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Digitally programmable microfluidic automaton for multiscale combinatorial mixing and sample processing†

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Abstract

A digitally programmable microfluidic Automaton consisting of a 2-dimensional array of pneumatically actuated microvalves is programmed to perform new multiscale mixing and sample processing operations. Large (μL -scale) volume processing operations are enabled by precise metering of multiple reagents within individual nL-scale valves followed by serial repetitive transfer to programmed locations in the array. A novel process exploiting new combining valve concepts is developed for continuous rapid and complete mixing of reagents in less than 800 ms. Mixing, transfer, storage, and rinsing operations are implemented combinatorially to achieve complex assay automation protocols. The practical utility of this technology is demonstrated by performing automated serial dilution for quantitative analysis as well as the first demonstration of on-chip fluorescent derivatization of biomarker targets (carboxylic acids) for microchip capillary electrophoresis on the Mars Organic Analyzer. A language is developed to describe how unit operations are combined to form a microfluidic program. Finally, this technology is used to develop a novel microfluidic 6-sample processor for combinatorial mixing of large sets ($>2^6$ unique combinations) of reagents. The digitally programmable microfluidic Automaton is a versatile programmable sample processor for a wide range of process volumes, for multiple samples, and for different types of analyses.

Introduction

Programmable microfluidic systems offer the unique ability to automate biomolecular assays on a common microchip format. A wide range of mechanisms have been employed for transporting and mixing nanoliter scale reagent volumes in microfluidic devices including droplet generation,¹ electrowetting,² and a variety of microvalve and pump technologies.^{3,4} Droplet mixing and splitting operations have been used to automate diverse assays including glucose determination⁵ and enzyme kinetic analysis.⁶ However, with some exceptions,⁷ these systems operate at the nanoliter scale, and can suffer from significant imprecision, limiting quantitation.⁸ Multilayer soft-lithography has been used to develop large reactor arrays for parallelized bioanalysis and combinatorial mixing of reagents.^{4,9,10} Although these systems can address a large number of reactors in parallel, they lack

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programmable sample processing capabilities for large volume samples. The monolithic membrane valves developed by our group¹¹ have enabled automation of assay protocols including pathogen detection,¹² DNA sequencing,¹³ and single nucleotide polymorphism detection.¹⁴ While these systems offer miniaturized sample processing capabilities, each system performs only limited sets of sample processing operations and significant design modifications are required for each new application. In analogy to electronic computing, these devices are similar to conventional hand held calculators - optimized for a few specific operations. A truly versatile, programmable microfluidic sample processing platform, analogous to a programmable computer, should provide facile automation of assays and also accommodate a wide range of volumes, the ability to actively control reagent processing, and convenient modular assembly of unit operations.

The ability to process large (μL scale) sample volumes is critical for many types of molecular diagnostic assays that require detection of low titer targets. For instance, HIV viral load detection typically requires several hundred μL sample volumes to achieve appropriate detection limits.¹⁵ The standard approach to the automation of these assays utilizes slow sample handling robots that are expensive and occupy large amounts of space. Alternatively, microfluidic systems have been developed to process larger sample volumes and trap target molecules or cells within microchambers using capture probe labeled surfaces,^{16,17} microspheres,¹⁸ gels,¹⁹ porous monoliths,²⁰ or other solid substrates.²¹ However, these systems lack the programmable sample processing capabilities necessary to conveniently automate diverse assay protocols on a single device. There is therefore a need for a programmable microfluidic platform that can automate sample processing operations for volume scales ranging from nanoliters to milliliters.

Due to the laminar flow profiles in microfluidic channels, sample and solution mixing is still one of the major challenges in microfluidic lab-on-a-chip applications. Microfluidic devices typically operate in the Stokes laminar flow regime so mixing only occurs *via* diffusion unless a mechanism to perturb the laminar flow profiles is employed. Microfluidic devices for combinatorially mixing streams of reagents have been developed using passive mixing elements.^{22,23} Although these systems can process large sample volumes, they are single-purpose and lack programmability. Active mixing has been demonstrated using microvalves to pump different reagents through a loop structure^{24,25} and by delivering solutions to a common reservoir followed by transfer to a second reservoir for active mixing.²⁶ These structures have been used to perform combinatorial mixing operations,⁹ but they lack large volume processing capabilities within short time-scales and programmable adjustable control of mixing proportions in a single architecture.

