

Chemical cytometry on a picoliter-scale integrated microfluidic chip

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An integrated microfluidic device has been fabricated for analyzing the chemical contents of a single cell (chemical cytometry). The device is designed to accomplish four different functions: (i) cell handling, (ii) metering and delivering of chemical reagents, (iii) cell lysis and chemical derivatization, and (iv) separating derivatized compounds and detecting them by laser-induced fluorescence. These functions are accomplished with only two valves, formed by multilayer soft lithography. A new kind of three-state valve and a picopipette are described; these elements are crucial for minimizing the reaction volume and ensuring optimal shape of the channel for electrophoresis injection. By using these valves, a reaction volume of ≈ 70 pl is achieved for the lysis and derivitization of the contents of a single Jurkat T cell (≈ 10 μm diameter). As a demonstration of the use of this integrated microfluidic device, electropherograms of amino acids from individual Jurkat T cells are recorded and compared with those collected from a multiple-cell homogenate.

Microfluidics has become an attractive tool for the analysis of single cells because its dimensions are comparable with the size of single cells and because of the potential for performing rapid analyses on a massively parallel scale. Recently, several reports have appeared on the separation of the contents of individual cells, or “chemical cytometry,” (1–4) by using microfluidics (5–7). These methods are innovative in the integration of several distinct functions, including cell transport, lysis, and separation of contents, but each of these methods relies on off-chip loading of fluorescent substrates into the cells. This procedure represents a limitation on the use of chemical cytometry because the only accessible analytes are those that can be derivatized by dyes that penetrate the cell membranes. Ideally, the derivatization reaction would be performed during or after lysis, which enables detection of any cellular analyte. This goal is particularly challenging because, during the span of a 10-min reaction, reagents may diffuse or be pumped toward an undesirable location by gravity-driven (8) or surface-tension-driven flow (9).

We report here a technique that integrates the major steps in chemical cytometry on a microfluidic chip: (i) isolating an individual cell from bulk suspension, (ii) accurately metering and delivering chemical reagents, (iii) performing cell lysis and chemical derivatization, and (iv) separating derivatized cell contents by micellar electrokinetic capillary electrophoresis (MEKC) with fluorescence detection. The critical step of fluorescent derivatization was accomplished without diffusion or unwanted flow by using valves formed by multilayer soft lithography (10–18). This work builds on our recent work toward using valved microfluidic devices for chemical cytometry (8, 19), with the addition of a three-state valve. Together with a conventional two-state valve, a reaction chamber with a volume of 70 pl is constructed, which helps to reduce unwanted dilution of the chemical contents of a single cell. The valves prevent diffusion or pumping of reagents from the chamber, which allows sufficient time for derivatization to reach completion. Separation of derivatized amino acids in a single Jurkat T cell is compared with those from a population of cells. This technique provides a general method for derivatization of chemicals and chemical

species in a cell within an extremely small volume, and for detection of each derivatized product. We believe the versatility of this technique will be useful in biological studies on cells.

Experimental Procedures

Chemicals and Materials. Amino acids, 10% SDS solution, and D-mannitol were from Sigma. Sodium borate, sodium phosphate (dibasic, heptahydrate), potassium phosphate (monobasic), sodium cyanide, crystal violet, concentrated hydrochloric acid (HCl), and DMSO were obtained from Aldrich. Naphthalene-2,3-dicarboxaldehyde (NDA) was purchased from Molecular Probes, poly(dimethylsiloxane) (PDMS) prepolymer (RTV 615A and 615B) was purchased from General Electric, photoresists (SU-8 50 and SPR 220-7) were purchased from MicroChem (Newton, MA), and silicon wafers were purchased from Silicon Sense (Nashua, NH). Jurkat T cells and cell culture reagents were purchased from the American Type Culture Collection and Invitrogen.

Fabrication of Photoresist Masters. Photoresist masters for soft lithography were formed by using standard procedures (20–22) with slight modifications. Patterns for photolithography were designed with computer-aided design software (FREEHAND 10, Macromedia, San Francisco) and printed on transparency films with a high-resolution [3,600 dots per inch (dpi); 1 in = 2.54 cm] printer (MediaMorphosis, Mountain View, CA). To form a master for the valves (valve master), we spin-coated a thin layer (≈ 40 μm) of negative photoresist SU-8 (2,000 rpm, 40 s) on a 4-in-diameter silicon wafer and soft baked it in two steps on a hotplate (65°C, 3 min; 95°C, 5 min). After cooling, the photoresist was exposed through a transparency mask for 25 s in a mask aligner (Karl Suss, Waterbury Center, VT) with a UV-light source (365–405 nm) and developed.

