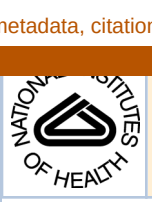

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Automated Capillary Electrophoresis System for Fast Single-Cell Analysis

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Abstract

Capillary electrophoresis (CE) is a promising technique for single-cell analysis, but its use in biological studies has been limited by low throughput. This paper presents an automated platform employing microfabricated cell traps and a three-channel system for rapid buffer exchange for fast single-cell CE. Cells loaded with fluorescein and Oregon green were analyzed at a throughput of 3.5 cells/min with a resolution of 2.3 ± 0.6 for the fluorescein and Oregon green. Cellular protein kinase B (PKB) activity, as measured by immunofluorescence staining of phospho-PKB, was not altered, suggesting that this stress-activated kinase was not upregulated during the CE experiments and that basal cell physiology was not perturbed prior to cell lysis. The activity of sphingosine kinase (SK), which is often upregulated in cancer, was measured in leukemic cells by loading a sphingosine-fluorescein substrate into cells. Sphingosine fluorescein (SF), sphingosine-1-phosphate fluorescein (S1PF), and a third fluorescent species were identified in single cells. A single-cell throughput of 2.1 cells/min was achieved for 219 total cells. 88% of cells possessed upregulated SK activity, although subpopulations of cells with markedly different SK activity relative to that of the population average were readily identified. This system was capable of stable and reproducible separations of biological compounds in hundreds of adherent and nonadherent cells, enabling measurements of previously uncharacterized biological phenomena.

INTRODUCTION

Upregulation of SK increases drug resistance in a number of cancers, including acute myeloid leukemia (AML), the deadliest form of adult leukemia.[1,2,3] SK converts sphingosine to sphingosine-1-phosphate (S1P), a stimulator of cell growth, differentiation, migration, and survival.[4] Inhibitors of SK activity have been found to decrease chemotherapeutic resistance, even in multi-drug resistant leukemic cells lines, and are currently in preclinical and clinical trials.[2,5] The cell-to-cell variability in SK-pathway dependence has important implications for the success of SK inhibitors as cancer therapeutics. Within a population of cells, a subset of cells may possess low SK activity and, therefore, exhibit inherent resistance to SK inhibitors. Indeed, the activity of many oncogenic kinases has been shown to vary drastically not only between the cancerous cells of different AML patients, but also by as much as 100-fold between individual cells in the

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SUPPORTING INFORMATION AVAILABLE

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same tumor.[6,7] Increasing evidence in other tumor systems suggests that the single-cell heterogeneity in regulation of oncongenic signaling-pathways is a general feature of most cancers.[8,9,10] Therapeutic inhibitors of SK activity currently in active development may have limited benefit if SK activity is highly variable among cells within a tumor. Thus, strategies to measure SK activity in large numbers of single tumor cells would assist in the characterization of cell-to-cell heterogeneity of this key signaling pathway while providing avenues to formulate individualized drug therapy for AML patients.

Traditional measurements of SK activity involve extraction of sphingosine and S1P from cell lysates comprised of millions of cells, labeling the molecules fluorescently or radiometrically, and quantifying the amount of sphingosine and S1P using high-performance liquid chromatography (HPLC),[11,12] thin-layer chromatography (TLC),[13] or fluorescence spectroscopy.[14] The detection limits of these technologies range from 0.3 pmol for HPLC-MS to 10 nmol for TLC. However, the predicted amounts of sphingosine and S1P within a single cell are (5 – 200) amol, below that of these analytical technologies. Capillary electrophoresis (CE), a separation technique that utilizes laser-induced fluorescence to achieve low limits of detection, has been used to perform single-cell measurements of lipids, including phosphatidylinositol phosphates and glycosphingolipids.[15,16] SK activity has been detected in as few as 10 cells using a fluorescent substrate, but single-cell measurements have not been achieved.[17]

Although traditional CE and chip-based CE (μCE) are powerful techniques for single-cell analysis, a major impediment to wider implementation of single-cell CE has been low throughput for biologically relevant analytes. Several strategies have been developed to address this problem, with the fastest single-cell CE platforms separating fluorescent dyes and biological analytes at rates of 1.8 cells/min[18] and 0.25 cells/min[19], respectively.[20,21,22] These systems performed serial analysis by sequentially injecting individual cells and then separating the cellular contents so that the analyte bands of sequential cells did not overlap at the detection window. These methods assessed a limited number of cells (≤30 cells) but incorporated important features such as facile switching between cell and electrophoretic buffers. Higher throughputs have been achieved using microfluidic-based electrophoresis, the fastest of which separated glutathione and reactive oxygen species at a throughput of 38 cells/min for a total of 76 cells.[23] Microfluidic devices have also separated fluorescent dyes in as many as 600 total cells.[24] However, to date, μCE has been used for limited biological applications due to the difficulty in analyzing adherent cells and adapting devices for electrophoretic separations of biological analytes. This is primarily due to the difficulty in exchanging the cellular contents from a physiologic buffer into an appropriate electrophoretic buffer for separation.[25] Although many single-cell CE and μCE systems expose cells to potentially stress-inducing phenomena such as electric fields, shear stress, and/or electrophoretic buffer, no systems have been characterized for their effect on cell homeostasis. This poses a potential challenge for this technology, as alterations in homeostasis have been shown to change basal cell physiology and can affect measurements of metabolic pathways and protein signaling cascades.

