Recent developments in microfluidic large scale integration

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In 2002, Thorsen et al. integrated thousands of micromechanical valves on a single microfluidic chip and demonstrated that the control of the fluidic networks can be simplified through multiplexors [1]. This enabled realization of highly parallel and automated fluidic processes with substantial sample economy advantage. Moreover, the fabrication of these devices by multilayer soft lithography was easy and reliable hence contributed to the power of the technology; microfluidic large scale integration (mLSI). Since then, mLSI has found use in wide variety of applications in biology and chemistry. In the meantime, efforts to improve the technology have been ongoing. These efforts mostly focus on; novel materials, components, micromechanical valve actuation methods, and chip architectures for mLSI. In this review, these technological advances are discussed and, recent examples of the mLSI applications are summarized.

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Recent advancements in microfluidic technologies have created new opportunities in the fields of biology and chemistry through miniaturization and automation of common processes, including mixing, metering, trapping, reaction, filtering, and separation, among others. Microfluidic large scale integration (mLSI) enables the fabrication of microfluidic chips containing hundreds or more of these functions using well-established lithography techniques, similar in principle to electronic LSI in the semiconductor industry [1]. Small feature dimensions, abundant spatial parallelism, and control over fluid flow provide mLSI technology with advantages such as high throughput, precise metering, automation and low reagent consumption. The implications of mLSI technology can especially be seen in recent advances in single cell, genomic, and protein analysis.

The on-chip valve is the key component of mLSI, much like the transistor in semiconductor LSI. Typically, microfluidic valves are fabricated by multilayer soft lithography (MSL) using PDMS and are actuated by external pneumatic controllers. A valve requires an elastomeric membrane that is deflected to control the fluidic resistance; when the valve is open, the fluidic resistance has a minimum value, determined by channel dimensions and when the valve is closed, the fluidic resistance is increased to infinity because flow rate is reduced to zero. In other cases, fluidic resistance can be set between these two extremes by controlling the pressure values or channel shape. The application of this flow control principle has led to the development of many higher-level components including peristaltic pumps, rotary mixers, multiplexers, and sieve valves. PDMS-based mLSI is widely used and applicable, however the technology has not found a '*killer application*' outside of a laboratory setting. Poor chemical resistance, a fundamental limitation of PDMS as a substrate material, causes swelling and defects when common solvents such as acetone or toluene are used as working fluids [2]. Another challenge is to realize facile valve actuation: although mLSI chips are small, pneumatic actuation requires complex external controls and a cumbersome chip-to-world interface, which has been compared, quite unflatteringly, to Medusa's head [3]. Therefore, there is a growing effort to make mLSI technology more compatible with chemically resistant elastomers and to reduce the external components required for actuation.

In the first mLSI review published in 2007 [4], Melin *et al.* discussed economies of scale, parallelization strategies, multiplexing and multistep biochemical processing and highlighted several components as building blocks of mLSI technology. In a later review in 2010 [5], Mark *et al.* summarized applications of PDMS-based mLSI chips and discussed the strengths and limitations of mLSI as a substrate technology. Here, new components and recent advances in architecture and design methods will be reviewed to present readers with a broader perspective regarding the capabilities and usage cases for the most advanced mLSI devices to date. Table 1 summarizes the requirements, components and applications of mLSI as covered in this review.

Component-level developments section reviews new materials for realizing fundamental mLSI components, different actuation methods, and the most recent spate of mLSI components. In *Architectural developments* section, higher density and miniaturized components requiring reduced external control elements are reviewed; when

Table 1

Summary of the mLSI requirements, components and applications discussed in this review

Microfluidic large scale integration (mLSI)		
Requirements	Components	Applications

• Reliable material
• User friendly actuation
• Higher density
• Less external components
• Flexibility and programming
• Automated design
	Fundamental:
	  • Push up, down, sieve valve
	  • Normally closed valve
	  • Structured valve
	  • Cell trap
	High level:
	  • Multiplexer, latch, pump, meter, mixer
	  • Gradient generator and selector
	  • Separation column
	  • Agar filled chamber
	  • Multilaminate mixer
		• Single cell culturing and imaging
		• Single cell genomic and protein analysis
		• Cell microenvironmental interaction
		• Protein synthesis and characterization
		• Chemical and biological screening
		• Solid phase liquid chromatography

integrated together in single-chip architectures, these technologies increase throughput, dynamic range, and sensitivity for many applications. *Automated mLSI chip design and layout* section reviews recent attempts to automate the design and layout of mLSI chips. Current practice is to draw each layer of a device manually using commercial software such as AutoCAD. In recent years, there have been several notable academic efforts to automate the design and layout process. The final section reviews recently reported biological and chemical applications of mLSI technology.