To form a master for the fluidic channels (channel master), a thin layer (≈ 10 μm) of positive photoresist (SPR 220-7, Shipley) was spin-coated (2,000 rpm, 40 s) on a 4-in-diameter silicon wafer and baked on a hotplate (105°C, 4 min). The photoresist was exposed through a transparency mask for 18 s and developed. The wafer was then baked on a hotplate (120°C, 2 min) to reflow the photoresist to form curved features.

The surfaces of the masters were made more hydrophobic by exposing them to perfluoro-1,1,2,2-tetrahydrooctyltrichlorosilane vapor (United Chemical Technologies, Bristol, PA) in a vacuum desiccator to prevent adhesion of the elastomer to the wafer or the photoresist structures during the curing step.

Fabrication of PDMS Microfluidic Devices. PDMS devices with valves were formed by multilayer soft lithography by using a variation on methods reported (10–19). The top layer of the device (valve layer) was formed by casting PDMS prepolymer (RTV 615 A:B

Abbreviations: MEKC, micellar electrokinetic capillary electrophoresis; NDA, naphthalene-2,3-dicarboxaldehyde; PDMS, poly(dimethylsiloxane).

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with a mass ratio of 10:1) against a valve master and curing the PDMS in an oven (60°C, 3 h). The PDMS layer (≈ 4 -mm-thick) was peeled from the master and holes were punched through it for connection to an external pressure controller.

The bottom layer of the device (channel layer) was formed by spin-coating (1,500 rpm, 50 s) PDMS prepolymer (RTV 615 A:B with mass ratio of 10:1) onto the channel master. After this channel layer was partly cured into a soft-gel state in an oven (70°C, 7 min), the valve layer of PDMS was aligned and affixed to the channel layer under a stereoscope. More PDMS prepolymer was added to completely cover the master, followed by another cure step in an oven (70°C, 1 h). This bonded PDMS layer was peeled from the channel master and holes were punched for connection to reservoirs. The PDMS piece was placed onto a flat PDMS that was spin-coated (≈ 500 rpm, 50 s) and cured on a glass slide to form an enclosed fluidic system. Short glass tubes (i.d. ≈ 5.5 mm and o.d. ≈ 7 mm) were glued to the system for reservoirs.

To form a separation microfluidic channel with “double-T” injection, PDMS was molded against a silicon master with photoresist pattern; after peeling off from the master, the PDMS mold was sealed against a flat piece of PDMS to generate the fluidic channel.

Cell Culture. Jurkat T cells were maintained at 37°C in a humidified atmosphere with 5% CO₂. Cells were grown in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin (100 μ g/ml) and were subcultured (1:5) every 7 days. Cells that had been subcultured more than eight times were discarded.

For analysis of single cells, a 1-ml aliquot of cells was centrifuged ($\approx 150 \times g$, 5 min) and the pellet was resuspended in PBS three times. After the final rinse, the cells were spun down and resuspended in a 2-ml aliquot of isotonic phosphate buffer (4 mM dibasic sodium phosphate/1 mM monobasic potassium phosphate/285 mM D-mannitol, pH ≈ 7.4) and immediately loaded onto a microfluidic chip (≈ 50 μ l loaded for each experiment). The final cell density, measured with a hemacytometer, was $\approx 1.4 \times 10^6$ per ml.

Fluorescent Derivatization. For off-chip derivatization of amino acids, reaction mixtures were prepared in 10 mM sodium borate buffer (pH ≈ 9.2 adjusted with 10 N sodium hydroxide) and 1% SDS. NDA (DMSO stock solution) and KCN were added so that the final concentrations of NDA, each amino acid, and cyanide were 1, 0.05, and 0.1 mM, respectively.

For off-chip derivatization of the contents of cell populations, a 10-ml aliquot of cells (with $\approx 10^6$ per ml density measured by a hemacytometer) was spun down and lysed with SDS (1%, 0.5 ml) for 10 min and ultrasonicated for 5 min. Lysate was passed through 0.1- μ m centrifuge filters (Millipore) and then mixed 1:1:1 with NDA (20 mM in DMSO) and sodium cyanide solution (30 mM).