Here, we present the design and optimization of a fully automated single-cell CE system capable of analyzing both adherent and nonadherent cells. The maximum throughput of the system for single-cell analysis was determined by separating the model dyes fluorescein and Oregon green. The separation performance of these analytes was compared to that performed with previously published single-cell CE and microfluidic systems. Activation of protein kinase B (PKB) was measured in cells to determine whether the experimental steps prior to cell lysis altered stress-related biological pathways, potentially altering the outcome of kinase measurements. The single-cell CE system was then used to measure the phosphorylation of a fluorescent reporter for SK in the U937 human myeloid leukemia cell

line. The peak efficiencies, resolution, and rate of single-cell separations of SF and S1PF were quantified. Over 200 cells were analyzed and the heterogeneity of SK activity between single cells was characterized.

EXPERIMENTAL SECTION

Materials

Sphingosine fluorescein (SF) and sphingosine 1-phosphate fluorescein (SF1P) labeled with the 5′ and 6′ isomers of fluorescein were purchased from Echelon Biosciences Inc. (Salt Lake City, UT) and were greater than 95% pure. Additional materials are listed in the Supporting Information.

Channel System Fabrication

Channels to contain the electrophoretic and physiologic buffers were fabricated from poly(dimethylsiloxane) (PDMS) (Sylgard 184). The length, width and depth of the electrophoretic buffer channel were 3 cm, 0.5 cm, and 1.5 mm, respectively. The channel containing physiologic buffer (135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, 10 mM glucose, pH 7.4) was 3.5 cm in length. The width of the narrowest region was 0.3 cm and the circular reservoirs at both ends of the channel were 1 cm in diameter. The electrophoretic and physiologic buffer channels were connected by an air-filled perpendicular channel 0.5 cm long and 1 mm wide. The PDMS channel system was bonded to a 1002F-coated coverslip and an array of cell traps fabricated into the 1002F was placed within the physiologic channel.[26] Each trap in the 10×10 array was 30 μm in diameter and20 μm in depth, and the distance between the centers of adjacent traps was 100 μm. The cell traps were fabricated as described previously.[21] The cell trap array was placed in the center of the physiologic buffer channel, in line with the air-filled channel.

Automation

An inverted microscope (Ti-E, Nikon (Melville, NY)) was fitted with a computer-controlled motorized stage (Ti-SER, Nikon). The channel system containing trapped cells was mounted on a temperature controlled stage insert, which was maintained at 37 °C. The address of each cell trap in the 10×10 array was calculated based on the manually determined positions of the four corner wells using customized software written in Python (Wolfeboro Falls, NH). The Python software also interfaced with the software controlling stage movement (Micromanager software, Vale Lab, UCSF) and single-cell electrophoresis system (LabVIEW, National Instruments, Austin, TX). The coordinates of each cell trap containing a single cell were manually identified, recorded, and used to readdress that cell. A customized program (TestPoint, Measurement Computing, Norton, MA) was used to measure analyte fluorescence at the capillary-detection window as well as the current across the capillary.

Capillary Electrophoresis

CE separation of fluorescein and Oregon green dyes was conducted at a field strength of 510 V/cm using a borate-SDS buffer (45 mM borate and 20 mM SDS at pH 9.4). Separations of SF and S1PF were performed at 450 V/cm using a phosphate-propanol buffer (27 mM sodium phosphate and 10% 1-propanol at pH 7.3). Fused-silica capillaries (Polymicro Technologies, Pheonix, AZ) with an inner and outer diameter of 30 and 360 μm, respectively, were used for the electrophoresis. The total length of the capillary was 35 cm and the length from the inlet to the detection window was 4 cm. The short effective length of the capillary was made possible by mounting a custom-built epifluorescence detection system on the microscope stage, as has been described previously.[18]

Cell Culture

PC12 rat pheochromocytoma cells were grown in Roswell Park Memorial Institute (RPMI) medium containing 15% FBS and 1% penicillin/streptomycin and were used for assay of phosphorylated PKB. Rat basophilic leukemia (RBL) cells were cultured in Dublecco's Modified Eagle Medium (DMEM) with 10% FBS and 1% penicillin/streptomycin and were used for the fluorescein/Oregon Green experiments. Phosphorylation of sphingosine was measured in U937 human acute myeloid leukemia cells grown in RPMI media with 10% FBS and 1% penicillin/streptomycin. All cells were maintained at 37 °C in a humidified 5% CO_2 environment.

Cell Loading with Fluorescent Reagents

RBL cells were loaded in cell traps in the channel system as described previously[18] and then incubated at 37 °C in 5% CO_2 for 2–3 h. Prior to CE analysis, RBL cells were incubated in physiologic buffer containing 250 nM Oregon green diacetate and 50 nM fluorescein diacetate for 25 min. The cells were then washed 5x with physiologic buffer and analyzed by CE.

Nonadherent U937 cells were incubated in physiologic buffer containing freshly diluted SF (5 μM) for 8 min, centrifuged for 3 min at 800*g*, washed 5x with physiologic buffer, and then loaded into the cell traps.