Component-level developments

Any candidate material to replace PDMS for mLSI technology must possess transparency and elasticity as fundamental properties. One early candidate was photocurable perfluoropolyethers (PFPEs) which have been used to make on-chip valves [2]. PFPEs have low surface energy, Young's modulus and toxicity, and their high chemical resistance and gas permeability makes them attractive for mLSI; however, sensitivity to oxygen makes their processing difficult. More recently, a commercially available silicone with a PFPE backbone (SIFEL) was processed by MSL to make standard size (100 μm) valves [6•]. These devices showed high solvent resistance while retaining the other favorable properties of PDMS, making them a promising candidate for future mLSI technologies. Viton [7], a commercial fluoroelastomer, can also be used as a membrane layer when bonded onto PMMA and cyclic olefin copolymer (COC) layers with plasma treatment and chemical assistance; the actuation pressure and bonding strength satisfied the requirements of mLSI.

Tuning the properties of PDMS by coating or doping can be a better strategy compared to replacing it with an alternative material especially when the goal is to reduce small molecule absorption; Parylene c coating [8] and silica nanoparticle doping by sol–gel treatment [9] have emerged as promising techniques. For example, the diffusivity of Rhodamine B can be reduced by a factor of 19 in Parylene c caulked PDMS when compared to native PDMS [8].

The cost of pneumatic external controllers is a significant impediment to the widespread adoption of mLSI technology. Potential replacement technologies include electrostatic [10], magnetic [11], torque generated [12], Braille pin [13], thermal [14,15] and shape memory alloy (SMA) [3] actuation.

The minimum requirements to render an actuation technology suitable for mLSI are scalability and a fast (<1 s) response time. Thermal methods are scalable but have slow response times. For example, phase change microvalves using ohmic resistors as heat sources have response times of minutes [14]. Replacing the phase change element with a thermo responsive polymer (poly(N-isopropylacrylamide)) and reducing the thermal mass of the responsive element (100 μm valve scale) decreased the response time to seconds [15]. Favorably, the pressure resistance of microvalves in COC was very high (1350 psi), and could be optimized by modifying the crosslinker and tetrahydrofuran composition; unfortunately, the quartz halogen lamp used for heating has a large area (diameter = 1.3 cm), which severely limits the scalability.

Gu *et al.* showed that 320 vertically moving Braille pins can be integrated with PDMS to realize pumps and mixers; although short response time (120 ms) makes it promising, large valve diameter (900 μm) limits the scalability [13].

Another alternative valve technology uses an electrostatically operated PDMS membrane with metallic flexures, which can be driven by CMOS integrated circuits at a voltage of 15–20 V and frequency of 5 MHz [10]. The size of these valves was around 200 μm, permitting large scale integration. SMA actuation, too, simultaneously offers fast response time and small valve dimensions and also can be assembled directly on printed circuit boards [3].