Programmable microfluidic systems should enable the performance of diverse sample processing operations on a single device. Modular microfluidic devices have been developed in which functional components can be rearranged to achieve different processing operations for μL -scale sample volumes.²⁵ However, the modifications required are labor intensive, and the devices do not permit program changes in real time. Grover *et al.* demonstrated a microfluidic device capable of replacing a conventional autosampler system.^{27,28} This device utilized a network of bus valves to select from a set of input reagents for continuous transfer to a suspended microchannel resonance sensor. While rapid reagent switching frequencies were achieved with this device, higher dimensionality versions of this sample processor are necessary to achieve a broad range of assay automation.

We previously introduced a digitally programmable microfluidic Automaton capable of automating assay protocols using nanoliter scale sample volumes.^{29,30} This system utilized an 8×8 rectilinear array of pneumatically actuated monolithic membrane microvalves. Basic operations were achieved by digital transfer of fluid between microvalves in the array.

Simple programs for reagent routing, mixing, rinsing, serial dilution, and storage/retrieval were developed enabling assay automation at the nL scale. However, this device was not capable of processing or actively mixing larger sample volumes, programs were not developed and demonstrated for continuous processing operations, nor was an approach for combinatorial operations on larger sets of inputs (>3) devised.

Here we present a novel version and use of the digitally programmable microfluidic Automaton that enables continuous-stream combinatorial processing of nanoliter to milliliter scale sample volumes. With this system, multiple reagents can be digitally mixed using novel combining valve operations to generate a continuous output of processed sample. Sample storage operations utilizing external holding reservoirs are developed to enable serial processing operations for μL scale samples. The digitally programmable microfluidic array transports reagents between the holding reservoirs and can simultaneously mix reagents during the process. We present the basic rules for assay automation using this system and the development of an effective language to describe the automated protocols. The utility of this system for serial dilution of large volume samples and for the automation of carboxylic acid sample labeling for capillary electrophoresis with laser-induced fluorescence detection is demonstrated. Finally, the extension of this technology to a novel 6-input combining valve that enables combinatorial μL scale mixing operations for large sets of reagents is demonstrated.

Materials and methods

Automaton fabrication and design

The digitally programmable microfluidic Automaton was fabricated as a 3-layer glass PDMS (polydimethylsiloxane) hybrid structure as previously described.²⁹ The pneumatically actuated 8×8 array of 4-way microvalves incorporates a rectilinear array of fluidic channels that enable discrete transfer of fluids between adjacent valves. Microvalves are actuated by vacuum (-87 kPa) and closed with an adjustable pressure applied through drilled inputs on the pneumatic layer *via* computer actuated solenoid valves. Twenty-four drilled fluidic reservoirs on the perimeter of the array serve as either inputs or outputs and are connected by microchannels to the microvalves on the perimeter. To increase the volume of the reservoirs, holes were punched into 3 mm thick PDMS pieces and aligned with the drilled inlets. When automated access to off-chip reservoirs was desired, PEEK tubing was threaded into a tight-fitting hole in a PDMS gasket and aligned with the drilled inlets. Fluid flow through the PEEK tubing was achieved *via* pumping programs using microvalves of the digitally programmable microfluidic Automaton. The Automaton device used here had fluidic features etched to a depth of 30 microns and pneumatic features etched to a depth of 70 microns.