Single-Cell CE

For automated serial single-cell analysis, a trapped cell was moved directly under the inlet of the capillary, which was positioned 50 μm above the plane of the cells. The physiologic buffer (1 mm/s flow velocity, 35 °C) and the electrophoretic buffer (2 mm/s flow velocity, 25 °C) were held at ground potential throughout the experiment. Excess buffer from both channels was removed by placing a vacuum line at the channel outlet ends. To analyze the contents of a cell, the cell was placed under the capillary and lysed by a focused laser pulse, as described previously.[27] The cell contents were electrokinetically injected into the capillary by applying −5 kV to the capillary outlet for 1 s. The voltage across the capillary was then set to zero for 1 s and the capillary was transferred through the air gap to the electrophoretic buffer. During buffer exchange, the capillary remained stationary and the channel system mounted on the microscope stage was moved. For the fluorescein/Oregon green analysis, a separation voltage of −18 kV was applied to the capillary outlet for either 14 s, for maximum throughput serial separations, or 52 s for low throughput serial separations. The separation voltage was −16 kV for 25 s for the SF/S1PF separation. The voltage was then set to zero for 1 s to move the capillary back to the physiologic buffer and position it at the address of the next single cell to be analyzed. This process was repeated under computer control until all single cells in the array were analyzed.

Activation of Cells with Nerve Growth Factor (NGF)

Glass coverslips or cell traps were treated with 1 M NaOH for 1 h, washed, and treated with 100 ng/mL poly-D-lysine for 1 – 12 h. PC12 cells were seeded on the treated coverslips or cell traps, and then incubated in RPMI media with 10% FBS and 5% FCS (24 h) and subsequently serum starved in RPMI media with 0.1% FBS (36 h). Cells were then stimulated with 100 ng/mL nerve growth factor (Millipore, Billerica, MA) for 15 min, as described previously.[28]

Immunofluorescence Assay for Phosphorylated PKB

The primary antibody used for the immunofluorescence assay was a phospho-PKB (Ser 473) rabbit mAb and the secondary antibody was a goat anti-rabbit IgG tagged with an Alexa

Fluor 488 conjugate, both purchased from Cell Signaling Technology (Danvers, MA). Blocking buffer (phosphate buffered saline (PBS) with 1% BSA) was purchased from Thermo Scientific (Waltham, MA). PC12 cells were fixed with 4% paraformaldehyde for 25 min and permeabilized by incubation in 0.1% Triton in PBS for 20 min. The samples were then incubated in blocking buffer (30 min), primary antibody diluted 1:200 in blocking buffer (1 h), blocking buffer (1 h), and finally in secondary antibody diluted 1:1000 in blocking buffer (45 min). Between incubations, the samples were washed 2x with PBS. Quantitative single-cell analysis of phospho-PKB (the average fluorescence intensity of a cell) was performed using fluorescence imaging, as previously described.[17]

RESULTS AND DISCUSSION

Design of a CE System for Single-Cell Analysis

To increase the throughput of single-cell CE while maintaining cell health, an automated CE system was designed and optimized (Figure 1A). An array of 100 microfabricated cell-sized wells was used to trap adherent or nonadherent cells at known addressable positions (Figure 1B). The cell trap array was positioned in an open PDMS channel where cells were continually exposed to fresh physiologic buffer (Figure 1C). Electrophoretic buffer was contained in a channel parallel to the physiologic buffer channel to prevent cell exposure to the cytotoxic electrophoretic buffer. These two channels were connected by a perpendicular channel filled with air that prevented mixing of the two buffer systems. The air was stably contained within the connecting channel because the surface tensions of the buffers were greater than the critical surface tension of the hydrophobic PDMS channel, which prevented wetting of the air channel. During analysis, cells were positioned under the capillary inlet, lysed, and electrokinetically injected into the capillary. The capillary then traversed the air channel to the electrophoretic buffer and separation of cellular contents was initiated by application of a voltage across the capillary. The channel remained in the electrophoretic buffer during electrophoresis, after which time, the capillary returned to the physiologic buffer channel to the address of another cell to be analyzed. The process was then repeated. Capillary movement and alignment over a new cell required less than 1 s.

Characterization of Separation Performance in the Absence of Cells

This system was designed for the repeated injection of cellular contents, which required multiple plugs of cellular contents to coexist within the capillary, all undergoing separation simultaneously. However, physiologic buffers possess high salt concentrations, in contrast to optimized electrophoretic buffers, which often feature low salt concentrations to minimize capillary current. Low ionic strength buffers enable efficient separations at maximal field strengths, but the differences in ionic strengths between the electrophoretic buffer and the physiologic buffer were expected to lead to destacking of the analyte plug, causing significant peak broadening. To determine how the presence of multiple high-salt plugs within the capillary impacted separation performance, an equimolar mixture of fluorescein and Oregon green in physiologic buffer was repeatedly injected into the capillary and separated. To fully mimic the events during cell analysis, the dyes were placed in the physiologic buffer channel. A buffer plug (1.6 nL) was loaded into the capillary, which was then moved into the electrophoretic buffer channel, and electrophoresis was initiated. After 10 s, the capillary was moved back into the physiologic buffer channel, a second plug injected, and the process repeated. Serial analyte injections were performed in this manner at a rate of 5.5 injections/min. Under these conditions, 23 high-salt analyte plugs were present simultaneously in the capillary (0.7 plugs/cm capillary). For each plug loaded, two peaks were visualized on the electropherogram, one of which co-migrated with fluorescein and the other with Oregon green (Figure S1, Table S1, Supporting Information). The resolution was 3 ± 0.5 and the peak efficiencies were $7{,}000 \pm 1{,}000$ (4-cm effective capillary length) for the

