continuity with currently accepted protocols. Many of these prototypes were produced using PDMS due to its low cost and simple fabrication. Fabrication strategies for materials more established for cell culture, such as glass or

polystyrene, will increase adoption by biologists and provide better chemical compatibility and a more stable surface chemistry than that of PDMS. Further advances in microdevices for cell culture will surely be geared towards further mimicking the *in vivo* environment in which biological processes naturally occur.

### 3.4 Controlling the cellular microenvironment

Novel platforms are permitting carefully controlled studies of the effects of environmental factors on cells. Much of a cell's physiology and function depends on the precise chemical milieu in which it exists. Microfluidic systems offer obvious advantages for this field, such as precise control of flow rates and concentration gradients, the ability to combine multiple chemical and physical signals, and small volumes to minimize the cost and time required for experiments. Recent advances using microfluidics to study environmental influences on cell physiology include studies of chemotaxis and drug action and resistance.

**Chemotaxis**—Chemotaxis, directed cell movement in response to a chemical gradient, has long been investigated by microfluidic platforms. While each year brings along a number of microfluidics studies in this area, we highlight investigations with impressive biological impact. Cancer is one area in which chemotaxis has high relevance, and devices have recently been used to investigate new paradigms in cancer cell chemotaxis, including the roles of naturally-occurring “source” and “sink” cells<sup>205</sup> and interstitial fluid flow across the extracellular matrix.<sup>206</sup> Microfluidics were also applied to study chemotaxis in other clinically relevant areas: burn injuries<sup>207</sup> and angiogenesis.<sup>208</sup> Neutrophils from burn patients showed a significant reduction in chemotactic speed compared to healthy controls, providing further evidence for neutrophil dysfunction in burn victims.<sup>207</sup> Another device used a collagen scaffold to assess the migration of microvascular endothelial cells when exposed to gradients of angiogenic factors.<sup>208</sup> These devices hold promise for studying migration of a variety of cell types in a 3D environment using a combination of gradients. Microfluidics were also applied to investigate the cellular mechanism of chemotaxis. A three-input microfluidic device generated gradients that reversed direction at a defined frequency.<sup>209</sup> Above a certain frequency, cells placed in the alternating gradient field became chemotactically “stalled,” allowing researchers to spatially confine and biochemically analyze the stall process. Microfluidics also contributed to studies of microbial chemotaxis. For example, a microfluidic study of complex stimuli on *E. coli* revealed that the overall chemotactic response depended on the ratio of the dominant chemoattractant receptors.<sup>210</sup> In other recent research, several marine microbes demonstrated chemotactic responses to dimethylsulfoniopropionate (DMSP), a phytoplankton-derived sulfur compound connected to the formation of atmospheric clouds and hence global climate patterns.<sup>211</sup> These microfluidics devices demonstrated the applicability of chemotaxis platforms to a wide range of cell types as well as their growing impact in addressing fundamental biological questions.

**Combining multiple environmental cues**—*In vivo* cells respond simultaneously to a multiplicity of incoming signals including soluble and immobilized ligands. New microdevice applications have sought to multiplex stimuli to better mimic the native cellular environment. One application spotted ligands alongside varying surface densities of extracellular matrix protein.<sup>212</sup> Another platform altered this approach to apply signaling cues from both the extracellular matrix and soluble factors. To study differentiation in alveolar epithelial cells, different concentrations of extracellular matrix protein were printed onto a device before cell seeding, after which soluble factors were supplied.<sup>213</sup> Similarly, an arrayed microfluidic device with a combinatorial perfusion system permitted high-throughput testing of up to 16 culture conditions comprised of 4 extracellular matrices and 4 soluble factors in media.<sup>214</sup> Combinatorial perfusion combined with monolithic digitally-

controlled valves in a thin PDMS layer allowed user-directed flow control of single culture wells.<sup>215</sup> This enabled precise delivery of environmental factors to cells for differing time spans as might occur within an animal.

**Drug action and resistance**—Microfluidic devices offer a variety of advantages in quantifying drug actions on cells, including the ability to assess a wide range of drug concentrations and perform multiplexed cell assays in a high throughput manner. For example, a simple microfluidic device integrated cell culture, protein measurements, and cell viability assays for both experimental and control cells used to study chemotherapy resistance.<sup>216</sup> An alternative device assessed chemotherapy effectiveness using a parallel array of cell culture microchambers.<sup>217</sup> An upstream architecture progressively diluted drug with culture media so that each microchamber was perfused with a different drug concentration, spanning six orders of magnitude, a practical requirement for most modern drug discovery applications. Importantly, this platform yielded an IC<sub>50</sub> (half maximal inhibitory concentration) value identical to that obtained from a traditional culture dish, establishing continuity between microfluidic and accepted macroscale assays. Other microfluidic studies improved on established methods by incorporating more realistic 3D cell culture conditions. Tumor spheroids, which may more accurately depict *in vivo* tumor microarchitecture were shown to be better suited for studies of chemotherapy and resistance than isolated cells.<sup>218</sup> Drug treatment of spheroids showed that these 3D tumor cultures were more drug resistant than monolayer cultures. Similarly, a bacterial biofilm cultured in a PDMS microchannel demonstrated the effect of the cellular community on drug resistance.<sup>219</sup> Regardless of the antibiotic used, higher concentrations were needed to eradicate biofilms compared to free-swimming bacteria. In the context of rapidly spreading antibiotic resistance and increasing knowledge about biofilms, this study represents a pioneering effort to modernize antibiotic susceptibility testing. Combined with the study of tumor spheroids, this research also demonstrates the effect that neighboring cells have on each other, the focus of Section 3.5.

### 3.5 Cell-cell interactions

Outside the laboratory, few cells exist in isolation, and studies of microfluidic co-cultures are elucidating how intercellular signals contribute to cellular communities. Microfluidic devices have been engineered to enable cellular interactions such as direct cell-cell contact, release and reception of soluble mediators, and transmission of electrical signals. One recent paper, for example, showed that the viability of a cell line was enhanced when it was co-cultured in microfluidic drops with another cell line known to secrete growth factor.<sup>220</sup> Another microfluidic device facilitated studies of quorum sensing in a population of *E. coli* genetically engineered to express GFP (green fluorescent protein) in the presence of neighboring cells; precise microfluidic control of the cells' environment coordinated oscillations in GFP-expression.<sup>221</sup> Advances in understanding cellular interactions were derived from both novel device fabrication, cell-patterning methods<sup>222</sup> and innovative biological applications. These studies have revealed new insights into neuronal and cancer cell communities.