Off-chip mixtures of cell contents and amino acid standards were allowed to react for 15–30 min before separation. For both reactions, the mixtures were prepared such that they contained at least 5% final concentration of DMSO to eliminate precipitation of derivatives.

Microfluidic experiments were carried out on an Axiovert 135 inverted microscope with a high-voltage power supplier and a pressure controller (see Movie 1, which is published as supporting information on the PNAS web site).

Supporting Information. The experimental setup (*Supporting Text*), a video of chemical reaction on chip (Movie 1), and a video of single-cell analysis on chip (Movie 2) are published as supporting information on the PNAS web site.

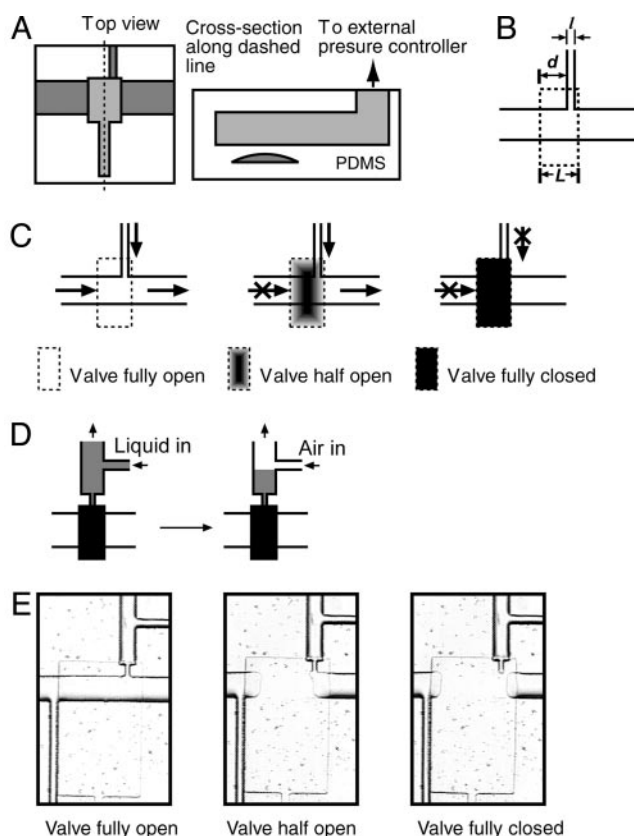


Fig. 1. Three-state valve and picopipette. (A) Schematic illustration of a three-state valve (top view and cross section). Channels on the upper layer (light shade) are filled with water and connected to an external pressure controller; channels of the lower layer (dark shade) are the microfluidic channels with smoothly curved surfaces. The pressure from the upper channel deforms the membrane between the layers and controls the opening and closing of the lower channel. (B) A simplified diagram of a three-state valve. (C) Schematic of the three states of this valve. (D) Scheme of the function of a picopipette. (E) Pictures of a three-state valve that is connected to a picopipette.

Results and Discussion

Three-Way Valve and Picoliter Pipette. Fig. 1A shows a schematic of a three-way valve that is used to control fluid flow. This valve is similar to other valve elements constructed by multilayer soft lithography in that it depends on the elastomeric property of PDMS. The upper layer contains a closed chamber that is connected to an external pressure controller; the lower layer contains the microfluidic network. Between the layers is a thin elastomeric membrane that deforms under pressure to close the fluidic channel. The three-state valve is different from conventional two-state valves in that the chamber of the upper layer in the three-way valve is aligned onto a T junction of microfluidic channels (Fig. 1B). The center of the valve is slightly shifted from that of the T junction but still covers all three channels, i.e., $L > d + l$ and $d + l/2 > L/2$. Under these conditions, this valve has three states as shown in Fig. 1C. These states are selected by controlling the pressure on the valve as follows: (i) when no pressure is applied, the PDMS membrane does not deform and the fluidic channel is open to flow in all directions; (ii) when a little pressure is applied, the membrane starts to deform and the left horizontal channel is blocked, but the side channel remains open to the right horizontal channel; and (iii) when more pressure is applied, the side channel is also blocked and no flow takes place through this valve in any direction. The three-state valve replaces the function of multiple two-state valves. The

three-state valve enables us to minimize the final reaction volume and also to achieve the desired geometric shape of the injection plug for subsequent electrophoretic separation.