fluorescein peak and 2,300 ± 900 for the Oregon green peak. For comparison, a single plug of the fluorescein/Oregon green mixture loaded into the capillary and electrophoresed exhibited peak efficiencies for fluorescein and Oregon green of 8,300 ± 600 and 4000 ± 400, respectively with a resolution of 8.2 ± 0.4. As expected, deterioration in separation performance occurred due to the multiple high ionic-strength plugs within the low-ionic-strength electrophoretic buffer. The separation performance of Oregon green was more markedly decreased than that of fluorescein, likely due to the higher charge of Oregon green, which made it more susceptible to destacking.[29] However, robust separation of the two dyes remained possible with good peak area (0.78 ± 0.02 for fluorescein and 1.09 ± 0.03 for Oregon green) and migration time (29.2 ± 0.3 s for fluorescein and 34.1 ± 0.2 s for Oregon green) reproducibility. These results suggested that separation of fluorescein and Oregon green from single cells would be possible with this system. Importantly, this simple test strategy could be utilized to predict which buffer/analyte combinations could be employed successfully with this single-cell analysis platform.

Separation of Fluorescein and Oregon Green from Single Cells

To determine whether the model dyes could be separated from single cells, cells were preloaded with the dyes and single-cell analysis was performed in an automated fashion at 1.2 cells/min and with 5 sample plugs in the capillary (0.1 plugs/cm). The electropherograms obtained from the single cells exhibited two fluorescent peaks with migration times corresponding to that of fluorescein and Oregon green (Figure S2, Table S2, Supporting Information). The migration times of the fluorescein and Oregon green peaks were not stable for the first 5 cell-lysate plugs loaded into the capillary with times of 28.5 ± 2 s and 34 ± 5 s (n= 5), respectively (Figure S3, Supporting Information). This was most likely due to the changing local field strengths within the capillary as more high-salt buffer plugs were introduced. After the first 5 plugs, the reproducibility in migration time improved: 26.3 ± 0.3 and 30.9 ± 0.5 s for fluorescein and Oregon green (n= 7), respectively. When the capillary was pre-loaded with 10 plugs of physiologic buffer prior to single-cell analysis, the migration times were 26.3 ± 0.3 and 31.4 ± 0.6 s, respectively (Figure S3, Supporting Information). The peak efficiencies of fluorescein and Oregon green peaks were 2000 ± 800 and 3000 ± 1000, respectively, with a resolution of 1.8 ± 0.5. Thus, the presence of the cellular constituents decreased separation performance relative to that of the dyes dissolved in the physiologic buffer solution. This was most likely due to binding of the dyes to cellular components and adsorption of cellular debris onto the capillary walls.[30]

To determine whether a higher single-cell analysis rate could be attained without degrading separation performance, cells were injected and separated at a rate of 3.5 cells/min, which corresponded to 15 sample plugs present simultaneously within the capillary (0.4 plugs/cm). For these experiments, the capillary was pre-loaded with 15 physiologic buffer plugs and then single-cell CE was initiated. The efficiency, migration times and resolution were similar to that attained at the lower rate of single-cell analysis (Table 1). Thus, the throughput of the automated system could be increased to 3.5 cells/min without compromising the system performance. The separation quality and cell throughput was compared to the separation performance of previously published serial-analysis capillary and microfluidic systems (Table 1).[18,31] Remarkably, this system brings the capillary-based approach close to the throughput of the microfluidic system with similar separation performance but with a much greater flexibility in the electrophoretic buffer utilized.

Measurement of Phosphorylated PKB (Phospho-PKB) in Cells Prior to Assay

Accurate measurement of model dyes from cells is possible irrespective of the cell's condition as long as the cell's membrane is intact so that the dyes remain intracellular. However, accurate measurement of many cellular properties, for example, kinase activation,

requires that the cell remain unperturbed until cellular reactions are terminated at the moment of lysis. Many enzymes, including SK, are activated in response to stresses placed on a cell, such as hypotonic-buffer exposure, shear forces, and chemical insult.[32] Thus, it was important to assess cell health following exposure to potential insults such as epoxy photoresist contact, adjacent cell lysis, electrophoretic buffer contamination, and other factors during the assay process. Phosphorylated PKB was measured as a marker for cell stress since phosphorylation is altered in response to a diverse array of cellular stresses including oxidative, hyperosmotic, hypoosmotic,[33] and nonphysiologic chemical exposure.[34,35,36] The cellular stressors introduced by this system were assayed by measuring alterations in single-cell basal phospho-PKB during: *i)* culture in the 1002F cell traps, *ii)* incubation in low concentrations of electrophoretic buffer, and *iii)* a mock single-cell CE assay, where cells were exposed to the full conditions of the assay prior to lysis, including the effect of laser-based cell lysis on nontargeted, adjacent cells.

1002F photoresist has previously been shown to be biocompatible in that cells can grow and divide when cultured on a 1002F surface.[26] However, more subtle effects on cellular health, such as induction of cell stress responses, have not been examined. Cells were cultured on glass surfaces or in 1002F cell traps for 60 h and phospho-PKB was measured by immunofluorescence. Cellular fluorescence, or phospho-PKB levels, were indistinguishable on the two surfaces (Figure S4, Supporting Information). As a positive control, cells on the two surfaces were stimulated using nerve growth factor (NGF), which is known to activate PKB. Phospho-PKB levels were identical in cells grown on the different surfaces (Figure S4, Supporting Information). These data demonstrate that culturing cells in 1002F traps do not elevate cell stress relative to culture on a glass surface.