**Neuronal cells**—Because of their ability to precisely control flow and pattern cells in discrete areas, microfluidic devices are helping researchers better understand interactions between neurons and nearby supporting cells. Neuron cell physiology is tightly connected to the microenvironment created by supporting cells, and so two devices that allow for either parallel or serial perfusion of culture chambers are highly useful tools. In these devices, glia or other neuronal support cells were stimulated in one chamber, and soluble factors released by the activated cells were directed to neurons grown in the adjacent chamber. One device controlled flow between adjacent culture chambers using a pressure-controlled valve, the

other by adjusting flow rates to each chamber.<sup>223,199</sup> A related study patterned microglia and neurons in a centrifugal device, demonstrating that microglial cells preferentially collected around degenerating neurons, suggesting a role for microglia in neurodegenerative disease states.<sup>224</sup> Other devices focused on neuron-neuron interactions and the formation of synapses. A simple two-compartment system housed neurons transfected with GFP in one compartment and RFP (red fluorescent protein)-expressing neurons in the other (Figure 9c).<sup>225</sup> Small connecting channels permitted synapse formation between neurons of the different compartments. Imaging of intracellular calcium concentrations demonstrated synapse-to-nucleus signaling between neurons. As a whole, these devices demonstrated the utility of microfluidics for precise studies of interactions between neurons and physiologically-relevant neighboring cells. Indeed, commercial microfluidic devices for some of these applications are now on the market.<sup>226</sup>

**Cancer**—An active area of cancer research focuses on the role that normal surrounding cells play in supporting the growth and progression of tumor cells, and microfluidic devices are well positioned to recapitulate the tumor microenvironment. A series of “sandbag” structures were developed to entrap tumor cells and then perfuse them with media conditioned by mechanically stimulated “donor” cells upstream.<sup>227</sup> Studies of signaling between cancer cells and nearby fibroblasts have used microfluidic devices to combine fibroblasts with liver,<sup>228</sup> salivary gland,<sup>229</sup> or lung<sup>230</sup> tumor cells (Figure 9d). These devices often featured user-defined flow paths and programmable flow networks that allowed researchers to investigate two-way communication between the different cell types.

### 3.6 Organs-on-a-chip

Many recent research efforts in microfluidics focus on the development of organ-on-a-chip technology, the microfabrication of increasingly “life-like” environments for cell culture. Such technology aims at a better approximation of *in vivo* cellular organization, function, and interactions than simple 2D tissue-culture systems without the complexity of an intact animal. The ability of microsystems to place cell layers in discrete locations and to apply stimuli in specified patterns at controlled times has yielded devices that successfully recapitulate many aspects of the pulmonary and cardiovascular systems. These miniaturized environments have also been utilized to model organ-organ interactions in ways not possible with “scaled up” macroscopic systems.

**Lung-on-a-chip**—Airway-shaped microchannels with attached pulmonary epithelial cells were used to simulate the lung. Movement of liquid plugs through the channels simulated occlusion and re-opening of airways.<sup>231</sup> Additionally, surfactants similar to those produced endogenously by the lung were shown to be protective against mechanical stresses and epithelial cell injury.<sup>232</sup> Similar stresses to the alveolus (air sac), arising from the “meniscus effect” of a migrating air-liquid interface, were investigated with a microfluidic “alveolus-on-a-chip” platform, which showed that a “moving meniscus” affected the attachment and viability of alveolar cells. These studies are a step towards a microfluidic model for lung conditions such as ventilator-induced lung injury and pulmonary edema (Figure 10a).<sup>233</sup> Another study of mechanical strain in the pulmonary system probed the interface between the alveolar cells and microvessel endothelial cells by culturing back-to-back monolayers of alveolar epithelia and endothelia. The utility of this model was demonstrated by studies of pressure-volume dynamics, host-defense processes, and pulmonary toxicology.<sup>4</sup> Significantly, the results obtained mirror those from studies in mice, making this platform a useful alternative to animal studies.

**Cardiovascular system**—Since microfluidic devices consist largely of channels with flowing liquids, these devices have been used to model the cardiovascular system. A



microfluidic platform enabled research on small arteries with user-controlled application of pharmacological agents to specific spatial regions of the blood vessel.<sup>234</sup> Leukocyte recruitment into new capillary buds during angiogenesis was studied by tracking leukocyte movement into a blind-ended channel branching away from a main channel filled with whole blood.<sup>235</sup> Future studies would be expected to include endothelial-lined channels to more closely mimic the vascular system. Another microfluidic device utilizing either pulsatile or constant flow was developed to monitor the production of reactive oxygen species in endothelial cells within the channels.<sup>236</sup> Two microfluidic devices, a microcardiac cell culture model ( $\mu$ CCCM)<sup>237</sup> and an endothelial cell culture model (ECCM),<sup>238</sup> also modeled the mechanical stresses from blood flow on developing cardiac myocytes and endothelial cells. Variations in the pressure and flow rate yielded flow conditions that mimicked heart failure, high and low blood pressure, fast and slow heart rate, and normal cardiovascular function. Consequently, this device promises to be an excellent tool for future studies of myocyte and endothelial cell physiology in cardiovascular disease. *In vivo*, however, cardiomyocytes receive input from the nervous system. Thus, a recent device allowed co-culture of cardiomyocytes and sympathetic neurons and enabled formation of functional synapses between the neurons and myocytes, a notable advancement towards a heart-on-chip that features a variety of regulatory inputs.<sup>239</sup> As these organ-on-a-chip platforms advance, a natural application is to adapt them for disease-specific studies, as evidenced by the myocyte/endothelial cell devices above.<sup>237,238</sup>

**Inter-organ interactions**—The ability to sequentially connect microfluidic culture chambers with fluid streams, much as organs are connected by blood vessels within the body, positions microfluidic devices for the study of inter-organ interactions, particularly in the context of drug absorption and metabolism. Recent systems have investigated how intestine-to-liver signaling affects the absorption and subsequent metabolism of an orally-administered drug (the “first-pass effect”). Intestine and liver slices in a two-compartment device with sequential fluid flow showed similar drug metabolism rates to that of organ slices in bulk culture yet a more physiological gene expression profile compared to the traditionally cultured organ slices.<sup>240</sup> A similar device used intestinal and liver cell lines for a “micro-total bioassay”, in which a supplied agent flowed through a channel coated with intestinal epithelial cells to simulate drug absorption (Figure 10b).<sup>241</sup> The agent then encountered cultured liver cells to simulate metabolism by the liver. Only after encountering these liver cells was the drug (or its metabolites) delivered to the downstream target tissue of interest. In this study, drug absorption and metabolism were assessed faster and with fewer cells than in conventional bioassays. Future work is expected to expand this technology to incorporate multiple, interacting organ systems on-chip, for example, a microchip version of the cardiovascular (heart) and pulmonary (lungs) systems. These systems will create interacting model systems in a highly controlled environment to explore networks of organs and tissues.

### 3.7 Organisms-on-a-chip

Microfluidic technology provides many advantages for studying whole organisms on-chip, including control of the environment (chemical, geometrical, and other), precise application of stimuli and chemical compounds, and higher throughput compared to macro-level analyses. Furthermore, use of whole organisms offers a complex interacting set of organ systems for study. Advances in this area have included work on the roundworm *Caenorhabditis elegans* (*C. elegans*), the plant *Arabidopsis thaliana*, the zebrafish (*Danio rerio*), and fertilized oocytes, embryos, and embryoid bodies.

**Roundworms**—*C. elegans* is well suited for microfluidic devices for several reasons. First, the organisms are small (approximately 1 mm in length) and thrive in aquatic

environments, including fluid-filled microchannels. Second, they are transparent, allowing easy examination by microscopy. Lastly, the organism's genome has been successfully sequenced, and *C. elegans* has long been a model organism in developmental and cell biology. Most platforms allow culturing, immobilizing, and observing single worms over their entire lifespan. A major theme in recent microfluidic experiments with *C. elegans* has been immobilization of worms for microscopy. Methods have included the use of "worm clamps,"<sup>242</sup> pressure-driven flow into a tapered aperture,<sup>243</sup> droplets to encapsulate the organism,<sup>244</sup> and a temperature-sensitive resin (Figure 10c).<sup>245</sup> In the "worm clamp," for example, worms were introduced into a series of microfluidic chambers through microchannels, early in their life cycle, and their rapid growth trapped the adult worms in the chambers for further studies.<sup>242</sup> On-chip platforms allowed a range of biological studies to be performed, from calcium imaging in single chemosensory neurons to the physiology of locomotion<sup>246</sup> and to whole worm behavioral analysis.<sup>247</sup> Future efforts could include stimulation of discrete regions of the organism, such as an anatomy-specific study of the effects of drugs on worm development, or "lineage-on-a-chip" studies, in which a single worm matures and reproduces on-chip with additional culture chambers downstream for its progeny.