In addition to the three-state valve, a metering structure that can deliver liquids on the scale of picoliters is important for the analysis of single cells. We adapted a design from Hosokawa *et al.* (23) and integrated it with a three-state valve. Fig. 1D shows schematically how this picopipette functions; liquid is injected from an inlet channel by pressure, with the three-state valve fully closed. Because PDMS is permeable to air, liquid is pushed toward the closed valve; as air is displaced through the PDMS, the chamber between the inlet and the valve is filled with fluid. A successive step of injecting air from the inlet forces most of the liquid from the channel and traps a small volume of liquid that can be loaded through the three-state valve into a reaction chamber. The volume of the remaining liquid is accurately defined by the channel dimensions and the distance from the inlet to the valve. This method can accurately deliver picoliter amounts of liquid (aqueous solution in air). Although fast evaporation of water makes it difficult to meter lower volumes accurately with an air–water interface, it might be possible to meter aqueous solutions with subpicoliter volume by using an oil as the second fluid to prevent evaporation.

The combination of the picopipette and the three-state valve enables us to perform chemical reaction of liquids with picoliter volumes on-chip. A three-state valve with a T-junction channel width of 100 μm and a valve chamber width of 400 μm has been fabricated and tested; its three states are shown in Fig. 1E. The membrane between valve and fluidic layers is ≈ 50 μm thick, and the pressures for these states are 0, 6, and 15 psi (1 psi = 6.89 kPa), respectively.

Microreactor and Its Integration with Capillary Electrophoresis. The three-state valve and the picopipette are combined with a two-state valve to form a six-port microreactor. Fig. 2A illustrates the procedure. The picopipette meters reagents and the three-state valve controls the direction of fluid flow so that the reagents only go into the reaction chamber.

The gas permeability of PDMS is useful for filling the pipette (21, 24, 25). Although it is possible to fill the pipette by opening (fully or half) the three-state valve to evacuate air, this procedure requires very careful control to prevent liquid from flowing beyond the valve. The gas permeability of PDMS also helps to remove bubbles that are accidentally trapped in the reaction chamber; by applying pressure from either valve, bubbles are displaced from the chamber in ≈ 1 min.

Fig. 2B contains frames from a video (Movie 1) depicting the use of the microfluidic reactor for an acid–base reaction. A basic solution of concentrated crystal violet, a pH indicator, is used to show the progression of the reaction. The solution color changes from blue to yellow when HCl is loaded into the chamber.

A capillary electrophoresis separation channel is integrated with the reactor; the design is shown in Fig. 3A. Five neutral amino acids are derivatized on-chip with NDA and separated by MEKC (1% SDS/10 mM borate buffer at pH ≈ 9.2) (Fig. 3B). The entire volume of the reaction chamber is injected for the separation. For comparison, off-chip derivatized amino acids are separated in the same conditions on a double-T injection with the same injection length (100 μm). The peaks from the on-chip reaction are broadened; we believe the width of the valves may increase the injection length and the perturbation from opening valves could also cause peak broadening.

Integrated Microfluidic Chip for Manipulation and Analysis of Single Cells. We fabricated a microfluidic chip (Fig. 4) for analyzing amino acids in individual cells. This chip consists of four sections: cell manipulation channels (channels 5–7), reagent introduction channels (channels 1 and 2), a reaction chamber (*Inset*), and a

