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Microelectrophoresis Platform for Fast Serial Analysis of Single Cells

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Abstract

A capillary-based microelectrophoresis platform for fast serial analysis of single cells is described. In this system, the capillary remains fixed and a two-channel flow system is used to rapidly switch the buffer surrounding the capillary inlet from a physiological buffer to an electrophoretic buffer. Single cells are retained in the physiologic-buffer channel utilizing an array of cell microwells patterned into the channel floor. The defined addresses of the cells on the array enable the sequential delivery of individual cells to the inlet of the capillary, where a focused laser pulse lyses the cell. The cell chamber is moved along a preordained route so that the inlet of the capillary is located in the electrophoresis buffer for separation and the physiological buffer during cell sampling. The throughput of the current system is limited by peak overlap between successive samples. Key characterizations of this system including the fluid flow rates, the cell array dimensions, and laser energies were performed. To demonstrate this system, 28 cells loaded with Oregon green and fluorescein were serially analyzed in under 16 min, a rate of 1.8 cells/min.

Keywords

Capillary Electrophoresis; Serial Analysis; Chemical Cytometry; Single Cell Analysis

1. Introduction

The study of individual cells yields rich information that may be obscured by the averaging which occurs when pooling cells for analysis. Measurements on single cells have enhanced the understanding of cellular biochemical pathways underlying signal transduction and other cell processes. Classic examples of biological phenomenon revealed by single-cell analysis include the temporal patterns of intracellular calcium concentration after receptor activation, and the switch-like behavior of signal transduction enzymes in the initiation of embryogenesis.[1,2] In recent years, technical progress has led to chemical separation techniques of sufficient sensitivity to enable analysis of single cells, a field known as chemical cytometry.[3] Chief among these separation technologies has been capillary electrophoresis (CE).[4] Initial single-cell analyses by CE were measurements of the amino acid and protein content of neurons and erythrocytes.[5–7] CE-based analyses in single cells have now been accomplished for analytes as diverse as carbohydrates, neurotransmitters, proteins, nucleotides, lipids, and other molecules.[8–13] CE has also been used to monitor individual mitochondrial properties, measure the activity of various enzymes, and develop

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protein maps from single cells.[12,14–22] CE has numerous advantages as an analytical tool for single cell analysis. The dimensions and volumes of the capillary are compatible with those of even small mammalian cells. The remarkably high resolving power of CE and the exquisite sensitivity made possible by laser-induced fluorescence detection allows monitoring of multiple analytes. Additional applications are continually being reported as the use of CE in cell-based assays expands to probe the intricate biochemistry of single cells.

A drawback of CE for chemical cytometry is its relatively low throughput, typically 5–35 cells per day.[4,12,15,19] Due to the large diversity in single cell behavior, the analysis of hundreds to thousands of individual cells is often necessary to generate the statistics needed to fully define many cellular properties, for example activation of signal transduction pathways.[2,23] Parallel and serial analysis strategies have been explored to enhance CE's throughput.[24–26] Parallel analyses increase throughput by relying on arrays of capillaries to increase the sampling population.[24,27] This approach requires simultaneous and accurate alignment of all capillaries with respect to the samples, which may be challenging with single living cells. Serial analysis increases throughput by sequential injections of analyte into the same capillary.[28] This method frequently relies on the injection of subsequent samples before the previous sample has been detected. One example of this method is a flow-based injection system used to assess the protein content of nonadherent cells at a rate of 10 cells in 40 minutes.[28] This method has the benefit of simplicity with good throughput. However, it is limited to nonadherent cells, utilizes a mixed physiological and electrophoretic buffer for separation, and possesses variable spacing of analyte plugs in the capillary. To develop serial analysis of adherent cells, Marc *et al* designed a coaxial system to rapidly modify the buffer composition surrounding the inlet of a capillary from a physiological buffer to a separation buffer.[29] The separation capillary mated with a coaxial capillary were placed adjacent to cells cultured in a physiologic buffer. After injection of the cell into the capillary, separation buffer flowed through the coaxial capillary providing a 100% exchange of separation buffer to the inner capillary during electrophoresis. After analyte separation, flow in the coaxial capillary was stopped and the inlet moved to the next cell for sampling. A steady stream of physiologic buffer prevented upstream cells from being adversely affected by the separation buffer between sampling. The analysis of 20 adherent cells within 40 min was achieved using this system. Although the coaxial CE system improved throughput, the instrumental setup was complex and required precision flow control. In addition, a 2 mm upstream incursion of separation buffer required relatively large distances between the cells to be sampled, limiting the number of cells that could be cultured and sampled using this platform.