**Zebrafish**—Early whole animal platforms to study simple vertebrates raise zebrafish (*Danio rerio*) from embryos through early developmental stages on microfluidic chips. Zebrafish are commonly used in drug screens and as disease models, inspiring a microfluidic platform to culture 32 independent zebrafish embryos (Figure 10d).<sup>248</sup> Optimizing flow rates resulted in 100% survival and successful development of embryos for 5 days on-chip, though embryos were shorter in length than embryos grown on traditional 96-well plates. Devices of this type have been used to study the effects of exposure to ethanol<sup>248</sup> and doxorubicin on zebrafish development, often including multiple analytical parameters<sup>249</sup> and producing results similar to those from a 96-well plate comparison control. Thus, these devices enable further use of the zebrafish model for development and drug toxicity studies in a cheap, high-throughput format with the ability to monitor a variety of parameters, from heart rate to morphology.

**Plants**—A recent "plant-on-a-chip" immobilized an *Arabidopsis thaliana* root segment within a multilaminar PDMS flow platform. This system delivered a synthetic stimulant based on a natural plant hormone using laminar flow to localize the hormone to a specific portion of the root, enabling good spatial resolution of the root-developing effects of the compound.<sup>250</sup> The result is a promising method with potential applications in botany, agriculture, and environmental sciences. Another plant-on-a-chip platform investigated plant reproduction, specifically pollen tube growth and guidance.<sup>251</sup> Unfertilized ova from *A. thaliana* were placed in alcoves of a microfluidic channel, and the growth of sperm-carrying pollen tubes from a pistil towards the ova was observed microscopically and quantitatively analyzed. These devices will enable further studies of plant reproduction using customized, controllable microenvironments.

**Oocytes, embryos, and embryoid bodies**—A number of investigators have applied the strengths of microfluidic chips to the handling and analysis of oocytes and embryos. Noting that *in vitro* fertilization (IVF) still relies on manual pipetting, one platform used a microwell array to perform integrated oocyte trapping, fertilization with sperm, and early culture of the resulting embryo.<sup>252</sup> A similar device featured an array of fenestrated wells for immobilizing oocytes and thin channels for sperm motility screening, a common practice in IVF.<sup>253</sup> Only motile sperm were able to migrate from the deposition chamber on the device periphery into the central chamber, where the oocyte array was located. This circumvented the need to manually pipette motile sperm, minimizing post-selection damage.

While the authors present these devices as tools for IVF, such platforms also have potential for basic science studies of spermatozoa function and motility and early events in embryogenesis.

Microfluidic devices were also used to study embryoid bodies, small collections of stem cells that mimic the differentiation of a true embryo. Previous studies demonstrated that the fate of an embryoid body depends strongly on its size, inspiring a microfluidic platform to seed embryonic stem cells using a precise resistance network for replicable cell deposition.<sup>254</sup> By controlling cell density, flow rate, and duration of flow, 60 embryoid bodies of homogenous size were formed. Since future work is likely to utilize microscopy to study development, the engineering advance made by an embryo-orienting microfluidic device is also significant.<sup>255</sup> This prototype used a series of “traps” to capture and orient over 700 *Drosophila* embryos, achieving high throughput, spatiotemporal imaging of developmentally-relevant signaling processes.

#### 4. Conclusions and Outlook

The past 18 months saw continued progress in developing an ever widening palette of materials and fabrication strategies for  $\mu$ TAS. Researchers can now tailor their fabrication methods to match their budget as well as their application. While fabrication methods have continued to develop and diversify, sample preparation on-chip continues to be too often neglected. This review purports to address micro *total* analysis systems, but few devices actually offer a complete analysis on-chip because most require substantial off-chip sample processing. Compared to advances in other areas of microscale operations, sample preparation has seen relatively little progress in recent years, perhaps because it is often challenging, but rarely glamorous. Nevertheless, major strides must be made, either to optimize on-chip sample preparation steps or to design devices compatible with crude real-world samples. These advances are critical to realizing the potential of  $\mu$ TAS and to promoting their adoption by life scientists, health care workers, and industrial labs.

In this year’s review, we chose to focus on  $\mu$ TAS applications in cell biology and biochemical analysis. Every year sees new publications on microfluidic systems for clinical applications and diagnostics; however, few of these devices have been adopted for routine use outside research laboratories. This is due in part to the fact that the field of clinical diagnostics is quite competitive, and many of the relevant tests have highly developed non- $\mu$ TAS protocols in place. Consequently, microfluidic alternatives must be quite sophisticated and offer substantial advantages to displace established methods. In contrast, recapitulation of biological systems on-chip is a rapidly emerging new field. Microfabrication and microfluidics offer unique advantages in this area. For example, organ-on-chip devices take advantage of precise fluid handling, independent control of mechanical, electrical, and chemical signals, and the capacity to span multiple biological scales from the molecule to the cell to the organ and organism. To date, few other technologies offer the same control over the physics, chemistry, and biology of living systems, and  $\mu$ TAS are likely to dominate this field in the future.

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**Doug Orloff** received his B.Sc. in Chemistry from the University of North Carolina-Chapel Hill in 2007, concentrating in biological and analytical chemistry. As an undergraduate, he helped characterize the molecular biology of nuclear receptors. After a postbaccalaureate fellowship at NIEHS studying virology and host-pathogen interactions, he entered into a combined M.D./Ph.D. program at the University of North Carolina School of Medicine and is currently a Ph.D. candidate in pharmacology. His graduate research is at the interface of microscale analysis and pulmonary physiology. His research interests lie in applying bioanalytical chemistry and microfluidics to fundamental problems in respiratory cell physiology.

**Yuli Wang** received his Ph.D. in Materials Sciences in 2003 from the University of California at Irvine. Since then he has worked with Nancy Allbritton in developing new lab-on-a-chip technologies to support biologists in addressing important biomedical problems. He is currently a research associate in the Department of Chemistry at the University of North Carolina-Chapel Hill. Besides his academic research, he is interested in entrepreneurship and has co-founded two startup companies: Intellego Corporation and Cell Microsystems. His current research involves developing platforms for studying intestinal and airway epithelial cells and tissues.