Figure 1
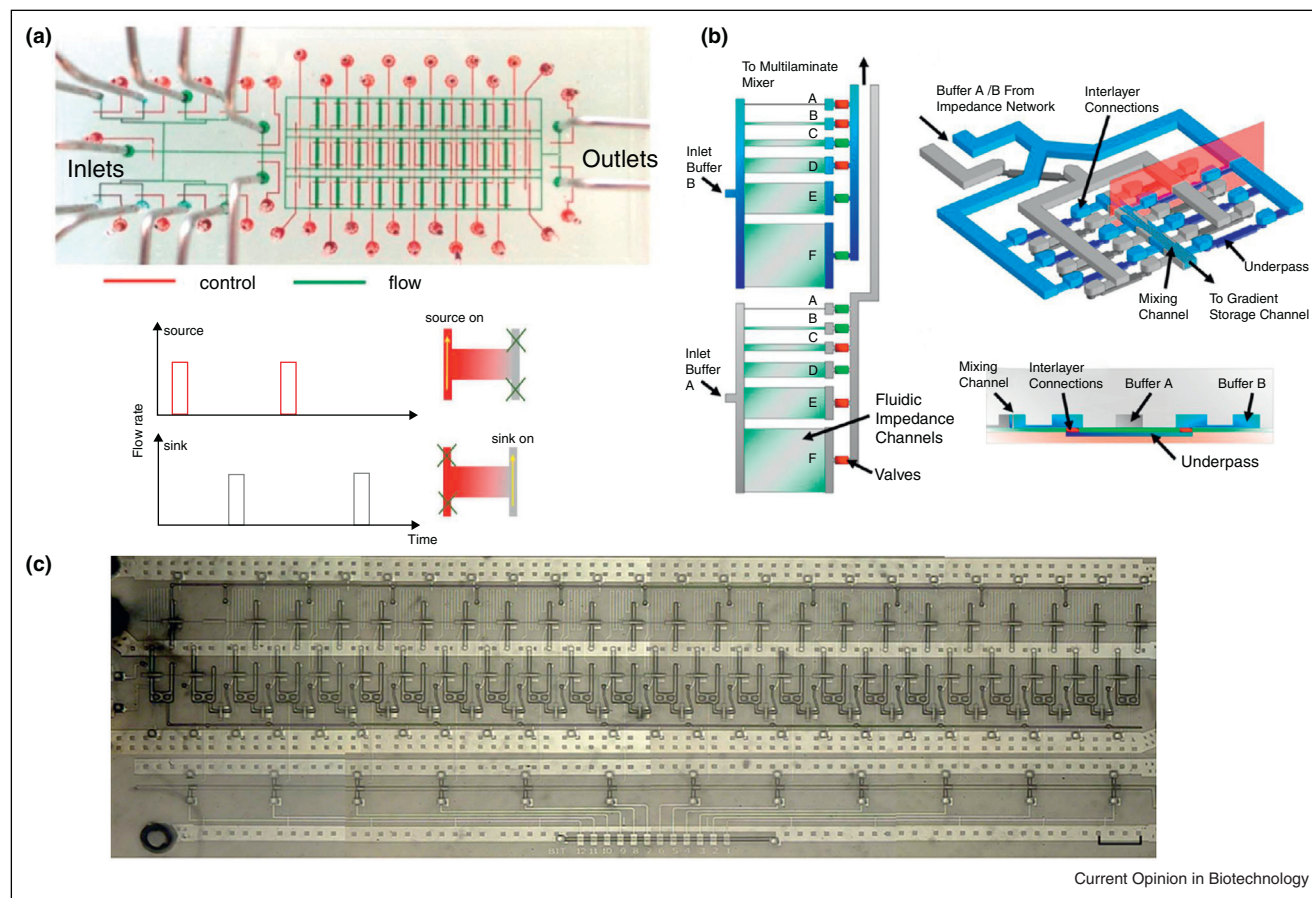
(a) Picture of the 30-plex chip for long term gradient generation (top) and the description of flow-switching which provides the stable gradient under no-flow condition (bottom), reproduced by permission [24]. **(b)** Schematic of the gradient selector (left), multilaminate mixer (top, right) and the cross section of the multilaminate mixer (bottom, right), reproduced by permission [23]. **(c)** Picture of a 12-bit fluidic shift register, reproduced by permission [16].

One limitation of SMA actuation is its low pressure resistance (5–15 psi); moreover, both electrostatic and SMA actuation are incompatible with MSL and require complex fabrication techniques.

Alongside the search for better materials and simpler actuation technologies, new mLSI components have been introduced using standard materials and techniques. Improved designs for *fundamental components* include normally closed valves (NCVs) [16••,17], structured valves [18], cell traps [19], and capacitors and diodes (Figure 1a) [20,21]; *higher-level components* that have been introduced include a chamber array [22], a high performance separation column [23••], a gradient selector (Figure 1b) [23••], a long term gradient generator [24•], a 2D spatial gradient controller [25], an agar filled chamber [26] and a mutilaminate mixer [27].

NCVs made of hybrid glass/PDMS materials [28] have been used for automated and quantitative single cell gene

expression analysis [29,30], but are millimeters in size, rendering them non-scalable. NCVs introduced by Devaraju *et al*. [16••] achieve scalability, but require a flash curable polymer; this technology was used for building static gain valves and consequently for realizing microfluidic logic circuits. Monolithic PDMS NCVs can also be fabricated through selective deactivation of oxidized PDMS, and have been demonstrated for large scale integration [17]. Structured valves incorporate topographical features on the roof of a standard valve, which augment standard valves with additional functionalities, such as surface patterning or trapping [18].