In the current work, a simple CE system utilizing a single capillary is integrated with a computer-controlled microscope stage for serial analysis of single cells. In this system, an open, two-channel flow system with physiological buffer in one channel and electrophoretic buffer in the alternate channel is fabricated and mounted on the motorized stage of an inverted microscope. By regulating the flow rate of the buffers, electrophoresis buffer is excluded from the cells which reside in microfabricated cell microwells within the channel filled with physiologic buffer. The microwells provide each cell with a precise and defined address to enable automated positioning of the capillary for single-cell sampling. A laser rapidly lyses an individual cell whose contents are loaded into the capillary. While the capillary remains fixed, the flow system is translated to position the capillary inlet in the channel containing electrophoresis buffer. After a defined period of time, the chamber is re-positioned to bring a new address containing the next cell to be sampled under the capillary inlet and the process is repeated to achieve serial analysis.

2. Materials and Methods

2.1 Materials

Precleaned glass slides (50 mm × 45 mm × 1.5 mm) were obtained from Fisher Scientific (Pittsburgh, PA). EPON resin 1002F (phenol, 4,4-(1-ethylethylidene) bis, polymer with 2,2-[(1-methylethylidene) bis (4,1-phenyleneoxymethylene bis-[oxirane]) was purchased from Miller-Stephenson (Sylmar, CA). SU-8 developer (1-methoxy-2-propyl acetate) was procured from MicroChem Corp. (Newton, MA). Carboxyfluorescein diacetate (fluorescein diacetate), fluorescein free acid (fluorescein), Oregon green 488 carboxylic acid diacetate 6-isomer (Oregon green diacetate) and Oregon green 488 carboxylic acid 6-isomer (Oregon green) were acquired from Molecular Probes (Eugene, OR). The Sylgard 184 silicone elastomer kit was obtained from Dow Corning (Midland, MI). All the other reagents were purchased from Fisher Scientific (Pittsburgh, PA).

2.2 Cell chamber with L-shaped channels

The open, L-shaped chamber containing the buffers was fabricated from PDMS (Sylgard 184) bonded to a glass coverslip. Each leg of the L-shaped PDMS chamber was 3 cm in length, 0.5 cm in width, and 0.2 cm in depth. The physiological cell buffer (135 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM Hepes, pH 7.4) and electrophoretic buffer (10 mM borate and 20 mM SDS, pH 9.4) each flowed into separate channels with the buffer streams joining at the channel intersection. The buffer reservoirs were connected to the channels via tubing and the flow rate of the buffer solutions was regulated by varying the height of the buffer reservoirs above the channel. The cell microwells were located in the channel with physiologic buffer 1.5 cm from the channel intersection. The microwells were fabricated as described previously.[30] During electrophoretic separations, the capillary was located in the channel with the electrophoretic buffer and was positioned 1.5 cm from the chamber intersection.

For electrokinetic sampling of fluorescent buffer, Oregon green (0.5 nM) and fluorescein (2 nM) were added to the physiologic buffer. At time zero, the capillary was placed in the region of the cell microwells and a voltage (5 kV) was applied through the capillary for 1 sec to electrokinetically load the fluorescent cell buffer, *i.e.* the sample. The voltage was then stopped for 3 sec during which time the flow chamber was translated to position the capillary inlet in the CE buffer channel. The electrophoresis voltage (15 kV) was then applied across the capillary for 24 s. After this time, the voltage was again reduced to zero and the stage repositioned in 3 sec to bring the capillary inlet over the cell array for the next electrokinetic injection. The process was then repeated to achieve serial sample injections. The velocities for the physiologic and electrophoretic buffers were 0.35 and 0.9 mm/s, respectively in these experiments. In these experiments, two groups of 20 serial sample introductions were performed. Fluorescence detection was accomplished as described in the Supplementary Data.