Electrophoretic buffers generally possess non-physiological chemicals, low ionic strengths, and non-neutral pH, all of which might alter cellular physiology and activate stress pathways. As the capillary is withdrawn from the electrophoretic buffer solution for transfer back to the physiologic buffer, a meniscus forms on the capillary end due to surface tension.[37] Therefore, small amounts of electrophoretic buffer were expected to be transported by the capillary into the physiologic buffer as the capillary was moved back and forth between the two buffer channels. To quantify the amount of liquid that the capillary transferred, the electrophoretic buffer channel was filled with a trypan blue solution and the physiologic buffer channel was filled with water. The capillary was repeatedly moved between these two solutions and the amount of trypan blue in the water-filled channel was measured using its absorbance at 607 nm. Each movement of the capillary between the channels transferred 8 ± 1 nL of electrophoretic buffer into the physiologic buffer channel (Figure S5, Supporting Information). For a physiologic buffer volume of 400 μL, 100 capillary movements would yield a 0.2% (vol/vol) contamination of electrophoretic buffer in the physiologic buffer.

To characterize the impact of low-level electrophoretic buffer contamination in the physiologic buffer, phospho-PKB was measured in cells incubated in physiologic buffer containing 0 – 1% electrophoretic buffer. Three common electrophoretic buffers with a variety of chemical constituents: Tris (100 mM Tris at pH 7.4), borate-SDS, and phosphate-propanol were assessed. Borate-SDS and phosphate-propanol buffer were additionally chosen because they were used for the fluorescein/Oregon green and SF/S1PF separations, respectively. The number of cells exhibiting phospho-PKB levels greater than 3x the standard deviation of the average phospho-PKB level in untreated cells was calculated. Increasing electrophoretic buffer contamination led to greater numbers of cells with increased phospho-PKB levels for all three buffers (Figure 2A). The borate-SDS buffer system yielded the highest levels of phospho-PKB at each of the concentrations tested, increasing phospho-PKB levels relative to untreated cells with as little as 0.1%

contaminating electrophoretic buffer. The phosphate-propanol buffer caused the smallest increase in phospho-PKB, requiring at least 1% buffer contamination to increase phospho-PKB amounts in the cells. These experiments suggest that exposing cells to even very low concentrations of electrophoretic buffer can alter signaling pathways. Although the separation performance of single-cell CE and μCE systems can be improved by injecting cells in electrophoretic buffer to prevent destacking, as several systems have previously done,[19,38] measurements made in these systems may not accurately reflect basal cellular physiology.

Laser-based cell lysis acts by creating an expanding and collapsing cavitation bubble that mechanically disrupts cells.[27] While bubble size is limited, fluid movement around the bubble is initiated and could impact nearby cells. To assess the effects of laser-based lysis of targeted cells on the adjacent nontargeted cells in cell traps, as well as the other possible stressors during the assay (electric fields during electrokinetic injection, shear stress from buffer flow, and electrophoretic buffer contamination) phospho-PKB was measured in cells during a mock cell-assay protocol. The capillary was positioned 100 μm from a trapped cell (over an empty well) and a focused laser pulse was delivered below the capillary, as would be performed for actual cell lysis. A voltage was applied to the capillary to load a buffer plug and the capillary was moved to the electrophoretic buffer channel. This process was repeated for each cell on the array that was adjacent to an empty cell trap. The mock assay had no detectable effect on phospho-PKB activity, compared to control cells without exposure to a nearby laser pulse or capillary (Figure 2B, 2C, and 2F). To test for phospho-PKB inhibition, a group of positive control cells were stimulated with NGF and the cells divided into a control and mock-assay group. These NGF-stimulated cells exhibited increased phosphorylation of PKB, with the control and test groups demonstrating similar numbers of cells with elevated phospho-PKB (Figure 2D, 2E, and 2F). These results suggested that the single-cell assay did not increase cell stress levels nor did the assay procedures inhibit appropriate cellular responses to stimuli prior to cell lysis.

Measurement of S1PF Formation in Single U937 Cells

Once a fast cell assay-system was designed and characterized, formation of S1PF could be measured in large numbers of cultured U937 cells, a myeloid leukemia cell line. Cells were incubated with SF, washed, and then loaded into the cell traps. Single cells were then serially assayed for fluorescent SF metabolites. The separation voltage was applied for 25 s after the contents of a single cell were loaded into the capillary, yielding a throughput of 2.1 cells/min for a total of >200 cells. The electropherograms of each cell possessed 1, 2 or 3 peaks (Figure 3A). A peak with an average migration time of 30 ± 1 s co-migrated with SF and a peak that migrated at 41 ± 2 s co-migrated with S1PF. A third peak, present in 7% of cells, migrated at 35.0 ± 0.9 s, between the SF and S1PF peaks. The resolution between the SF and S1PF peaks was 3 ± 1.5 and the efficiency of the SF and S1PF peaks was 1300 ±800 and 2000 ± 1000, respectively. The molar amounts of SF, S1PF, and the unknown compound in cells, calculated by comparing peak areas measured in cells to those measured using a standard solution with known concentrations of SF and S1PF, varied from approximately 0.1 – 10 amol with an average of (0.5 ± 2) amol and (6 ± 8) amol for SF and S1PF, respe ctively (Figure 3B). The percent of phosphorylated reporter ($S1PF_{moles}/(SF_{moles} + Unknown_{moles} + S1PF_{moles})$) in a cell ranged from 0 to 100% with an average of 90 ± 30% and did not depend on the total amount of reporter loaded into the cells ($R^2 = 0.003$) or the time of the measurement ($R^2 = 0.06$) (Figure 3B and Figure S6, Supporting Information). Thus, most cells demonstrated a large capacity to convert SF to S1PF without saturating SK. A comparison of the percent of SF phosphorylated in single cells revealed that population average did not capture the distinct subpopulations of cells present (Figure 3C). The majority of cells (83%) had greater than 80% S1PF. 11% of the cells possessed 1 to 80%