High-level components comprise multiple valves or chambers to realize a specific function. For example, agar filled chambers for cell culturing [26] and high-performance separation columns for chromatography [23••] require viscous liquids to fill in small microchambers. Such operations are realized using a series of valves that fill PDMS chambers with appropriate materials, and are

then closed permanently for sealing. Likewise, valves placed in various configurations can generate temporal [24•] or spatial control [25] over concentration gradients, while mutilaminate mixers provide fast and efficient mixing using underpass channels (Figure 1b) [27].

As fluid volumes decrease, evaporation becomes a challenge, requiring hydration elements [31]. When chip complexity increases, on-chip nozzles [32•] can assist with product recovery. Such mLSI components play an important supporting role in larger and more comprehensive architectures.

Architectural developments

The term architecture is interchangeably used to define the chip cross-section or circuit design. Architectural improvement strategies focus on increasing *chip density*, decreasing the *reliance on external controllers* and designing *programmable chips* to reduce process time and complexity.

Chip density can be increased either through component miniaturization or the introduction of additional layers. The Quake research group at Stanford University has recently demonstrated integration of 1 million control elements onto a single chip is possible through microfluidic very large scale integration (mVLSI) [33•]. mVLSI has the potential to realize high dynamic range and reconfigurable chips for digital-PCR, digital-MDA or digital-ELISA applications.

It is possible to integrate more functionality into a fixed-area device by increasing the number of layers [23••,34••,35]. Inter-layer connections can be realized through lithographical methods [34••,35] or laser ablation [27]. Lithographical methods are simpler than laser ablation, but require fine-tuning of fabrication conditions when the device geometry changes.

Reducing the *dependency on external pneumatic controllers* can reduce the complexity of the chip-to-world interface for mLSI; this was the subject of two recent review papers [36,37]. Microfluidic logic circuits can eliminate the requirement of one external controller for each independently controlled valve set by utilizing a fluidic shift register [16••,35,38]. Researchers at Fluidigm recently demonstrated microfluidic NOT, NAND and NOR gates, flip-flops, oscillators, a self-driven pump and a 12-bit fluidic shift register (Figure 1c) [16••].

A pressure-based serial digital to analog converter (DAC) [20] can eliminate the need for multiple pressure sources by generating arbitrary pressure values. Similarly, a frequency dependent flow control circuit can control several fluidic pathways with a single AC input. Leslie *et al.* demonstrated such a device which can select between

two fluids depending on the frequency of a time modulated pressure source (0.2 Hz or 7 Hz) [21].