2.3 Loading cells into cell microwells

The cell microwells were loaded with rat basophilic leukemia (RBL) cells and cultured as described previously.[30] Of the microwells on the array, $83 \pm 10\%$ ($n = 5$) were occupied with single cells. Most of the remaining microwells were vacant and $3 \pm 2\%$ of the microwells possessed two cells. No cells were present on the 1002F layer between the microwells. The channel with entrapped cells was then placed in an incubator overnight prior to use.

2.4 Automated software identification of cell microwell addresses

An inverted microscope (Ti-E, Nikon, Melville, NY) was fitted with a computer-controlled motorized stage (Ti-S-ER, Nikon) on which the channel with cell microwells was mounted. The channel region with cell microwells was scanned and imaged using Metamorph software version 7.6 (MDS Inc., Canada). The address of each cell microwell was identified by its area and shape using a customized software routine (Metamorph). The coordinates of each cell microwell center were recorded and used to readdress that cell microwell.

2.5 Single-cell sampling

Cells were loaded with fluorescein and Oregon green, and then lysed by a laser, as reported previously.[31] Briefly, a diode-pumped, passively Q-switched Nd:YAG laser (532 nm, 750 ps pulse with a vertically polarized beam, PowerChip NanoLaser; JDS Uniphase Corp., Milpitas, CA) was steered through a polarizer (LPVIS100 Linear Polarizer; Thorlabs Inc., Newton, NJ) to control the pulse energy from 1 to 6 μ J. For cell lysis, a single laser pulse was focused (40 $\times$, 0.60 N.A., Plan Fluor, Nikon, Melville, NY) onto an entrapped cell. During cell lysis, the inlet of the capillary was centered 50 μ m above the cell microwell. Focusing of the microscope to position the focal point of the laser beam at the cell:glass interface was accomplished manually.

3 Results and Discussion

3.1 Design of the two-channel flow chamber

The goal of this work was to design a robust, automated system for fast serial analysis of single, live cells by capillary electrophoresis. The buffer in which cells reside is not optimal for chemical separations and the electrophoresis buffer is toxic to cells. Thus, a rapid means for switching from the cell buffer to the CE buffer for the separation step was required. This was accomplished by designing an “L”-shaped, open, two-channel flow system to enable the buffer channels to be repositioned with respect to the capillary inlet while holding the capillary stationary. The physiological buffer and electrophoretic buffer streams moved through the separate channels so that the buffer flows joined at the channel intersection. A vacuum line connected to this intersection served as the outlet for both channels. Cells to be sampled resided in microfabricated cell microwells in one leg of the flow system where they remain surrounded by the physiologic cell buffer at all times (Figure 1A). After cell sampling the capillary remained stationary while the microscope stage supporting the channels moved to position the capillary in the channel with flowing electrophoretic buffer. A voltage was applied to the capillary for electrophoresis of cellular contents. To sample a second cell, the voltage was returned to zero and the channel/stage moved to position the capillary over the cell array. After cell sampling, the channel/stage moved so that the capillary was returned to the electrophoretic buffer channel and electrophoresis was resumed.

3.2 Characterization of the two-channel flow system

To prevent cells from being exposed to electrophoretic buffer during the experiments, the flow rates of the two buffers were optimized to prevent the electrophoretic buffer from moving into the region containing the cells. To test the system, trypan blue (0.1%) was added to the electrophoretic buffer to visualize the flow and mixing of the fluids in the two channels. The velocities of the buffers were then varied from 0.45 to 0.9 mm/s for the electrophoretic buffer and 0.1 to 0.35 mm/s for the cell buffer while evidence of trypan blue movement into the cell chamber was monitored (Fig. 1B). Buffer velocities suitable for excluding the electrophoretic buffer from the cell array are shown in Fig. 1C. Due to the potential for shear induced stress on cells, the lowest physiologic buffer velocity that

excluded the electrophoretic buffer from the cell channel was used. This value was determined to be 0.13 mm/s with a corresponding electrophoretic buffer flow of 0.45 mm/s. This cell buffer velocity was less than one-tenth of that required for the aforementioned coaxial system (1.7 mm/s).[29] Additionally when fluorescein (1 nM) was added to the cell buffer and the capillary was placed in the electrophoretic channel 1.5 cm from the channel intersection, no increase in fluorescence was observed in the capillary.