phosphorylation, and another 6% demonstrated less than 1% phosphorylation. These categories were also independent of the amount of SF loaded into the cell and the time of the measurement. Thus, significant heterogeneity existed amongst the cells in terms of S1PF formation as well as the amount of total reporter loaded. The high levels of S1PF formation in the majority of these cultured cells is consistent with prior work demonstrating that SK inhibitors can decrease AML cell survival.[2] However, the existence of cell subpopulations with low to moderate SK activity indicate that SK inhibitors might not be equally effective on all cells, suggesting that alternative therapies may be necessary to target AML cell populations not dependent on increased SK activity.

CONCLUSION

A robust, fully automated system for serial single-cell CE was developed and characterized. The system presented here performs faster analyses of fluorescent dyes and biologically relevant analytes than any prior capillary-based designs. The flexibility in electrophoretic buffer selection opens the door for the analysis of a wide range of biological compounds in single cells without stimulating a cell stress-response. The system was sufficiently robust to analyze >200 cells and to identify subpopulations of SK activity in the cultured U937 cells. This examination of heterogeneity in SK upregulation at the single-cell level adds to existing evidence that indicates SK inhibition could be an effective method for treating leukemia, although it also suggests that for total annihilation of leukemia cells, combination therapies will be important. Our long-term goal is to enable targeted drug therapy for AML patients with upregulated SK activity. Additionally, this single-cell CE system will enable higher throughput measurements of a wide range of biological compounds in future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Ogretmen B, Hannun Y. Nat Rev Cancer. 2004; 4:604–616. [PubMed: 15286740]
2. Paugh S, Paugh B, Rahmani M, Kapitonov D, Almenara J, Kordula T, Milstien S, Adams J, Zipkin R, Grant S, Spiegel S. Blood. 2008; 112:1382–1391. [PubMed: 18511810]
3. Siegel R, Naishadham D, Jemal A. CA-Cancer J Clin. 2013; 63:11–30. [PubMed: 23335087]
4. Cuvillier O, Pirianov G, Kleuser B, Vanek P, Coso O, Gutkind J, Spiegel S. Nature. 1996; 381:800–803. [PubMed: 8657285]
5. Edmonds Y, Milstien S, Spiegel S. Pharmacol Therapeut. 2011; 132:352–360.
6. Gallay N, Dos Santos C, Cuzin L, Bousquet M, Gouy VS, Chaussade C, Attal M, Payrastre B, Demur C, Recher C. Leukemia. 2009; 23:1029–1038. [PubMed: 19158829]
7. Irish JM, Anensen N, Hovland R, Skavland J, Borresen-Dale AL, Bruserud O, Nolan GP, Gjertsen BT. Blood. 2007; 109:2589–2596. [PubMed: 17105820]
8. Marusyk A, Almendro V, Polyak K. Nat Rev Cancer. 2012; 12:323–334. [PubMed: 22513401]
9. Bendall S, Nolan G. Nat Biotechnol. 2012; 30:639–647. [PubMed: 22781693]
10. Gerlinger M. New Engl J Med. 2012; 367:976–976.
11. Butter J, Koopmans R, Michel M. J Chromatogr B. 2005; 824:65–70.
12. Lan T, Bi H, Xu S, Le K, Xie Z, Liu Y, Huang H. Biomed Chromatogr. 2010; 24:1075–1083. [PubMed: 20352614]
13. Olivera A, Barlow K, Spiegel S. Method Enzymol. 2000; 311:215–223.