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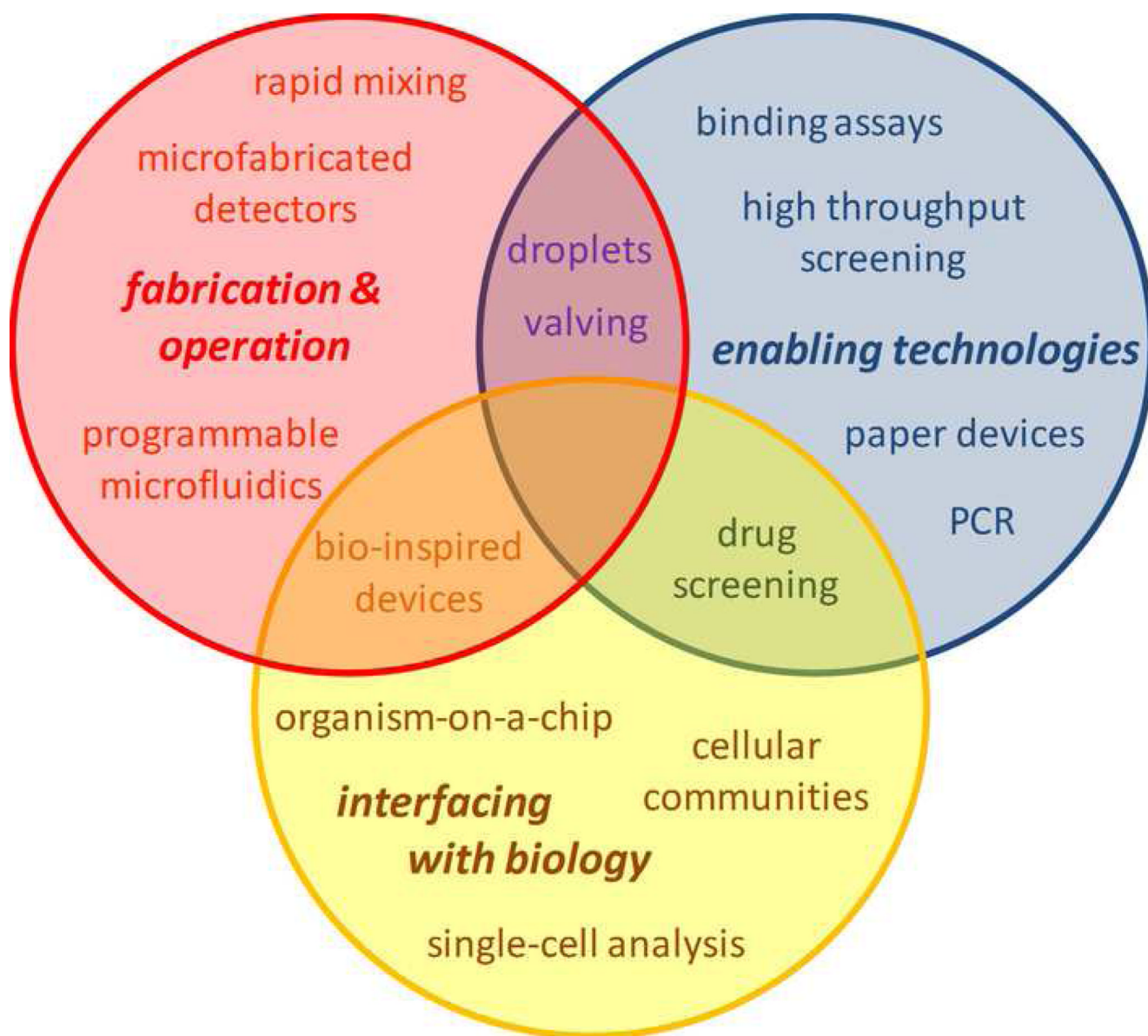