3.2 Characterization of serial injection for electrophoretic separations

To increase throughput, each sample is loaded before prior samples have reached the detection window. Successive injections must be timed to prevent analyte peak overlap and peak parking in the detection zone. Peak overlap occurs when the fastest migrating analyte of one sample overtakes the slowest species from the previous sample. This occurrence can confound peak identification and analyte quantitation. Since the voltage across the capillary is set to zero during channel movement, a peak positioned or parked in the detection window during this time will not be quantifiable due to the prolonged illumination of the analyte band relative to the illumination time for other analyte bands (data not shown). The time interval between sampling and analyte detection consisted of the following four periods: electrokinetic injection (1 sec), movement of the channel to place the capillary into the CE buffer (3 sec), electrophoresis (variable time -- dependent on the analytes), and movement of the channel to place the capillary back at the cell array (3 sec). The migration time difference of fluorescein and Oregon green was 19.9 ± 0.7 sec ($n = 20$). Thus, if the time for electrophoresis was set at 24 s, the analyte bands of sequential samples should not overlap. Furthermore, this time should also prevent peak parking in the detection zone since the migration time of fluorescein and Oregon green was 52.6 ± 1.5 s and 67.6 ± 2 s, respectively. With an electrophoresis period of 24 s, a sample could be injected every 31 s.

To evaluate this serial sampling method, experiments were performed with cell buffer containing Oregon green and fluorescein and the cell buffer was electrokinetically injected into the capillary. The sampling and analysis protocol began with the capillary positioned above the cell microwells in the channel containing the fluorescent cell buffer. The fluorescent buffer was loaded into the capillary and the channel translated to place the capillary in the electrophoretic buffer. This process was repeated to achieve serial injection and separation of the fluorescent cell buffer (Figure 2 and Table 1). Peak overlap was not observed in these experiments and the fluorescein and Oregon green peaks were readily identified based on their migration times (Fig. 2). For comparison, data from single electrokinetic injections of the dyes were also performed by permitting the dyes to elute from the capillary prior to introduction of the second sample (Table 1). The peak areas of the Oregon green and fluorescein were nearly identical when injected singly or serially. This suggested that peak parking at the detection window did not occur. In contrast both the resolution of the two peaks as well as the separation efficiency suffered for the serially injected peaks relative to that for the singly injected peaks. This was most likely due to the presence of multiple high salt buffer plugs in the capillary during electrophoresis of the serially introduced samples. The high salt buffer plugs locally altered the electroosmotic mobility as well as the analytes' electrophoretic mobility in the capillary. In addition, the high salt plug resulted in destacking of the analytes as they entered the capillary. These factors all contributed to the decreased resolution and efficiency.

A long term goal of this project is to measure the peak area ratio of the substrate and product of an enzyme in a single cell. Thus, an important measure of system performance is the reproducibility of the peak area ratio of Oregon green and fluorescein. The peak area ratios for the two analytes were nearly identical for serially and singly injected analytes. In both instances, the peak area was reproducible and independent of the injection voltage with an

RSD of 3%. When 95 serial electrokinetic injections were performed, the RSD of the peak area ratio measured was 3.5% (Fig. 2 inset).

3.3 Cell microwells for addressable cell sampling

For single-cell analysis by CE, positioning the capillary above a cell is often slow, particularly when the cells are randomly located. Microwells have proven useful to pattern cells at known addresses for analysis.[32–35] In prior work by the Allbritton group, single cells trapped in microwells were lysed by applying an electric field across an electrode beneath the cell and a ground electrode placed in the aqueous media above the well.[30] Although this electrical method was robust, scaling up to a large array of single cells was complex due to the large number of required electrical connections. To simplify the design and fabrication of the microwell array, laser-based lysis of the cells was chosen for the current work.[31] Laser-based cell lysis relies on the formation of a cavitation bubble generated by a focused microbeam.[36] The maximum density of the cell array depends on the size of the cavitation bubble or energy of the laser pulse. The cavitation bubble can not extend to adjacent microwells or these nontargeted cells may be lysed along with the intended cell. To determine the lowest laser energy for lysis, RBL cells were cultured in cell microwells for 6 h, loaded with fluorescein as a vital dye, and visualized by brightfield and fluorescence microscopy (Fig. 3A,B). The fluorescence intensity of each cell was recorded and a single focused pulse of varying energy was delivered to the glass:cell interface in the microwell. Lysis was judged by comparison of the brightfield and fluorescence images before and after the laser pulse as well as the loss of the fluorescein from the cell (Fig. 3C,D). At 1 μJ , 80% of targeted cells ($n = 10$) were lysed. When the energy was $\geq 2 \mu\text{J}$, 100% of targeted cells were lysed ($n = 10$). An energy of 2 μJ was therefore used in the experiments for serial cell analysis.