14. Billich A, Ettmayer P. Anal Biochem. 2004; 326:114–119. [PubMed: 14769343]
15. Jiang DC, Sims CE, Allbritton NL. Faraday Discuss. 2011; 149:187–200. [PubMed: 21221426]
16. Whitmore CD, Hindsgaul O, Palcic MM, Schnaar RL, Dovichi NJ. Anal Chem. 2007; 79:5139–5142. [PubMed: 17567107]
17. Lee K, Mwongela S, Kottegoda S, Borland L, Nelson A, Sims C, Allbritton N. Anal Chem. 2008; 80:1620–1627. [PubMed: 18197698]
18. Jiang D, Sims C, Allbritton N. Electrophoresis. 2010; 31:2558–2565. [PubMed: 20603824]
19. Chen S, Lillard S. Anal Chem. 2001; 73:111–118. [PubMed: 11195493]
20. Marc P, Sims C, Allbritton N. Anal Chem. 2007; 79:9054–9059. [PubMed: 17979298]
21. Marc P, Sims C, Bachman M, Li G, Allbritton N. Lab Chip. 2008; 8:710–716. [PubMed: 18432340]
22. Boardman A, McQuaide S, Zhu C, Whitmore C, Lidstrom M, Dovichi N. Anal Chem. 2008; 80:7631–7634. [PubMed: 18717573]
23. Xu CX, Yin XF. J Chromatogr A. 2011; 1218:726–732. [PubMed: 21185567]
24. Phillips K, Lai H, Johnson E, Sims C, Allbritton N. Lab Chip. 2011; 11:1333–1341. [PubMed: 21327264]
25. Kovarik M, Lai H, Xiong J, Allbritton N. Electrophoresis. 2011; 32:3180–3187. [PubMed: 22012764]
26. Pai JH, Wang Y, Salazar GT, Sims CE, Bachman M, Li GP, Allbritton NL. Anal Chem. 2007; 79:8774–8780. [PubMed: 17949059]
27. Sims C, Meredith G, Krasieva T, Berns M, Tromberg B, Allbritton N. Anal Chem. 1998; 70:4570–4577. [PubMed: 9823716]
28. Borgatti P, Martelli A, Tabellini G, Bellacosa A, Capitani S, Neri L. J Cell Physiol. 2003; 196:79–88. [PubMed: 12767043]
29. Beckers JL, Bocek P. Electrophoresis. 2000; 21:2747–2767. [PubMed: 11001282]
30. Jin W, Jiang L. Electrophoresis. 2002; 23:2471–2476. [PubMed: 12210205]
31. McClain M, Culbertson C, Jacobson S, Allbritton N, Sims C, Ramsey J. Anal Chem. 2003; 75:5646–5655. [PubMed: 14588001]
32. Gude D, Alvarez S, Paugh S, Mitra P, Yu J, Griffiths R, Barbour S, Milstien S, Spiegel S. Faseb J. 2008; 22:2629–2638. [PubMed: 18362204]
33. Kippenberger S, Loitsch S, Guschel M, Muller J, Kauffman R, Bernd A. Febs Lett. 2005; 579:207–214. [PubMed: 15620715]
34. Oehler-Janne C, von Bueren A, Vuong V, Hollenstein A, Grotzer M, Pruschy M. Biochem Bioph Res Co. 2008; 375:399–404.
35. Zundel W, Giaccia A. Gene Dev. 1998; 12:1941–1946. [PubMed: 9649498]
36. Dai R, Chen R, Li H. Int J Oncol. 2009; 34:1749–1757. [PubMed: 19424594]
37. Fishman HA, Scheller RH, Zare RN. J Chromatogr A. 1994; 680:99–107. [PubMed: 7952011]
38. Greif D, Galla L, Ros A, Anselmetti D. J Chromatogr A. 2008; 1206:83–88. [PubMed: 18657818]

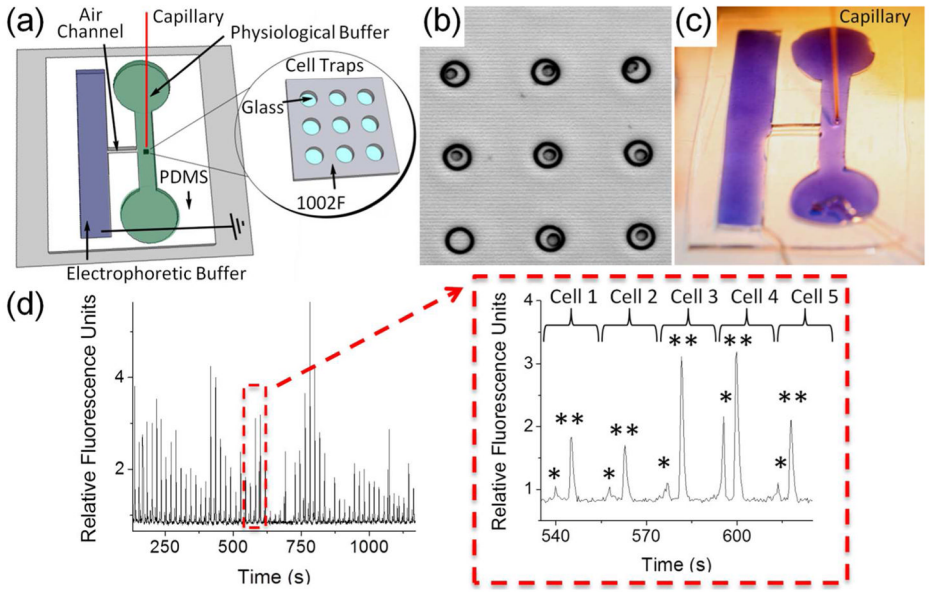

Figure 1.

System for serial assay of single cells. (A) Schematic of the channel system with 9 of 100 cell traps shown in the inset. (B) Bright-field image of 9 cell traps of which 8 are loaded with cells. The lower left trap is empty. (C) Image of the channel system with both channels filled with a trypan blue solution. The capillary is positioned over the cell traps. (D) Electropherogram of serial single-cell analysis of 59 cells pre-loaded with fluorescein (*) and Oregon green (**). The inset shows an expanded region of the electropherogram