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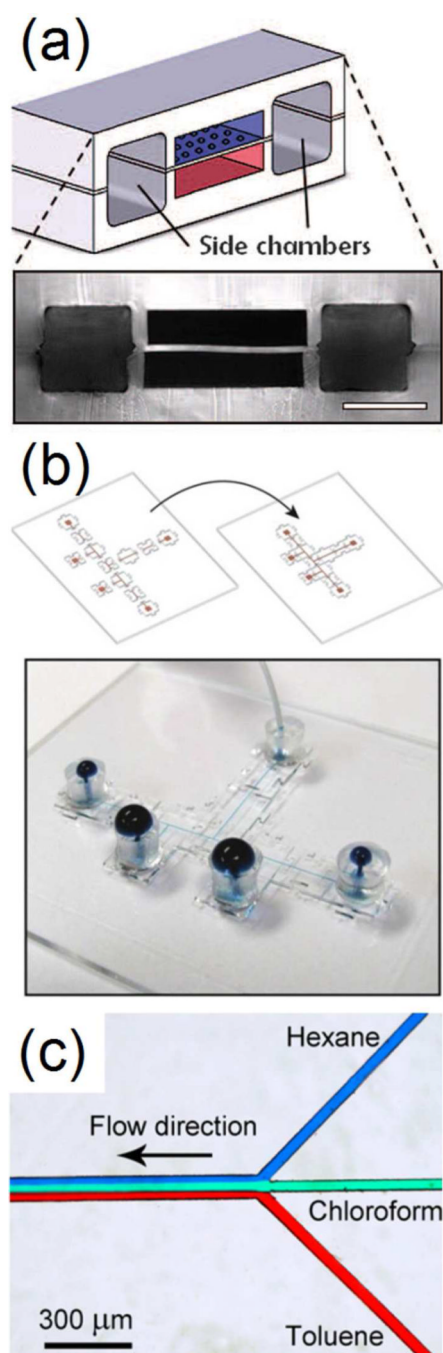
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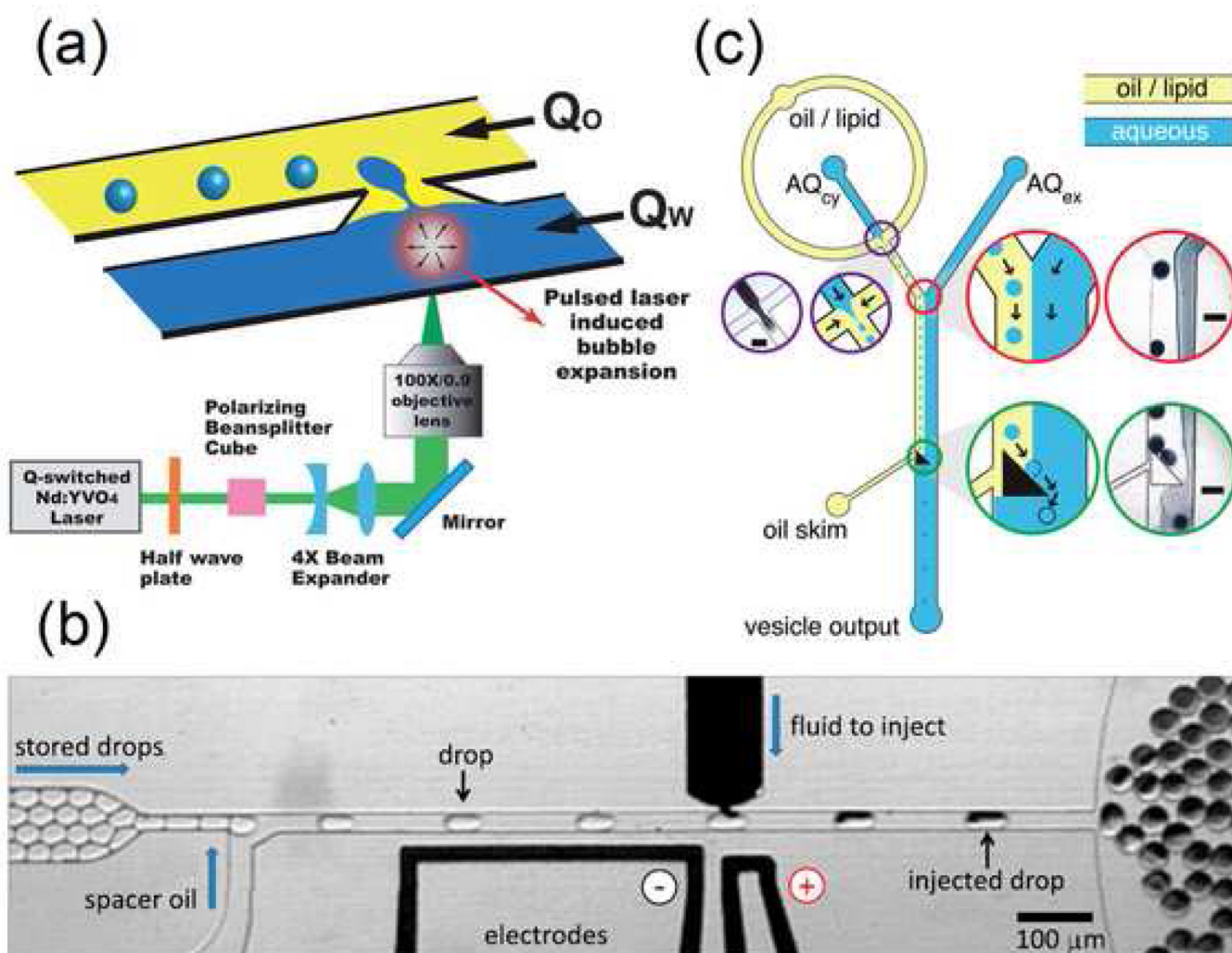
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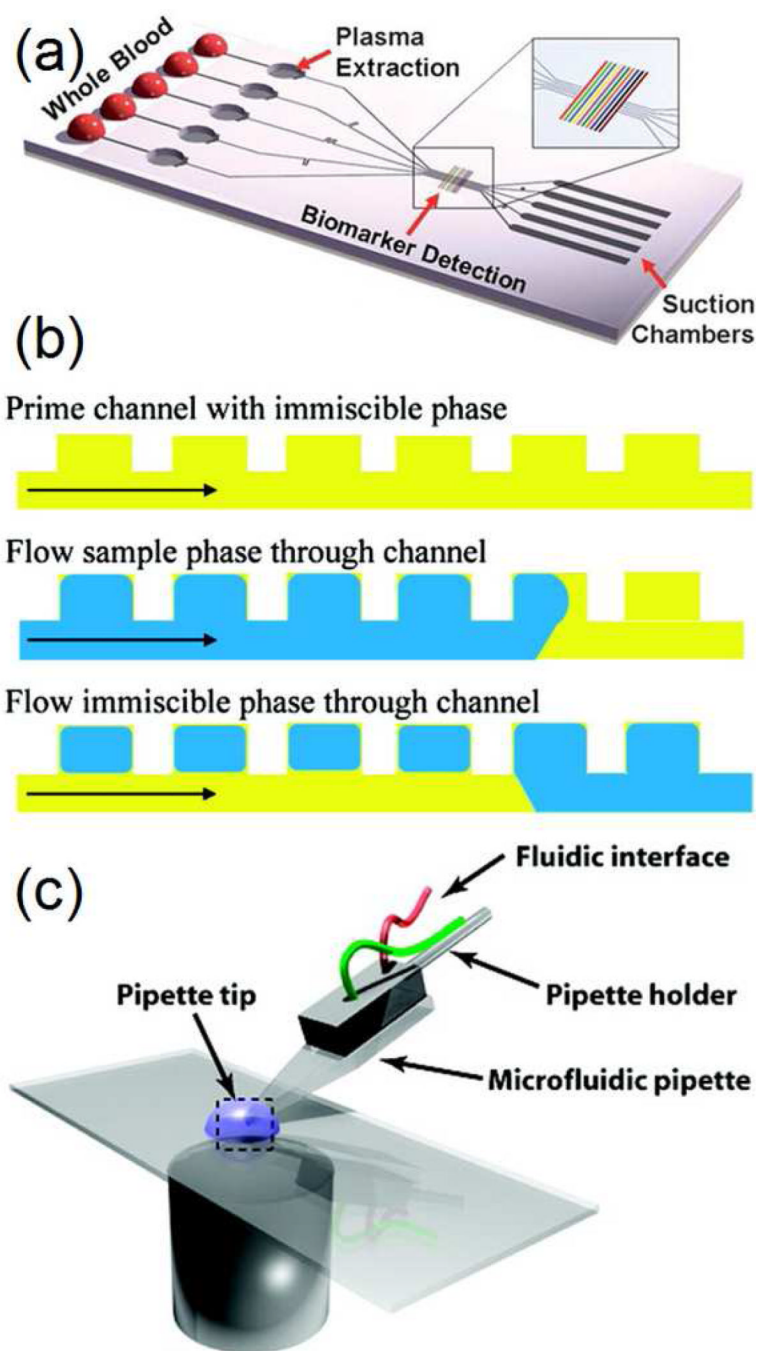
**Figure 1.** We highlight recent contributions to  $\mu$ TAS in three interlocking areas: fabrication & operation, enabling technologies, and interfacing with biology.



**Figure 2.** Microfabricated devices. Recent advances in microfabrication include (a) improved replication of *in vivo* conditions, (b) development of modular units, and (c) the use of solvent-resistant materials. (a) A lung-on-a-chip microfluidic device was composed of two large side chambers and a central microchannel separated by a 10- $\mu\text{m}$  thick porous PDMS membrane. Scale bar, 200  $\mu\text{m}$ . (b) A selection of pre-fabricated microfluidic assembly blocks were produced (top left), followed by assembly and bonding (top right) into a complete device (bottom). (c) A whole-Teflon microfluidic chip showed robust solvent compatibility using laminar flow of dyed organic solvents. Figures reproduced with permission from references 4, 9 and 10.