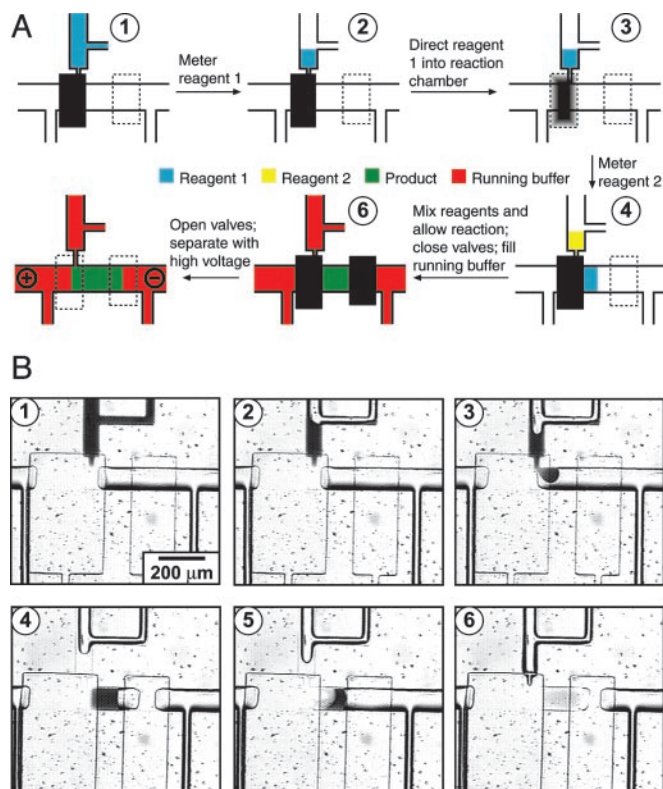


Fig. 2. On-chip reaction. (A) Schematic of the process of on-chip reaction. (B) Frames of a video that correspond to the steps of A. The circled numbers show the order of the frames and each frame corresponds to the schematics in A with the same number. A concentrated solution of crystal violet (a pH indicator) in 0.1 N sodium hydroxide is loaded into the microchamber with the three-state valve half-open; loading an equal amount of 1 N HCl into the chamber turns the solution from blue to yellow.

separation channel (channel 3). A three-state valve regulates flow of cells and reagents, and a two-state valve connects the reaction chamber to the separation channel. Fig. 5A schematically illustrates the process of manipulation and analysis of single cells with this chip. The corresponding video frames (see Movie 2) that recorded the manipulation of cells and derivatization of amino acids in a single cell are shown in a series in Fig. 5B.

Five steps are involved in the process of analyzing individual cells by chemical cytometry. First, a suspension of cells is introduced into inlet 7. To minimize the effects of high salt concentration from a normal PBS buffer that decrease the separation efficiency, the cells are suspended in an isotonic phosphate buffer with mannitol. Second, a single cell is moved into the reaction chamber. Cells flow from channel 7 to channels 5 and 6; once a cell is observed to be near the three-state valve (the region joining channels 5 and 6), the valve is opened to introduce the cell into the chamber. Because a minimal volume of cell media is preferred to be in the chamber, the valve can be half-opened to draw out extra cell media from the chamber without losing the cell (see Movie 2). Third, the cell is lysed and the buffer pH is raised by delivering a plug of 20 mM borate buffer (pH ≈ 9.2) with 1% SDS and 30 mM sodium cyanide to the reaction chamber by the picopipette. SDS lyses the cell immediately and the basic pH is required for derivatization. Fourth, the cell contents are derivatized with NDA (20 mM in DMSO), which is delivered to the reaction chamber by the picopipette. Cyanide and NDA concentrations are sufficiently high to ensure that all relevant species are labeled. Finally, running buffer is filled and the contents of the reaction chamber

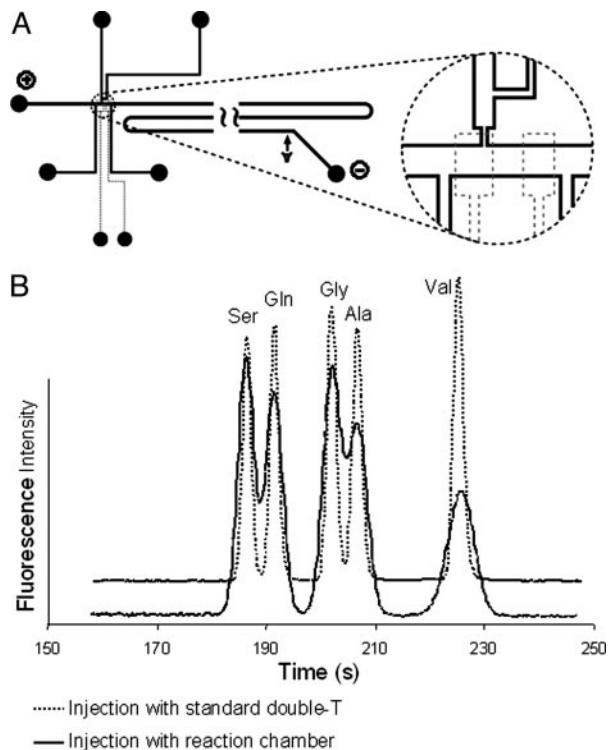


Fig. 3. On-chip reaction and separation. (A) Schematic of a microfluidic chip that is used to test the three-state valve and the picopipette. The upper layer of valves is shown in gray and a zoom-in diagram shows the details of the reaction chamber. A channel that is 16 cm long (detection is 1 cm away from the end reservoir) is used for MEKC separation. (B) Electropherograms of MEKC separations of five NDA-derivatized amino acids on the chip in A and on a double-T standard separation chip with the same dimensions. The concentration of each amino acid is 0.05 mM. The conditions for both separations are 10 mM borate buffer with 1% SDS at pH \approx 9.2 and 2 kV/16 cm.