A cross-sectional view of a microvalve array is presented in Fig. 1A. Each monolithic membrane microvalve consists of an etched displacement chamber in one glass wafer and a discontinuous fluidic channel structure in a second glass wafer. The wafers are reversibly bonded together using a featureless $254 \mu\text{m}$ thick PDMS elastomer membrane (HT-6240, Rogers Corp.). Application of a vacuum to the displacement chamber through a control channel pulls the PDMS membrane away from the discontinuity, allowing fluid to fill the chamber and/or flow across the discontinuity in the fluid channel. The maximum volume contained by a microvalve in the array is 120 nL, corresponding to 84% of the volume of the pneumatic displacement chamber.²⁹

Multiscale sample processing operations

Fig. 1A illustrates the mechanism of fluidic transfer within the Automaton. The basic program for the transfer of fluids between microvalves in a rectilinear array begins with a single open microvalve filled with fluid. An adjacent microvalve is opened, drawing fluid from the first valve. The first valve is then closed with an applied pneumatic pressure, forcing the remainder of the fluid into the second valve. A 120 nL bolus of fluid is thus transferred between adjacent microvalves. Reagents can be transferred from any input reservoir to any cell or output reservoir in the array, and repeated cycles enable the transfer of adjustable volumes between reservoirs.

Combining operations are performed by precisely metering multiple reagents into a single microvalve or set of adjacent microvalves in the array. This is achieved by opening a combining valve connected by open pathways to multiple inlets or filled adjacent valves. Fig. 1B illustrates a program using a combining valve operation. In this example, reagents from reservoirs *c1* and *d2* are directed into combining valve C2 and then digitally transferred to output *b1*. The microvalves used to load the combining valve are closed prior to the transfer of the contents of the combining valve to a new location. A complete description of this program is included in the ESI.†

Fig. 1C illustrates the language developed to efficiently describe on-chip autonomous fluidic operations. Valves are indicated by their position in matrix format, with columns designated by numbers and rows designated by letters. Reservoir inputs are labeled with the lowercase letter in italics according to the valve they are attached to. For example, an input reservoir to valve D3 is denoted *d3*. The basic operations used to automate assays are denoted by the following operators: C = combine, T = transfer, and F = flutter. The transfer operator indicates the locations between which fluid is being transferred, with the input location listed first, followed by the output location. The combining operator indicates the input locations followed by the valves in which the combining operation occurs. Both types of operations are repeated the number of times indicated after the square brackets that enclose the operation. The total volume of sample processed is adjustable based on this parameter. Fluttering operations are used to enhance mixing speed and efficiency. This is defined as repeated opening and closing of a microvalve in a transfer pathway with 50 ms actuation times. The flutter operator defines the specific valve that is fluttered and is performed continuously throughout a program. Descriptions of all operations presented in this work using this programming language will be found in the ESI.†

To characterize the operations of combining valves and complex serial processing operations including standard curve generation, fluorescent dyes including fluorescein, ROX, and Pacific Blue succinimidyl ester were loaded in different configurations on the Automaton. These dyes served as inputs to combining valves and were programmably transferred to an output reservoir. Actuation times of 250 ms were utilized for these programs unless otherwise indicated with a closing pressure of 50 kPa. Dilution factors and the proportions of reagents loaded into combining valves were determined by transferring samples from the outlet to a fluorescence plate reader (Flx800, Bio-Tek Instruments Inc.) and quantified using a standard curve. Back contamination of the inlet reservoirs was also tested by analysis of buffer in the inlets reservoirs with a fluorescence plate reader. Epifluorescence images were acquired with a Nikon Eclipse E800 using a 2× objective, 0.06 aperture and an output power at the excitation band of 4 mW.

Carboxylic acid sample labeling for capillary electrophoresis

To demonstrate the utility of the Automaton for reaction chemistry, specifically on-chip fluorescent derivatization, we designed a protocol for the automation of a previously

developed carboxylic acid sample labeling process for capillary electrophoresis.³¹ Cascade Blue hydrazide (CB, Invitrogen) was dissolved in triply-distilled Millipore water to 10 mM. EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, Sigma-Aldrich) was dissolved to 20 mM in triply-distilled acetonitrile (MeCN, Sigma-Aldrich), divided into 100 μ L aliquots, evaporated to residue, and stored at -20°C until rehydration to the appropriate concentration in 30 mM borate, pH 3. Carboxylic acid labeling was conducted by combining a carboxylic acid standard (200 μ M formic acid, 400 μ M each acetic, propanoic, butanoic, pentanoic, hexanoic and heptanoic acids, and 600 μ M octanoic acid, Sigma Aldrich) with 4 mM CB and 10 mM EDC, all in 30 mM borate, pH 3, in a 1 : 1 : 1 ratio. In the manual control process, after a 15 min incubation, the reaction mixture was diluted 1 : 3 with 30 mM borate, pH 9.5 for analysis.