For commercialization and mass production of mLSI, it is attractive to use a single software-*programmable chip* to execute any desired protocol. One such device was first developed by Jensen *et al.* [29] and recently improved to handle continuous processes with a greater number of inputs [39]. However, the larger hybrid PDMS/glass valve technology differentiates itself from mLSI and is more suitable for microliter scale fluid volume processing (the chamber volume is 120 nL); moreover, this technology requires one external valve per chamber, and it seems unlikely that any reduction is possible. Fidalgo *et al.* demonstrated an mLSI fluidic processor which has a 300-pL chamber volume, and uses 15 external valves to control 64 chambers [34••]. The device demonstrated electrofluidic display, surface immunoassay, microformulation and cell culturing capabilities. Another recent programmable chip combines droplets and flow-based microfluidics using 95 individually addressable nL-volume storage chambers with pL-volume droplets; water immiscible oil is used as a carrier fluid, and the device supports 8 different reagents through a specialized metering module [32•]. The innovative introduction of bypass channels at the entrance of each chamber prevented undesirable droplet wetting, which eliminated cross-contamination. This device was successfully used for single cell sorting, culturing, genotyping and whole genome amplification (WGA) experiments. Specifically, environmental samples are used to explore genomic relationships within natural microbial communities.
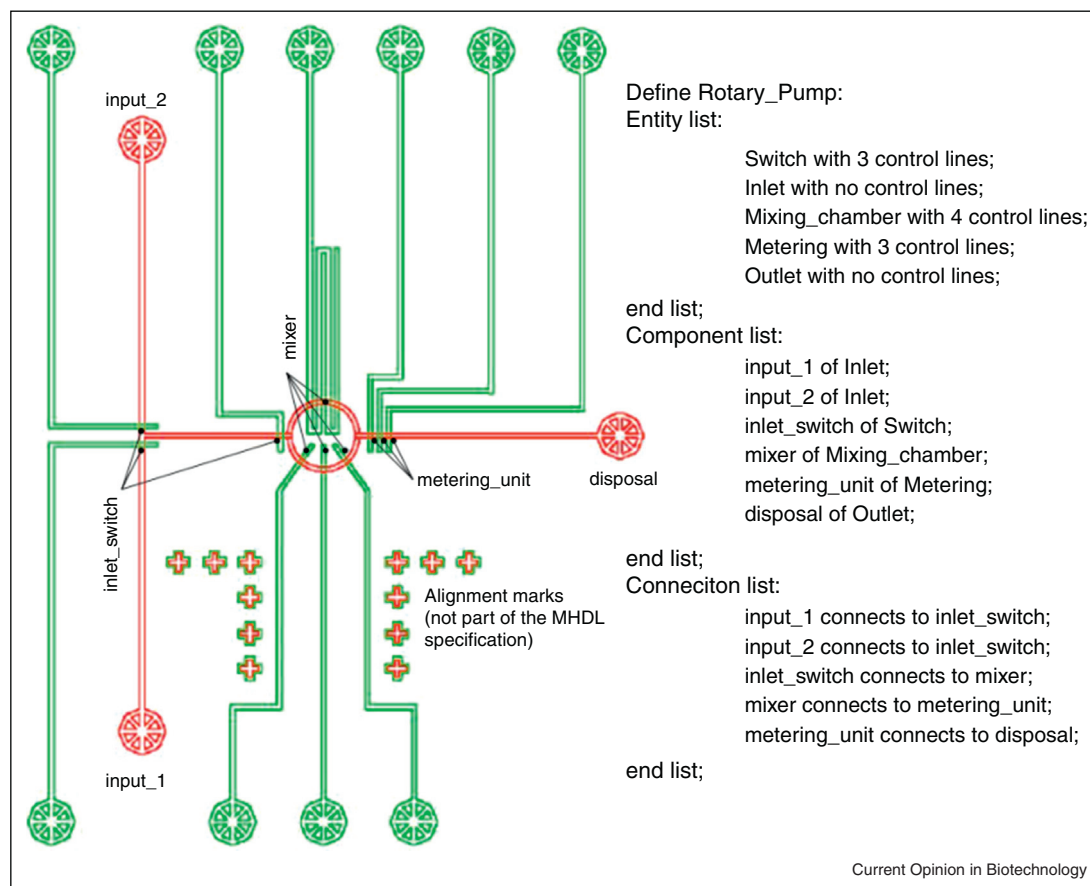
Automated mLSI chip design and layout

Starting in the early 1980s, the semiconductor industry pioneered computer-aided design of semiconductor technology, otherwise known as electronic design automation (EDA) [40]. There have been recent academic efforts to develop EDA software for mLSI chips. These research efforts are still early-stage, and have not yet produced working software tools. Nonetheless, the potential for increased mLSI designer productivity is readily evident, and user-friendly mLSI EDA software will hopefully be released within the next 1–2 years.

The first relevant issue is how to specify the architecture of a mLSI chip; *hardware design languages (HDLs)* such as *VHDL* and *Verilog* have been semiconductor industry standards for decades; no such standard exists for mLSI chips. One recent academic proposal is the *Microfluidic Hardware Design Language (MHDL)* [41], which borrows much of its syntax from VHDL. An MHDL specification of an mLSI chip includes a list of components (e.g. valves, mixers, etc.) followed by a list of fluidic interconnections between components, which will become microchannels once the device is physically laid out. An example can be seen in Figure 2.

Figure 2

Define Rotary_Pump:
Entity list:

    Switch with 3 control lines;
    Inlet with no control lines;
    Mixing_chamber with 4 control lines;
    Metering with 3 control lines;
    Outlet with no control lines;

end list;
Component list:

    input_1 of Inlet;
    input_2 of Inlet;
    inlet_switch of Switch;
    mixer of Mixing_chamber;
    metering_unit of Metering;
    disposal of Outlet;

end list;
Conneciton list:

    input_1 connects to inlet_switch;
    input_2 connects to inlet_switch;
    inlet_switch connects to mixer;
    mixer connects to metering_unit;
    metering_unit connects to disposal;

end list;

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An AutoCAD layout of a simple mLSI chip (left), and an MHDL description of the same chip (right).