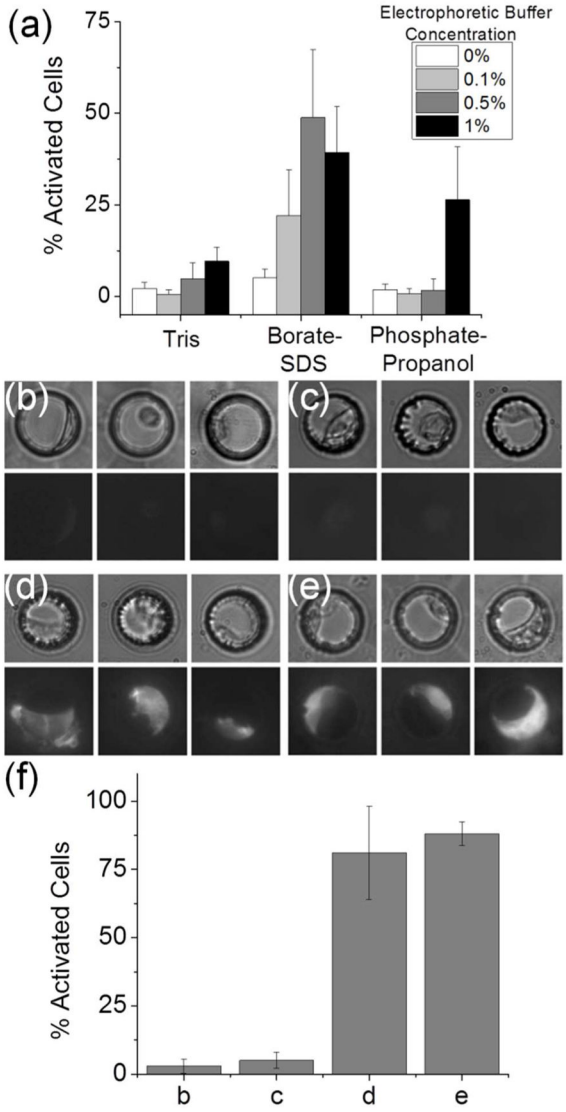

Figure 2.

Measurement of phospho-PKB in cells. (A) The percent of cells with elevated phospho-PKB when varying concentrations (0–1%) of electrophoretic buffers were intermixed with physiologic buffer. Each bar is the average of 3 experiments consisting of ≥ 50 cells each. The error bars represent the standard deviation. “Borate” represents the borate-SDS buffer while “Phosphate” describes the phosphate-propanol buffer. (B) Bright-field and fluorescence images of cells incubated in 1002F cell traps. (C) Bright-field and fluorescence images of cells incubated at a distance of 100 μm from regions in which a focused laser pulse was fired and a buffer plug was electrokinetically loaded into the capillary. (D) Cells treated as in panel B but with prior exposure to NGF. (E) Cells treated as in panel C but with prior exposure to NGF. (F) The percent of cells with elevated phospho-PKB corresponding to the experimental conditions shown in panels B to E. Each bar is the average of 3 experiments consisting of 100 cells. The error bars represent the standard deviation.

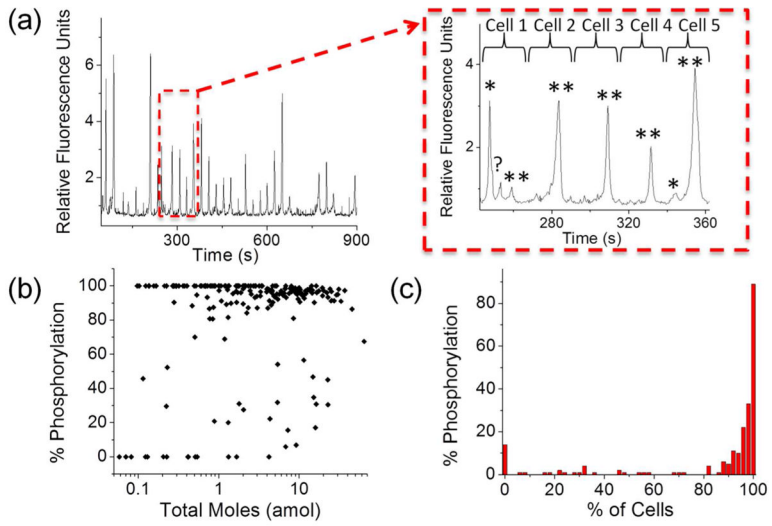

Figure 3.

Measurement of SK activity in single U937 cells. (A) Typical electropherogram of the serial separation of SF (*), S1PF (**), and an unknown (?) in 35 single cells. (B) Percent of phosphorylated reporter ($S1PF_{moles}$ / (SF_{moles} + $S1PF_{moles}$ + $Unknown_{moles}$)) plotted against the total moles of reporter loaded into the cell (SF_{moles} + $S1PF_{moles}$ + $Unknown_{moles}$) for each cell (n = 219). (C) Histogram of the percent of cells with different levels of phosphorylated reporter ($S1PF_{moles}$ / (SF_{moles} + $S1PF_{moles}$ + $Unknown_{moles}$)).

Table 1

Comparison of the separation of fluorescein (Fl) and Oregon green (OG) from single cells using capillary or microfluidic systems.

	Peak Efficiency ($N \times 10^4$)				
Chemical Cytometry System	**Fl**	**OG**	**Resolution**	**T_m (%RSD)**	**Throughput (cells/min)**
Automated Capillary (current system)	0.2 ± 0.1	0.3 ± 0.2	2.3 ± 0.6	3.4	3.5
Jiang *et al.* Capillary[18]	0.3 ± 0.1	0.2 ± 0.09	2.7 ± 0.5	3.5	1.8
Microfluidic Device[31]	*	0.38	*	4.8	7 – 12

T_m is the average migration time

* Information not provided