are separated by MEKC. A typical experiment requires <1 h. Before analysis, channels 5–7 are coated with BSA (5 mg/ml for 10 min), which greatly reduces the adherence of Jurkat T cells to the channel walls. By the end of an experiment, the cell-handling channels usually become narrowed or blocked by cell aggregations and cell debris. Consequently, a new chip is used for each experimental run.

The reaction chamber in this device has a volume of 70 μ l; devices with smaller reaction chambers should also be feasible. This volume is estimated from the dimensions ($70 \times 100 \times 10 \mu\text{m}^3$, Fig. 4) of the photoresist patterns, from which the microfluidic device was made. The Jurkat T cells in this experiment had a diameter of $\approx 10 \mu\text{m}$ and a volume of ≈ 1 pl. The dilution from a single cell in this experiment is ≈ 70 -fold, comparable with the dilution factor of our cell population studies (≈ 30 -fold).

Fig. 6 presents an MEKC electropherogram of derivatized amino acids from a single T cell; for comparison, an electropherogram of the contents of a population of cells with off-column derivatization is also shown. A few cells have been analyzed by this method and their electropherograms are similar. Peaks are identified by the method of standard addition in cell population electropherograms. We believe that the biggest peak in the single-cell curve is from insoluble cell debris, because the only difference between chemical cytometry and cell population methodologies is that the lysed contents of the cell population were filtered before derivatization. The resolution of the chemical cytometry electropherogram is not as good as that from the

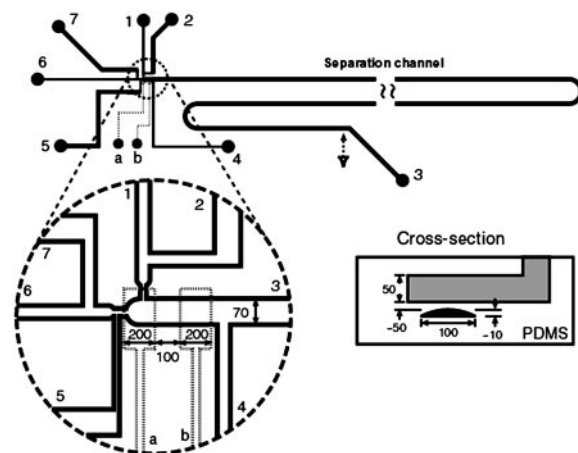


Fig. 4. Schematic illustration of a microfluidic chip used for single-cell analysis. The valve layer is shown in gray and a zoom-in diagram shows the details of the reaction chamber. The seven reservoirs (1–7) and both valves (a and b) are labeled. The dimensions are not to scale, and all numbers of dimension are in μm . A 16-cm-long channel is used for MEKC separation (detection is 1 cm away from the end reservoir).

cell population experiment. This resolution loss may have been caused because (i) analytes in the single-cell study are more dilute, (ii) some type of destacking might have occurred owing