The minimal distance between microwells needed to prevent cells in microwells adjacent to the target cell from being disrupted was measured. RBL cells were cultured in arrays with varying well-to-well distances (distance between the centers of two wells). A single laser pulse was delivered to a microwell. The fluorescence change of cells in adjacent microwells was measured before and after pulse delivery ($n = 20$) and compared to a control in which the laser was not fired. At well-to-well distances of $\leq 80 \mu\text{m}$, a decrease in fluorescence intensity was observed for cells in wells adjacent to targeted wells (Fig. 3E). At a distance of 100 μm , no change in the fluorescence intensity of nearby cells occurred. RBL cells were then cultured on an array with microwell distances of 100 μm and the energy of the laser pulse used for lysis was varied. The fluorescence change of the cells adjacent to those receiving the laser pulse was measured. For laser energies up to 6 μJ , the fluorescence intensity of the nearby cells did not change ($96 \pm 3\%$ of original fluorescence intensity). Thus, 100 μm was used as the well-to-well distance for the microwell array in subsequent experiments.

3.4 Serial analysis of single cells

To assess the optimal cell-content loading voltage during cell lysis, cells cultured in microwells and loaded with Oregon green and fluorescein were lysed and the voltage across the capillary (1 s) was varied. Two peaks with migration times similar to those of fluorescein ($52.9 \pm 1.1 \text{ sec}$) and Oregon green ($67.5 \pm 1.8 \text{ sec}$) were observed for all cells. No differences in analyte peak areas was observed when the loading voltage was $\geq 5 \text{ kV}$ (Fig. 4A). Thus, subsequent experiments employed 5 kV as the cell-content loading voltage.

RBL cells were again cultured in a microwell array, loaded with fluorescein and Oregon green, and then sequentially analyzed in an automated fashion. Prior to serial cell analysis, a computer algorithm was used to identify the addresses of the microwells (and cells). The

capillary was placed over the initial cell well. The computer then initiated the sequence of events described in section 3.1. This process was repeated until 28 cells were serially analyzed (Fig. 4B). The total analysis time for these cells was 932 sec yielding an analysis rate of 1 cell per 33 sec. This represents a significant improvement over the prior rate of 1 cell per 120 sec.[29,30] A faster throughput rate (1 cell per 8 sec) was achieved using a microfluidic device; however, that technology was limited to the analysis of non-adherent cells.[37] The current data demonstrated two peaks for each cell with peak migration times similar to that of Oregon green and fluorescein. There was substantial variability in the amount of each dye per cell as has been previously demonstrated.[29,31,37,38]

4 Concluding remarks

An automated microelectrophoresis system with buffer switching and cell lysis was developed to rapidly analyze the contents of adherent cells in a serial manner. An array of cell microwells was used to position the cells in a channel at known addresses so that a capillary could be repeatedly and accurately positioned above a cell. Fluorescein and Oregon green were separated and detected from single cells at a rate of 108 cells/h an improvement on the prior fastest capillary-based system which possessed a rate of 30 cells/h. The current system was demonstrated for adherent cells but should be equally applicable to nonadherent cells since the cells were entrapped in microwells. Improvements in the current system should further increase the rate of cell analysis. For example, placing the cell array closer to the channel intersection will shorten capillary travel time. A shorter distance between the capillary inlet and detection window would permit faster sampling times without peak overlap. In the current system the laser was fired via the computer, but was focused manually increasing the time in which the capillary was parked over the cell array. Addition of an automated focusing routine will substantially diminish the time that the capillary spends at the cell array site. The ultimate goal of this technology is to enable the application of chemical cytometry to large numbers of single cells for a greater understanding of cell biology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Two-channel cell and electrophoretic buffer chamber. (A) Side view of the two-channel system with physiological and electrophoretic buffer flowing into the indicated channels. The circular region in the channel with CE buffer marks the location of the capillary during electrophoresis. (B) Photo of channel juncture with physiological buffer (0.13 mm/s) and electrophoretic buffer (0.45 mm/s). The electrophoretic buffer contains Trypan blue. (C) The electrophoretic and physiologic buffer velocities were varied and the presence (x) or absence (closed circle) of electrophoretic buffer in the channel with the microwells was recorded. The solid line depicts the boundary for which greater physiologic buffer flow rates blocked movement of the electrophoretic buffer into the microwell channel.