An on-chip automated program was also developed to 1) mix EDC, CB, and standard using combining valve technology, 2) transfer the mixture to an output for a 15 min incubation, 3) dilute the mixture 1 : 3 with 30 mM borate, pH 9.5 and 4) transfer the processed sample to a second output for analysis. All samples were analyzed by transferring 30 μ L processed sample directly to the inlet reservoir on the Mars Organic Analyzer (MOA), a portable μ CE analysis system described in detail elsewhere,^{32–36} *via* a PEEK tubing interface. It should be noted here that the reprogrammability of the Automaton microdevice and the facile PEEK tubing interface enabled the production of results within 1 h following conception of the experimental plan.

Six-sample combinatorial processor

To extend the combining valve concept to larger sets of inputs, a 6-sample combinatorial processor was fabricated using a 3-layer glass-PDMS (polydimethylsiloxane)-glass hybrid structure (Fig. 2). Etch depths were identical to those of the Automaton. In this design, a central 2 mm diameter microvalve is radially connected by microchannels to 7 check valves controlling corresponding inlet reservoirs. An additional channel extends to a series of 2 mm mixing valves. The inlet across from and in line with the mixing valves contains buffer for rinsing cycles. The contents of any subset of the 6 remaining inlets can be loaded into the combining valve by opening corresponding subsets of check valves during operation. A program was developed to cycle through all possible (64) input sets with a rinse cycle between input states. For both the automaton and the 6-sample combinatorial processor, rinsing is achieved by simply transferring buffer through the valves in the network to a waste output for one minute or less to remove residual sample. The selected inputs are indicated by a binary code. For instance, the selection of inputs 1, 3, and 5 is indicated by the string 101010. Sample loading and transfer operations were performed with a 250 ms valve actuation time, and a closing pressure of 50 kPa.

Results

Combining operations can be used to precisely meter multiple reagents to an individual microvalve. To explore the capabilities of combining valve operations, programs were run to combine two reagents from parallel inputs (Fig. 3A), two reagents from orthogonal inputs (Fig. 3B), and three reagents (Fig. 3C). Epifluorescence images were acquired immediately after opening the combining valve to determine the overall flow profile of the inputs as the combining valve is filled. To determine the proportions of the inputs loaded into the combining valves, the programs illustrated in Fig. 3 were performed using fluorescein dye and buffer as inputs. Outputs were quantified using a 96-well fluorescence reader, and errors were determined by performing multiple runs. The proportions for two inputs at 180° are $50.3 \pm 0.8\%$ Input *d2*, and $49.7 \pm 0.8\%$ Input *d4*. The proportions for two inputs at 90° are $50.6 \pm 0.8\%$ Input *d2*, and $49.4 \pm 0.8\%$ Input *c1*. The proportions loaded in the case of three reagents are $33.4 \pm 0.8\%$ Input *c1*, $37.9 \pm 0.5\%$ Input *d2*, and $28.7 \pm 0.5\%$ Input *d4*. The

differences in proportions of the three inputs are due to different fluidic resistances in pathways from the inlets to the combining valve. These mixing proportions are highly repeatable (1–2% relative error) for a given combining valve operation, but the proportion must be characterized for each combining-valve location on the device for high accuracy assays.

The rate at which processed sample is generated depends on the volume of microvalve displacement chambers, the valve actuation rate, the number of microvalve volumes transferred per cycle, and the fluidic resistance of microchannel connections. While smaller microchannels reduce the dead volume between microvalves, they increase the time necessary for complete transfer of the contents from one microvalve to the next. The rate at which sample is processed can be increased by decreasing the actuation times of the microvalves until incomplete membrane deflection impedes transfer of fluid. With valve actuation times as low as 50 ms, the proportions of reagents loaded into a combining valve are not significantly affected by the processing speed.