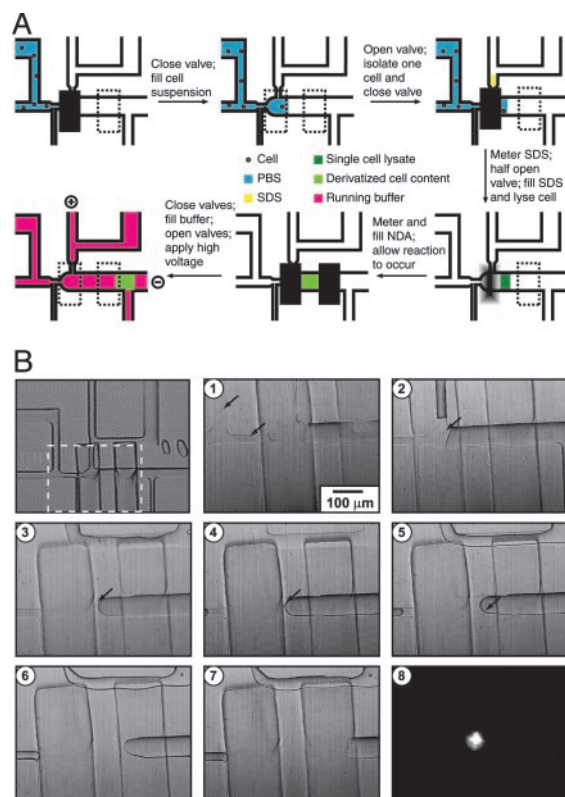


Fig. 5. Single-cell lysis and derivatization. (A) Schematic of the process of single-cell analysis on an integrated microchip. (B) Frames of a video of single-cell analysis corresponding to A. The first picture shows the overview of the system with all inlets; the dashed box denotes the area of the video that has been taken. The numbered video frames are in order of appearance. The last frame shows the fluorescence of the NDA-derivatized contents of a single cell in the reaction chamber when illuminated with a focused 405-nm diode laser. Arrows point to the positions of individual cells.

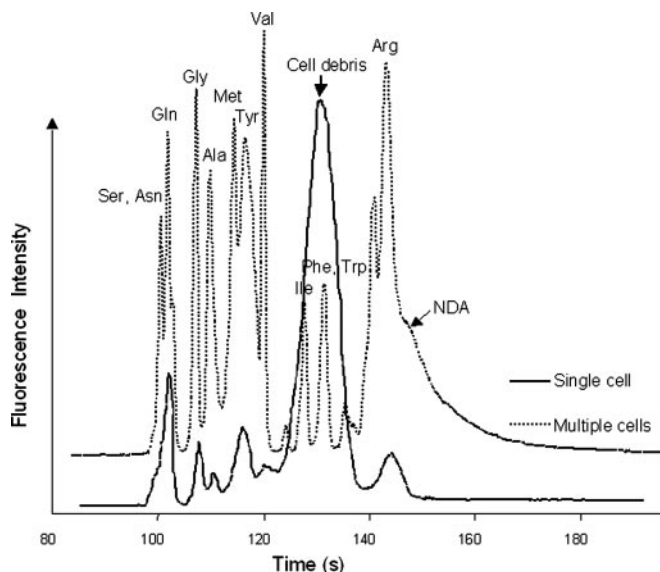


Fig. 6. MEKC electropherogram of NDA-derivatized amino acids from the lysed contents of a single cell. Also shown for comparison is the MEKC electropherogram obtained from the lysed contents of a cell population by using a "double-T" standard separation chip having the same dimensions. The separation conditions in both cases are 10 mM borate buffer with 1% SDS at pH \approx 9.2 and 4 kV/16 cm.

to concentration differences, and (iii) the double-T injection with the same injection length has better performance than the injection from the reaction chamber, as observed in the amino acid derivatization data shown in Fig. 3.

We have demonstrated chemical cytometry on an integrated

microfluidic chip with a reaction chamber having a confined volume of only \approx 70 pl. This volume compares favorably with the nanoliter volumes reported for derivatization reactions in capillary-based chemical cytometry methods (1–5), although smaller volumes have been reported (26). The capacity to confine a lysing, reacting plug of cell and reagents is an advantage that motivated prior work by us (8) and others (18) to use valves formed by multilayer soft lithography to confine approximately nanoliter volumes. To our knowledge, the 70-pl volume described here is the smallest reported for valve-confined reactions involving cells.

The total time for the analysis of one cell (cell injection, fluorescence derivatization, and separation) is $<$ 1 h. The relatively long analysis time is caused by cell manipulation (approximately a few minutes), cell lysis and derivatization (\approx 10 min for full reaction in the chamber), and capillary electrophoresis separation (a few minutes). We use a new PDMS chip for each experiment; fabrication of a batch of chips takes only a few hours, resulting mainly from curing time of the polymer. The merits of fast fabrication, low cost, and one-shot usage are a few reasons chips formed from polymeric substrates are becoming popular for many applications (27, 28).

In the future, the system could be optimized (e.g., automation of cell manipulation, removal of cell debris after lysis, and quantitative analysis) and cells could be injected continuously or multiple samples could be run in parallel. Although the present work analyzed cells through nonspecific fluorescent labeling of amino acids, any of a myriad of optical probes might be used (29). Alternatively, the device might be mated to a mass spectrometer for analysis (30). This device could be used generally for applications that involve reactions and separations of picoliter volumes of liquids.

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