Figure 2. Serial electrophoresis of fluorophore standards. (A) Typical trace of demonstrating the separation of fluorescein (*) and Oregon green (**). (Inset) The ratio of the peak areas of fluorescein and Oregon green plotted against the sample number.

Figure 3.

Laser-based lysis of single cells in microwells. (A) Shown is a brightfield image of two microwells each with a single cell. (B) Shown is a fluorescence image of the same cell in panel A. (C) Shown is a brightfield image after a focused laser pulse was delivered to the upper microwell. (D) Shown is a fluorescence image of panel C. (E) The fluorescence intensity ratio of cells adjacent to a targeted cell/microwell before and after delivery of a laser pulse (2 μJ) is shown. The x-axis shows the distance between the centers of adjacent wells. No laser pulse was delivered to any microwells for the control sample.

Figure 4.

Single-cell analysis. (A) The area of the Oregon green peak was measured as the cell-content loading voltage was varied. Shown is the average of the data points ($n = 10$) with the error bars representing the standard deviation. (B) Serial lysis of 28 cells and separation of their contents. Each cell possessed a peak for fluorescein (*) and Oregon green (**). After the final cell was lysed and the electrophoretic channel was moved to position the capillary in this buffer, no further movement of the channel was initiated. The x axis displays the time since the start of the experiment and thus includes the time for electrophoresis plus the time during which electrophoresis is stopped to either lysis a cell or move the stage beneath the capillary.

Table 1

Comparison of single and serial sample injection.

Injection	Sampling Voltage	Peak Area		R	N ($\times 10^4$)		Area ratio
		FI	OG		FI	OG	
Standard	2 kV	0.17 $\pm$ 0.02	0.15 $\pm$ 0.02	4.40 $\pm$ 0.38	2.98 $\pm$ 0.81	1.05 $\pm$ 0.30	1.16 $\pm$ 0.02
Serial		0.17 $\pm$ 0.01	0.15 $\pm$ 0.01	3.79 $\pm$ 0.30	1.88 $\pm$ 0.81	0.65 $\pm$ 0.14	1.16 $\pm$ 0.02
Standard	3.5 kV	0.28 $\pm$ 0.01	0.25 $\pm$ 0.01	3.59 $\pm$ 0.23	2.24 $\pm$ 0.98	0.46 $\pm$ 0.09	1.14 $\pm$ 0.02
Serial		0.28 $\pm$ 0.02	0.24 $\pm$ 0.01	3.15 $\pm$ 0.17	1.71 $\pm$ 0.70	0.34 $\pm$ 0.06	1.17 $\pm$ 0.03
Standard	5 kV	0.40 $\pm$ 0.03	0.37 $\pm$ 0.06	2.61 $\pm$ 0.31	2.2 $\pm$ 0.88	0.29 $\pm$ 0.04	1.15 $\pm$ 0.02
Serial		0.43 $\pm$ 0.06	0.38 $\pm$ 0.07	2.29 $\pm$ 0.22	1.25 $\pm$ 0.23	0.20 $\pm$ 0.02	1.18 $\pm$ 0.03
Standard	7.5 kV	0.59 $\pm$ 0.05	0.51 $\pm$ 0.04	2.20 $\pm$ 0.32	1.59 $\pm$ 0.64	0.15 $\pm$ 0.02	1.16 $\pm$ 0.01
Serial		0.59 $\pm$ 0.04	0.49 $\pm$ 0.04	1.96 $\pm$ 0.12	1.08 $\pm$ 0.23	0.16 $\pm$ 0.03	1.20 $\pm$ 0.03

Shown is the average and standard deviation of the data points.