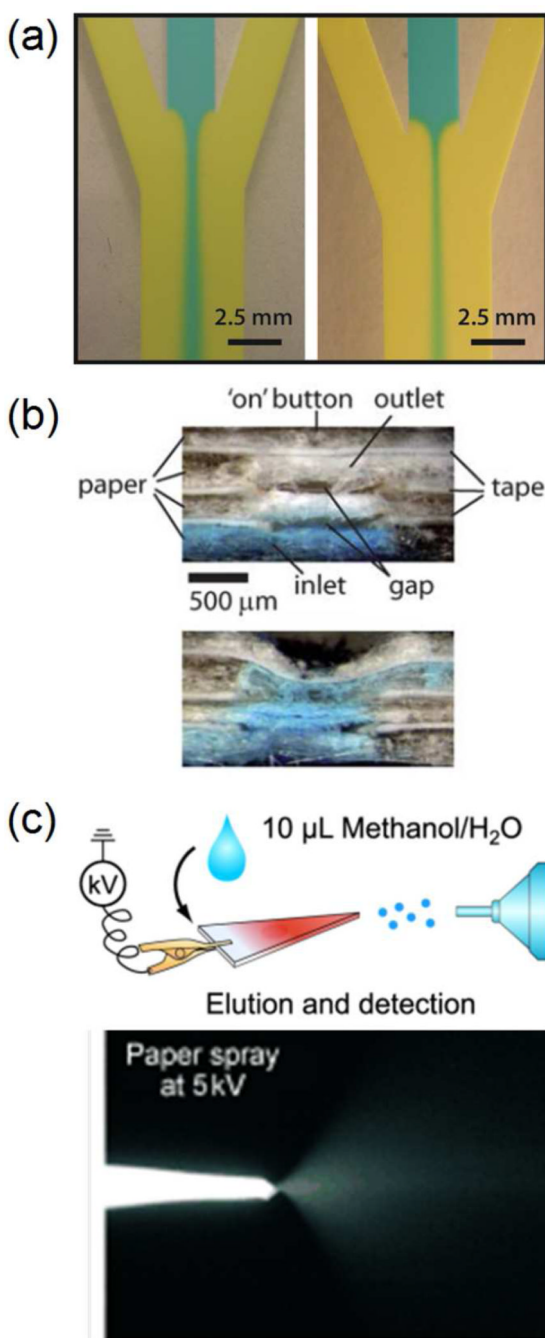


**Figure 3.** Droplet microfluidics. Recent advances (a) generate droplets on-demand, (b) synthesize lipid bilayer-enclosed droplets, and (c) selectively add reagents. (a) On-demand droplet generation by laser pulse used two microfluidic channels connected by a nozzle-like opening. A highly focused intense laser pulse induced a rapidly expanding cavitation bubble to push the nearby water into the oil channel for droplet formation. (b) Giant unilamellar vesicles were synthesized on a microfluidic assembly line that sent aqueous droplets through an oil phase to a “skim”, which sheathed them in a lipid bilayer before their reintroduction to an aqueous environment. (c) Picoinjectors added reagents to drops by merging them with a pressurized channel containing the reagent. Figures reproduced with permission from references 40, 45 and 48.

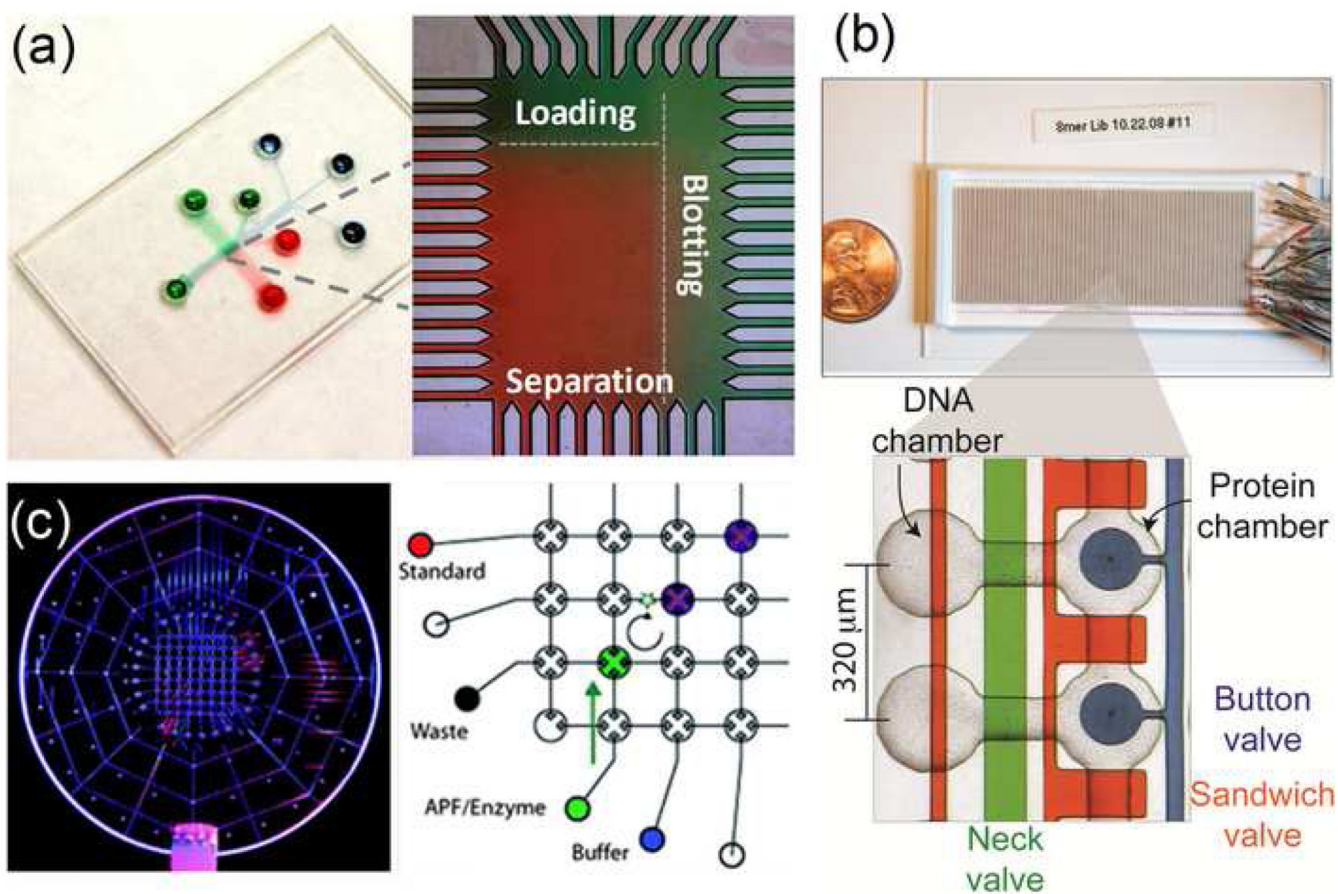


**Figure 4.** Simple, rugged devices. New innovations simplify sample (a) transport, (b) handling, and (c) dispensing. (a) A self-priming, self-contained, tether-free system integrated volume metering, plasma separation from whole-blood, multiple biomarker detection, and suction chambers for fluid propulsion. (b) A self-digitization platform automatically dispensed aqueous samples into an array of discrete volumes. (c) A microfluidic pipette with a circulating liquid tip selectively treated specific surface-immobilized biological cells within a culture. Figures reproduced with permission from references 71, 72 and 73.