The second issue is how to physically lay out the mLSI chip, starting either from an MHDL (or equivalent) specification of the architecture, or alternatively, from a higher-level specification of a protocol for which the chip will be designed. Several academic research groups have investigated different aspects of these issues.
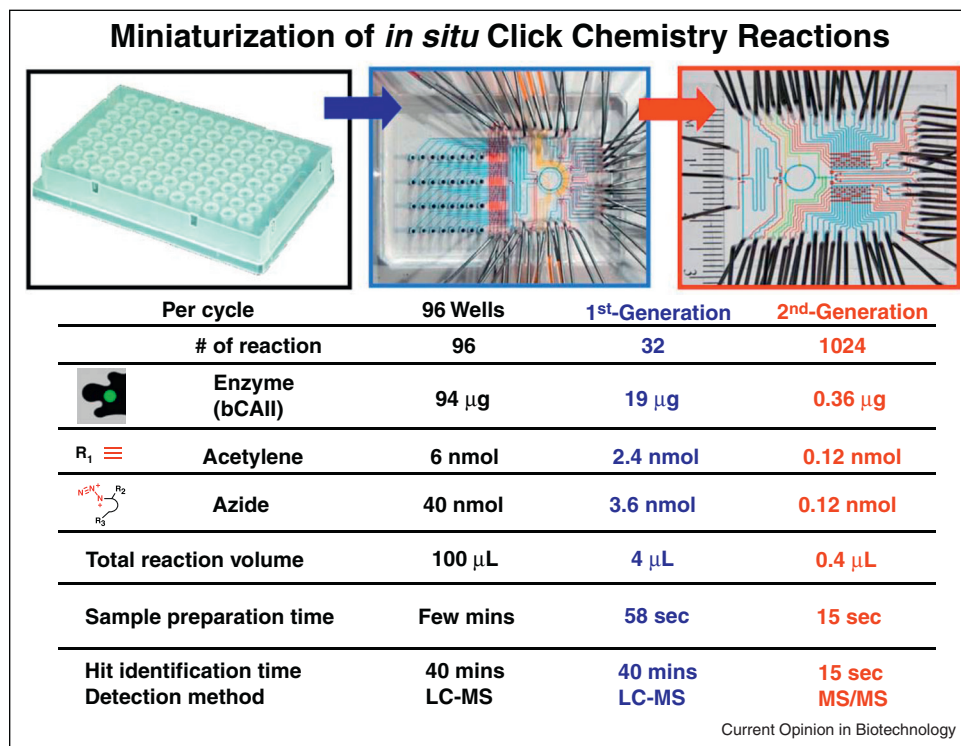
The first academic effort was Micado [42•], an AutoCAD plug-in that generates the control layer of an mLSI device. Given a physical layout of the flow layer and a description of the fluid transfers to perform, Micado automatically generates the control layer by first, placing valves; second, determining independently activated subsets of valves that can share a control line; and third, generating the control layer routing network, which connects each control input to the valves that it controls. Although it performs useful optimizations, Micado falls short of being a complete end-to-end solution for the design and layout of mLSI chips.

A second effort is an ongoing research project carried out at the Technical University of Denmark [43–46], with supporting efforts from researchers in Taiwan and Japan [47–49], which provides an algorithmic skeleton of an end-to-end design flow for mLSI chips. Starting from a high-level description of a biological protocol, algorithms are presented to: first, derive a graph-based representation of an mLSI chip that can execute the protocol [45,47–49]; second, automatically synthesize the protocol onto a graph-based representation of an mLSI chip [43,44]; third, physically place components in the flow layer, and route control channels between them [45]; and fourth, automatically generate the control layer [46]. Although complete in principle, this work has not yet been used to design a real-world mLSI chip that has been fabricated.

These research efforts have demonstrated the feasibility of the development of EDA tools for mLSI technology. The next step will be to produce a working tool that can increase mLSI designer productivity. In the long-term, the objective of such an effort would be to empower mLSI designer to create fully integrated chips whose complexity is several orders of magnitude beyond what

Figure 3

Miniaturization of *in situ* Click Chemistry Reactions

Per cycle	96 Wells	1st-Generation	2nd-Generation
# of reaction	96	32	1024
Enzyme (bCAII)	94 µg	19 µg	0.36 µg
Acetylene	6 nmol	2.4 nmol	0.12 nmol
Azide	40 nmol	3.6 nmol	0.12 nmol
Total reaction volume	100 µL	4 µL	0.4 µL
Sample preparation time	Few mins	58 sec	15 sec
Hit identification time Detection method	40 mins LC-MS	40 mins LC-MS	15 sec MS/MS

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Performance comparison between the conventional 96-well approach and the two generations of click-chemistry screening platforms, reproduced by permission [62].

is feasible using the manual design methodologies that are prevalent today.