The contents of a combining valve are partially mixed as they are transferred through a series of microvalves to an output. The mixing efficiency can be significantly enhanced by continuously fluttering one of the microvalves (with 50 ms actuation times) in the transfer pathway from the combining valve to the output. To visually evaluate the mixing efficiency, 10 μ M fluorescein and 10 μ M ROX were loaded into *d4* and *d2*, respectively, and a program was run (Program 1 in Fig. 4 and in the ESI†).

Fig. 4B presents epifluorescence images of the dye solutions as they are loaded into combining valve C3, and then transferred between microvalves to output *a1* in a single cycle of the program. Program 1 results in complete mixing of the two dyes by the time they reach A1. Program 2, which did not include fluttering, results in significantly less mixing of the two dyes in each cycle of the program, as illustrated by distinct green and red fluorescence regions in the valve A1 and A2 images. Microvalve fluttering is therefore an effective approach to enhancing mixing speed and efficiency for large volume processing operations on the Automaton.

To quantify the efficiency of mixing, Program 1 was performed using 10 μ M fluorescein and 1 $\times$ TTE buffer pH 8.3 as inputs. Fluorescence line profiles were acquired across valve A1 intersecting the direction of flow when fully opened (Fig. 4C). The first moment for each profile was calculated to measure the symmetry of the distribution of the fluorescence across the microvalve. Complete mixing is indicated by a distribution centered at 50% of the microvalve width. The mixing time was defined as the time required for complete transfer of the reagents from microvalve B3 to A1, and was adjusted by varying the microvalve actuation time. With an 800 ms mixing time, the fluorescence distribution center ($48 \pm 1\%$ microvalve width) was identical within the error to that of a mixture prepared off chip and run through the same program ($49 \pm 1\%$ microvalve width). The fluttering step included in Program 1 therefore results in complete mixing of reagents in less than 800 ms for each cycle of the program.

The speed of mixing for this program compares quite favorably to other microfluidic mixing approaches. For instance, we previously demonstrated a program to cyclically transfer two reagents within a loop of four microvalves in the array, requiring 2.5 s to achieve complete mixing.²⁹ Traditional microfluidic loop mixers require even longer times (<10 s) to achieve complete mixing.²⁴ Actively mixing solutions by transfer between fluidic reservoirs can take minutes.²⁶ Our approach to rapid, on-chip mixing may be useful for a broad range of applications that require complete mixing of reagents on-chip on either the nanofluidic (nL) or microfluidic (μ L) volume scales.

Multiscale serial dilution program

Since the output of a step in a fluidic program can be used as an input for a subsequent program step, multiscale serial processing operations can be performed on the Automaton. We previously demonstrated nanoliter scale, binary serial dilution in which the contents of a single microvalve are sequentially diluted with valve-based aliquots of buffer, resulting in nanoliter scale outputs.³⁰ The combining operations demonstrated here now enable arbitrary dilution factors with adjustable input and output volumes. As an example, we demonstrate the serial dilution of fluorescein to generate a 5-level standard curve (Fig. 5A) with adjustable output volumes. Fluorescein (10 μ M) and TE buffer (10 mM Tris, 1 mM EDTA) pH 8.3 were loaded into *c1* and *d2*, respectively. In the first step of the program, fluorescein and buffer are combined in both C2 and B2 for improved processing speed, and transferred to reservoir *b1* to produce *Out1*. Each subsequent standard curve level (Out2-Out4) was generated by combining one valve volume of fluorescein sample with one valve volume of buffer and transferring to the designated output. Program 2 (ESI⁺) was used to perform the overall process.