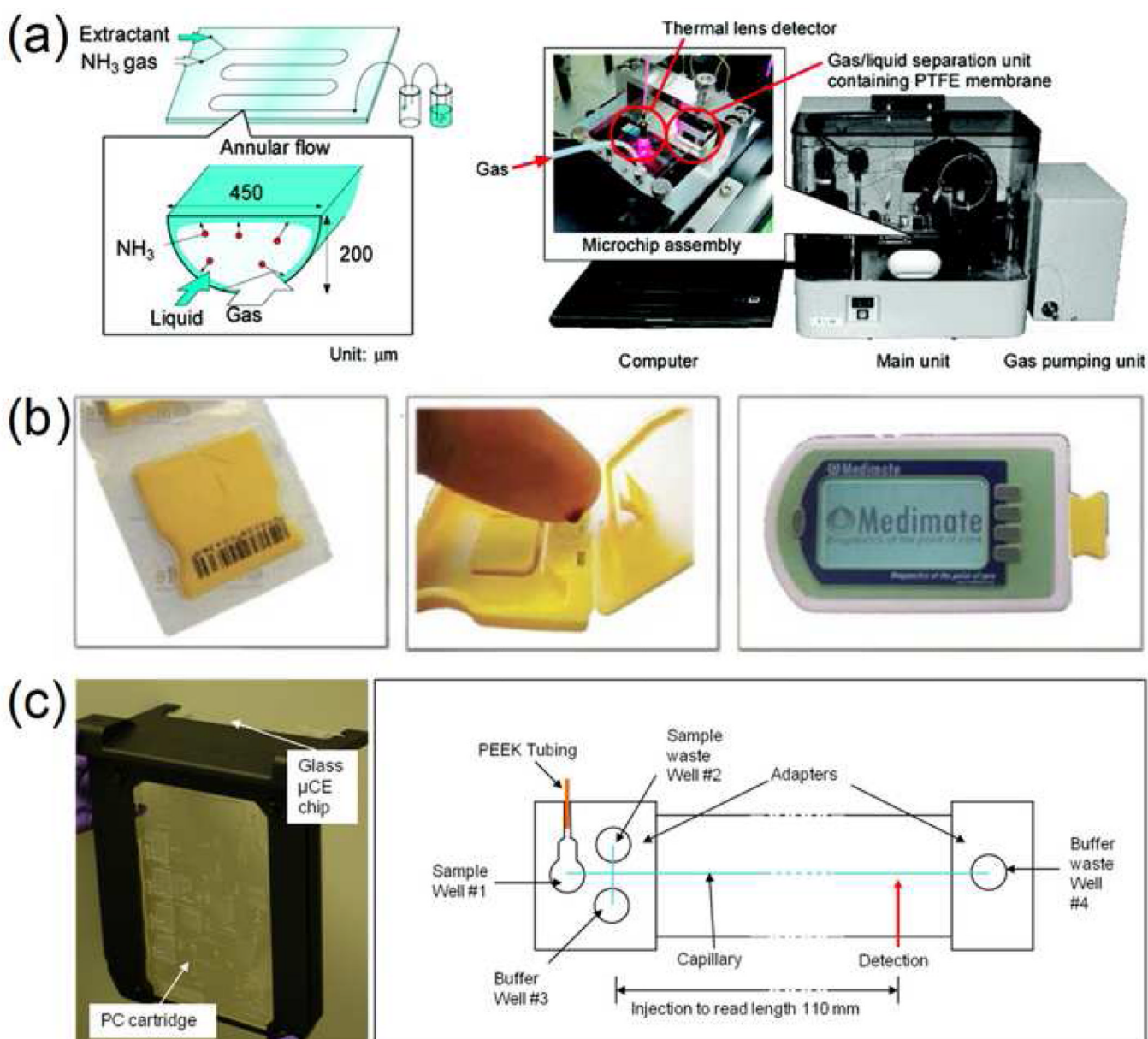




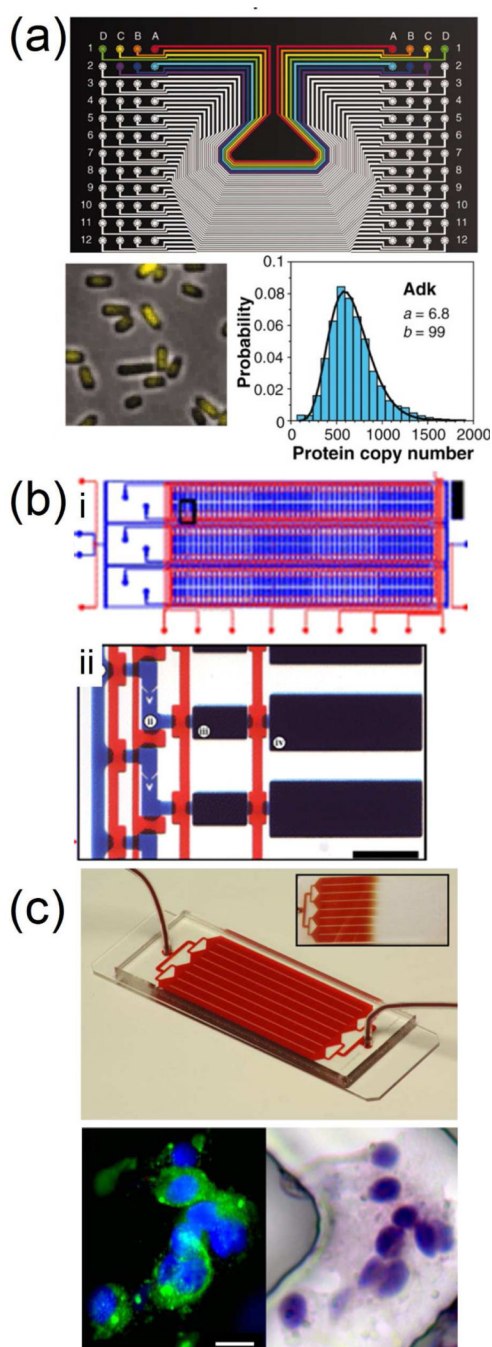
**Figure 5.** Paper microfluidics. Recent advances in this area include (a) recapitulation of fluid handling functionalities from traditional microfluidics, including laminar focusing, (b) integration of programmable switches, and (c) coupling to mass spectrometry using paper spray. (a) Laminar focusing was demonstrated in a paper device. (b) Tape and paper were used to fabricate a programmable 3D paper device. When the “on” button was depressed, a new flow path formed. (c) Analytes from whole blood were electrospayed directly from paper. Figures reproduced with permission from references 81, 85 and 38.



**Figure 6.** Rapid analyses. Microfluidic devices dramatically increase the speed at which biochemical information can be obtained by (a) decreasing analysis time, (b) multiplexing, and (c) automation. (a) A microfluidic device for western blotting cut analysis time to just 5 min using a discontinuous gel. (b) A massively parallel device screened transcription factor binding using arrays of valves. (c) A third device automated biochemical assays, including generation of a standard curve and sample analysis. Figures reproduced with permission from references 92, 102 and 63.