Biological and chemical applications

This chapter summarizes recent applications of mLSI components and technologies that have enabled scientists to develop a deeper understanding of a variety of chemical and biological phenomena. mLSI is particularly well suited for single cell analysis and manipulation; several excellent review articles have already summarized recent studies [50–52], which generally focus on single cell imaging [24•,26,31,32•,53–55] or single cell genomic/proteomic analysis [19,30,56•,57•,58,59].

An mLSI chip developed by Taylor et al. can perform 256 simultaneous time-lapse imaging experiments in 2048 single yeast cell traps for 32 different conditions [55]; however, in order to track cells over longer time scales, large chamber volumes and immobilization of cells under perfusion are required. In order to solve the immobilization problem, Lecault et al. used inverted chip geometry to image mammalian cells in 1600, 4 nL-volume chambers for over 60 hours [31]. In a more recent study, Frank et al. demonstrated generation and maintenance of thirty parallel gradients (ten independent chemical formulations in three repetitions) for 14-hours under no-flow conditions; a high-throughput, diffusion based gradient is

realized by temporal control provided by the on-chip valves. The chip is used to observe NFkB nuclear localization events in single mammalian cells for varying signaling molecule concentrations. The device enabled measurement of cell secretion effects at low concentrations [24•]. In another example, Falconnet et al. tracked yeast cells for multiple generations using a chip with polymerized agarose gel in 128 chambers for yeast cell immobilization, which allows tracking of 8 different genotypes under 16 different conditions [26].

For single cell genomic analysis, sorting, trapping, and lysing of the cell are typical mLSI chip functions. In a 2010 study, Fan et al. identified and sorted metaphase cells, released the chromosome by protein digestion of cytoplasm, and then randomly separated them in 48 chambers and amplified homologous copies of each chromosome from a single cell, following which the product is retrieved for genotyping [56•]. Whole genome haplotyping of single human metaphase cell as described in this study has applications in personal genomics.

Single cell protein secretion is another application of mLSI technology. Ma et al. realized multiplexed (>10 proteins) and quantitative measurement of secreted proteins from single cells [57•]. Immunosandwich assays of spatially encoded antibody barcodes in 1040,

3 nL-volume chambers are used for concentration measurements and multiplexing. A full barcode was representing a complete panel of protein assays and duplicate barcodes enabled statistics at the single cell level. The mLSI chip is used to assay the polyfunctionality of tumor antigen-specific T cells in the setting of an adoptive cell transfer therapy clinical trial for melanoma.

mLSI technology can also enable the study of microenvironmental interactions of cell populations. Liu *et al.* used a chip with 4 chambers connected by optional communication channels for monitoring cell-cell interactions. This experiment demonstrated the passive role but collaborative response of stromal cells while responding to signals from carcinoma cells [60].

Another field of application of mLSI is protein synthesis and characterization. In addition to high throughput, when married to DNA-to-protein arrays, mLSI has enabled multiplexed protein expression and utilization of advanced detection technologies, as summarized in the recent review by Maerkl on protein biochemistry [61].

Two recent examples of chemical applications are solid phase liquid chromatography [23••] and *in situ* screening of click-chemistry [62]. Huft *et al.* integrated high performance separation columns with on chip sample loading, gradient generation, detection and sample recovery. The chip allows packing of resin slurry in 10 min under 30 psi pressure. This technology is promising for sequencing library preparation, cloning and sample fractionation for diagnostics. Wang *et al.* performed 1024 *in situ* click chemistry reactions in a proof of concept study. This second generation platform provides 20–50-fold sample economy when compared to 96-well plates. Figure 3 summarizes the advantages of mLSI for click-chemistry screening.

In conclusion, the high throughput and sample economy advantages of mLSI technology are highly attractive for single cell imaging and genomics, protein biochemistry and chemistry studies. Innovative architectural developments with the existing material, actuation combination and by using manual design strategies produced rapid results and enabled several noteworthy applications. In the meantime, researchers continue with advancements in material and actuation aspects for widespread and easier use of mLSI. Although promising, the implications of these advancements in real-world applications are yet to come.

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