Six μ L of each standard level was generated with a total runtime of 7 min. Based on the predicted dilution factor of 2 for each level, a highly linear standard curve was generated ($R^2 = 0.996$). Each standard curve level was quantified using a standard curve prepared off-chip. The actual dilution factors produced were 2.27 ± 0.10 , 2.02 ± 0.11 , 1.96 ± 0.15 , and 1.93 ± 0.14 for levels Out1-Out4, respectively. The error was estimated based on four separate runs and was within the range previously reported for operations on the Automaton.²⁹ While the effect of variations in fluidic resistance must be quantified in some device configurations particularly for high-fidelity quantitative operations, variations are minimal when using this operational protocol on this device. Although each of the standard levels uses two microvalves for combining operations, the different input configurations and geometries affect the final proportions of the two inputs loaded. For instance, to produce Out1, buffer and dye are simultaneously loaded into C2, and then B2 is opened drawing fluid from the two input reservoirs. A larger volume of buffer is drawn into the combining valves due to the asymmetry of the pathways between the two inputs and B2, resulting in a dilution factor slightly greater than two. These differences are repeatable and can be quantified for assay calibration; further modeling of microvalve flow should enable precise prediction of these dilution factors. These results demonstrate the utility of our system for generating adjustable volumes of serially diluted sample for quantitative chemical and biochemical analysis.

Cascade Blue labeling of EDC-activated carboxylic acids

Cascade Blue labeling of carboxylic acids was performed with an automated program on the Automaton and analyzed on a μ CE microchip with the MOA. The Automaton program depicted in Fig. 6A combines CB from reservoir *c1*, carboxylic acid standard from reservoir *d2*, and EDC from reservoir *d4* in combining valve C2. The combined fluids are mixed by serial transfer *via* B2, A2, and A1 to the reservoir *a1* where it undergoes a 15 min incubation. After the 15 min incubation, the contents of reservoir *a1* are diluted by combining with pH 9 borate buffer in combining valves B3 and B2, and then transferred to reservoir *b1*. This program step is alternated with transfer of one microvalve volume of buffer to reservoir *b1* to achieve a dilution factor of 3. Program 3 (ESI⁺) was utilized to automate this process.

The assay was performed by transferring 30 μ L of the output of reservoir *b1* to the MOA CE microdevice and separated as described previously.³¹ An electropherogram of autonomous on-chip carboxylic acid labeling and dilution is compared to a fully manual protocol in Fig. 6B. The processing conditions were evaluated by comparing reaction efficiency as quantified by the peak areas and separation quality as quantified by the peak efficiencies.

The peak areas obtained by on-chip reaction are 40–50% larger than those obtained by a similar manual off-chip reaction with a run-to-run error of less than 5%. The reaction efficiency variation is likely due to a difference in mean reaction time. While the manual reaction was allowed to run for precisely 15 min, the automated reaction time varied from 15 min to 25 min because of the 10 min required to generate and transfer 30 μ l of labeled sample through the PEEK tubing. Since it has previously been demonstrated that EDC-activated CB labeling of carboxylic acids requires >8 h to proceed to completion,³¹ the observed peak areas are expected to increase with increased reaction times. The autonomously labeled samples exhibited only a 1% difference in peak efficiency compared to the manual process, which is within the normal run-to-run variation. This successful coupling of a versatile programmable sample processing Automaton having multiscale sample volume processing capability with a microchip CE analyzer is an important step toward the development of a fully integrated autonomous biomarker detection platform for space exploration based on the MOA.

Evaluation of 6-input combinatorial processor

A program was utilized to generate all possible combinations of reagents using the 6-input combinatorial processor. Fig. 7 illustrates combining valve operations for all possible permutations of 6 inputs dyes. For each case, all of the selected input dyes are precisely metered into a single combining valve. The proportions loaded for each input configuration were quantified by running the program described above with fluorescein dye and buffer as inputs and analyzing the outputs on a fluorescence plate reader (ESI†). In cases where only two inputs are selected, the results are similar to the two-input combining valve operations on the Automaton. Among these cases, the average dilution factor is 2.0 ± 0.2 . For larger sets of reagents, the proportions loaded are more highly dependent on the configuration of inputs. Further device characterization and potential modeling of the fluid flow within microvalves should enable precise prediction and calibration of the loading proportions. These results demonstrate that reagent sets larger than 4 can be controlled and combinatorially processed within a digital microvalve array by increasing the number of fluidic connections or inputs to the combining valves.