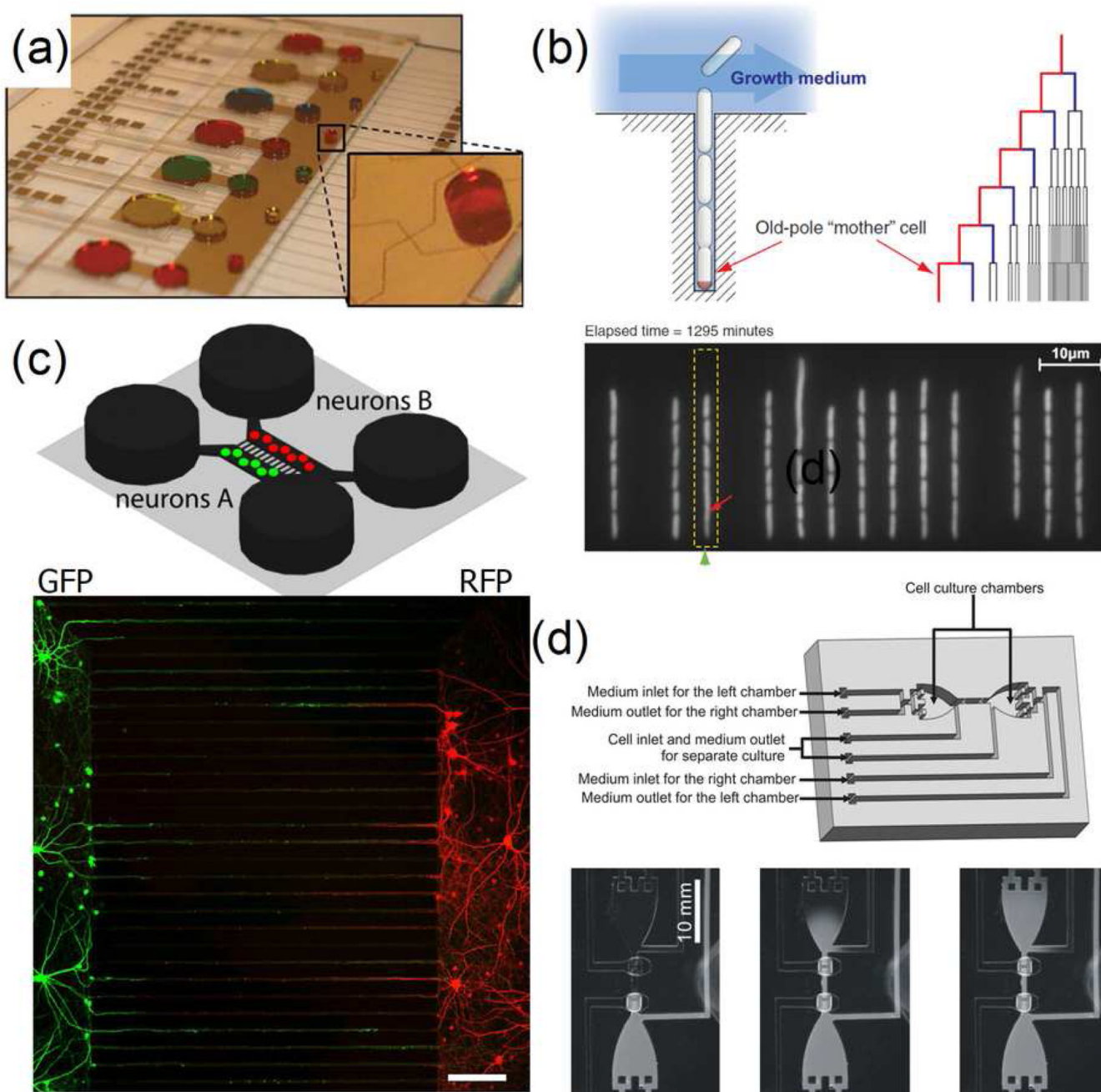


**Figure 7.** MicroTAS. True  $\mu\text{TAS}$  systems have been developed for (a) ammonia gas monitoring in clean rooms, (b) point-of-care determination of lithium in blood, and (c) forensic DNA analysis. (a) Annular flow extracted  $\text{NH}_3$  gas for colorimetric determination in the control and detection system shown to the right. (b) A sealed cartridge accepted whole blood for electrophoretic separation and electrochemical detection of lithium levels. (c) DNA was extracted from buccal swabs and amplified by PCR in the polycarbonate (PC) cartridge, then transferred to the glass chip for separation and detection. Figures reproduced with permission from references 125, 129 and 143.



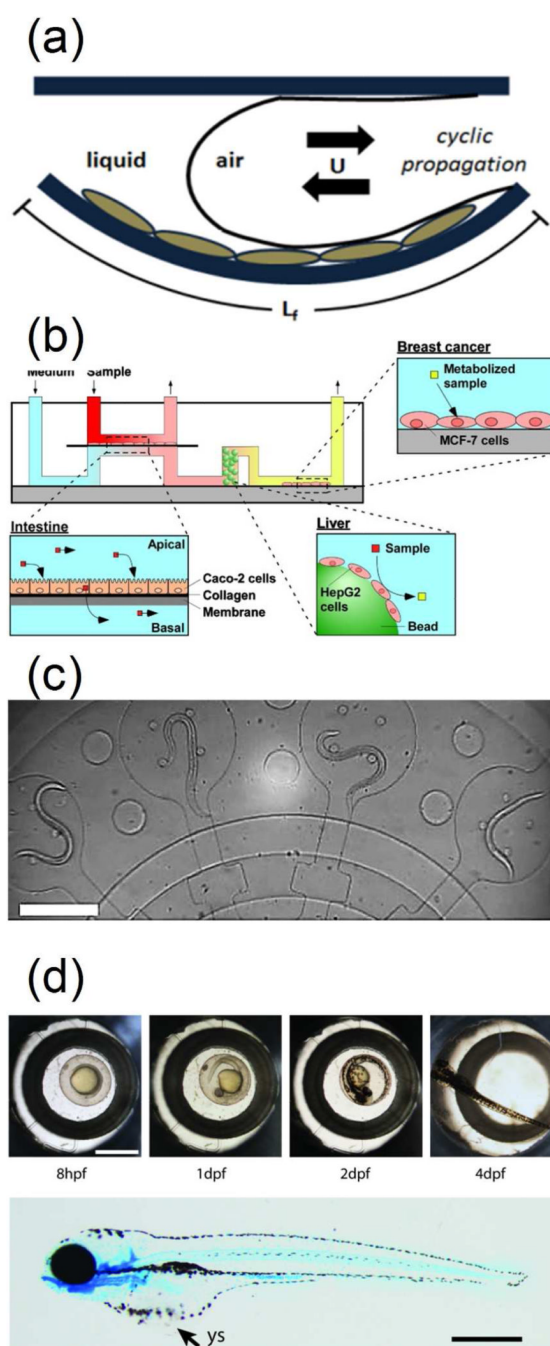
**Figure 8.** Single-cell analysis. Microfluidics benefit single-cell assays by (a) increasing throughput, (b) facilitating intracellular contents analyses, and (c) isolating rare cells. (a) A 96-channel assay chip allowed high-throughput analysis of over 1,000 proteome transcriptions in cultured *E. coli* cells. (b) A second device multiplexed analyses of the intracellular contents of individual cells. Single cells were isolated in 300 parallel channels, lysed to release cellular contents, and then analyzed by RT-qPCR. Scale bar, 400  $\mu\text{m}$ . (c) The precise fluid control and large effective surface area of microdevices facilitated sorting of specific cells from a heterogeneous population. The “herringbone chip” maximized cell-substrate

interactions to efficiently isolate CTCs from whole blood. Scale bar, 10  $\mu\text{m}$ . Figures reproduced with permission from references 168, 166 and 184.



**Figure 9.** On-chip cell culture. Microfluidic systems (a) automate cell culture, (b) direct cell growth, and (c–d) control cells’ microenvironments and interactions. (a) Cell culture procedures were automated by controlled addition of reagents on this digital microfluidic platform, which automated passage of cells. (b) Channel-directed cell growth allowed several generations of *E. coli* to be observed. Seeding single *E. coli* cells into the microchannels retained the “mother cell” in its original orientation as progeny filled the thin microchannels. (c) GFP- or RFP-expressing neurons formed synapses through small channels in the middle of the device. Scale bar, 150 μm. (d) Independently operated pneumatic valves separated two culture chambers that were perfused independently or sequentially (top) to precisely

apply media conditioned by cells cultured in the other chamber (bottom). Figures reproduced with permission from references 194, 201, 225 and 230.



**Figure 10.** Organ- or organism-on-chip systems. Microfluidic systems are (a–b) recapitulating organ systems *in vitro* and (c–d) manipulating entire microscopic organisms. (a) A microfluidic model of the pulmonary alveolus cultured alveolar cells on a flexible membrane within a microfluidic device. Application of cyclic pressure simulated the pressure changes experienced by alveolar cells *in vivo* during the breathing cycle. (b) In a “micro-total bio-assay” for drug studies, a drug sample was first applied to the upper channel, where it was absorbed and transported across the membrane by intestinal epithelial cells. The drug then moved through the bottom channel to liver cells that metabolized the drug before finally reaching the target cells. (c–d) Microfluidic devices for growth of whole (c) *C. elegans* and



(d) zebrafish (*Danio rerio*) embryos on-chip allowed researchers to monitor the growth and development of specific organisms over time. Scale bars are 200  $\mu\text{m}$  (c), 1 mm (d, top), and 500  $\mu\text{m}$  (d, bottom). Figures reproduced with permission from references 233, 241, 245 and 248.