Discussion

The digitally programmable microfluidic Automaton presented here is a versatile platform for combinatorial sample processing operations and automation of diverse protocols that require sample volumes ranging from the nanoliter to microliter scale. The compact design (2.5 cm $\times$ 2.5 cm microvalve array footprint) of this microchip processor should enable significant miniaturization of laboratory automation robots that perform similar functions. Such systems typically require minimum sample volumes of a few microliters and the use of costly disposable pipettes tips. While acoustic droplet ejection systems have been developed for transfer of nanoliter to microliter scale sample volumes into microtiter plates,³⁷ they lack the serial processing capabilities demonstrated here. Our platform can both serially and precisely process sample volumes ranging from a few nanoliters to the milliliter range, without the requirement for expensive disposables.

The large volume serial processing operations of the digitally programmable microfluidic Automaton are enabled by the use of combining valve technology. No back contamination of reagents was observed during experiments performed herein. Combining valves are therefore a valuable alternative to bus valves, which have been used in previous work for contamination-free reagent delivery.^{14,38} The extension of combining valve technology to larger sets of inputs in the 6-sample combinatorial processor enables the integration of operations of greater complexity involving large reagent sets on smaller digital microvalve arrays. Further work will be required to characterize carry-over fractions when biological

samples including whole blood, serum, DNA, urine, *etc.* are processed. Additionally, the use of “stop” valves to control the input to the processing array from a single input may be required for full contamination-control when multiple samples and highly complex processing routines are utilized.

Design modifications can be implemented to improve processing speed when faster sample processing is necessary. The volume transferred between microvalves can be increased by modification of the diameter of the microvalves or the microvalve etch depths.¹¹ For instance, doubling the etch depth and diameter would result in microvalve displacement chamber volumes greater than 1 μL and processing speeds up to eight times faster than demonstrated here.

The proportions of reagents loaded into a combining valve are affected by up to 15% due to variations in the fluidic resistance between the combining valve and the geometrically different fluidic inputs. For instance, in the three input case (Fig. 3C), less fluid from *d4* was loaded in the combining valve compared to reagent from *c1*. This can be explained by the longer pathway and correspondingly higher resistance between input *d4* and the combining valve. This difference was highly repeatable (1–2% relative error), and can be quantitatively characterized to calibrate operational protocols for individual fluidic resistance variations. Additionally, modeling the fluidic resistance and flow profiles of these circuits should enable prediction of the reagent proportions resulting from combining operations and rebalancing through design or operational changes.

The novel ability to process large sample volumes on the digitally programmable microfluidic Automaton will enable the coupling of this system to a wide range of analytical testing devices. We previously demonstrated automated concentration of target biomolecules on derivatized magnetic microspheres using the Automaton.³⁰ This platform could therefore be coupled to particle detections systems such as flow cytometers. Grover *et al.* previously demonstrated the efficiency of coupling the autosampler device to downstream analysis systems using the PEEK tubing method.²⁶ In addition, PEEK tubing interconnections have been used for automated sample loading onto the Mars Organic Analyzer, both in previous work³³ and here. PEEK tubing interconnections between the drilled outputs of the Automaton and the inputs of other analytical devices will enable programmable processing and delivery of samples to a wide range of analytical systems such as mass spectrometers, surface plasmon resonance detectors, or DNA microarrays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A Fluidic Transfer

B Example

Operation

F(B2)
[C(c1,d2→C2)
T(C2 → b1) 1]100

C. Fluidic Program Language

Combining operations:	$C(x,y \rightarrow z)$	Combine inputs x and y within valve z .
Transfer operations:	$[T(x \rightarrow y) i]n$	Transfer input x to output y with i valve volumes per cycle. Repeat n times.
Flutter operations:	$F(x)$	Continuously flutter valve x .

Fig. 1.

(A) Cross sectional view of a microvalve array showing the steps for transfer of fluids between microvalves. (B) Schematic of a combining operation showing labeling of valve array and inputs. Inputs from wells $c1$ and $d2$ are drawn in *via* valves $C1$ and $D2$, combined in valve $C2$, and transferred to output well $b1$, with a total fluidic transfer of one valve volume per cycle. The cycle is then repeated 100 times. (C) Fluidic program language. Combining operations indicate inputs ($x,y...$) followed by the microvalve or microvalves where combining occurs (z). Transfer operations indicate the microvalve or storage well input (x) followed by the output (y), and the number of microvalve volumes transferred per cycle (i). Operations within brackets are repeated n times. Flutter operations are performed continuously and indicate the microvalve fluttered (x).

Fig. 2.

Six sample combinatorial processor. Fluidic inputs 1–6 can be precisely transferred to the combining valve and digitally transferred through a series of valves to an outlet for storage. Check valves at each fluidic input enable combinatorial selection of any subset of the reagents in a given program cycle, and prevent cross contamination of the reagents. Using this system, 2^n multiscale reagent combinations are enabled by $n + 4$ microvalves. The selected reagent sets can be represented as a binary code. For instance, selection of inlets 1, 3, and 6 is denoted by the binary string, 101001.

Fig. 3. Epifluorescence images of loaded combining valves and schematics of multiscale sample processing operations on the digitally programmable microfluidic Automaton. (A) Combining valve C3 is loaded with of two fluorescent dyes from parallel input channels. The contents of C3 are then digitally transferred between B3-A1 to a1 in this example. (B) Combining valves loaded with two reagents from orthogonal inputs, and (C) with fluorescent dyes from three different inputs. The contents of a combining valve can be transferred to any microvalve or output in the array.

Fig. 4.

(A) Illustration of the program for continuous digital mixing of two reagents. ROX and fluorescein dyes are drawn into combining valve C3 and then digitally transferred through valves B3-A1 to an output well. (B) Epifluorescence images of each step of two different mixing programs. Program 1 includes continuous fluttering (repeated opening and closing of A3 with 50 ms cycles) throughout the program, while Program 2 is run without fluttering. (C) Fluorescence line profiles obtained across valve A1 intersecting the direction of flow during the performance of Program 1 with fluorescein and pure buffer inputs. The vertical lines indicate the location of the first moment of the fluorescence distribution; a sample

mixed off chip results in a center at $49 \pm 1\%$ of the microvalve width. With a 200 ms valve actuation time, complete mixing occurs within 800 ms during transfer from B3 to A1.

Fig. 5. Multiscale serial dilution program implemented on the Automaton. (A) The first dilution is achieved by combining sample and buffer in two microvalves (dashed box). The contents of two microvalves are then transferred to Out1 at each cycle, resulting in faster generation of large volumes. Each subsequent standard concentration level (B–D) is generated by combining sample from the previous standard curve level and buffer in the two indicated microvalves. (E) Results of the standard curve program generating 6 μL of each concentration level within a total runtime of 7 min. Error is estimated based on four separate runs. An R^2 of 0.996 is achieved with a predicted dilution factor of 2 for each level.

Fig. 6.

(A) Schematic of the program for labeling carboxylic acids performed on the Automaton. Sample containing a standard set of carboxylic acids is loaded into a combining microvalve with Cascade Blue (CB) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (B). At each cycle, the contents of the combining valve are transferred to Out1 with an output rate of $0.06 \mu\text{L s}^{-1}$. (C). After a 15 min incubation, the contents of Out1 are combined with 30 mM borate buffer, pH 9.5 for dilution and transferred to Out2. Approximately $30 \mu\text{L}$ of labeled sample is generated for analysis by microcapillary electrophoresis on the Mars Organic Analyzer or MOA (D). The standard contains $200 \mu\text{M}$ formic acid, $400 \mu\text{M}$ each

acetic, propanoic, butanoic, pentanoic, hexanoic, and heptanoic acids, and 600 μ M octanoic acid.

Fig. 7. Bright field images of all possible reagent subsets loaded into the combining valve of the 6-bit combinatorial mixing device. Green, blue, clear, red, yellow, and black dyes were loaded into reservoirs 1,2,3,4,5, and 6, respectively.