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Sampling Efficiency of a Single-Cell Capillary Electrophoresis System

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Abstract

Capillary electrophoresis (CE) combined with a laser-induced fluorescence (LIF) detection scheme is a powerful approach for single-cell analysis. For measurements requiring a high temporal resolution, CE–LIF is often combined with cell lysis systems based on pulsed lasers. Although extremely rapid, laser lysis has raised some concerns about the efficiency at which the cell contents are sampled. We have assembled a single-cell CE-LIF mounted on the stage of a microscope. This system was coupled with a nanosecond pulsed laser for cell lysis. We have analyzed green fluorescent protein (GFP) expressed in single mammalian cells and developed a novel approach to estimate the cell sampling efficiency (SE) based on the use of fluorescent calibration microspheres and flow cytometry. A significant advantage of this method is that it does not require any knowledge or assumption regarding the cell volume. We have evaluated the SE for different laser pulse energies (from 2 to 9 μJ) and two different pulse focal positions in the *xy* plane (0–10 μm from the center of the cell). We found the maximum SE at the lowest energy (2 μJ), with the pulse focused directly on the cell. We have demonstrated the utility of a novel method to measure the SE of a single-cell CE system. The measurements presented in this study indicate that rapid cell lysis with nanosecond lasers requires careful optimization of pulse parameters for maximum sampling of the cell contents. © 2007 International Society for Analytical Cytology

Key terms

capillary electrophoresis; single-cell analysis; cell-sampling efficiency; cell lysis; pulsed lasers; green fluorescent protein

SINGLE-CELL measurements present two significant advantages over bulk measurements from a biological point of view. First, fundamental cell-to-cell differences are preserved since the response is not averaged across the population (1). Second, single-cell analysis enables biochemical measurements on rare cell populations. Capillary electrophoresis (CE) is a separation technology that is capable of high resolution separations of analytes based on size and charge from small volumes (<pL). As such CE combined with the enhanced detection sensitivity of a laser-induced fluorescence (LIF) detection scheme represents a powerful approach for the analysis of single mammalian cells (2–7). Proteins and their activity in single cells have been sampled via this method by creating green fluorescent protein (GFP)-fusion proteins (8), labeling with fluorophore-conjugated antibodies (4,7,9,10), or using peptide based cell-permeable fluorescent enzyme reporters (6). A crucial element required for this analytical approach is a means to manipulate single cells. For monitoring cellular processes which require a high temporal resolution (e.g. signaling), sampling must be performed such that single cells can be selected and lysed before stress pathway activation occurs. The use of highly focused laser pulses for selective cell lysis has been used successfully in several studies to achieve cell lysis on a millisecond time scale as a means to rapidly lyse single cells and stop biochemical reactions by rapid dilution of the cell contents and turbulent mixing (2,3,5,6,11). Laser lysis is performed with Nd:YAG nanosecond pulses focused through high numerical aperture

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lenses (2,3,5,6). Focused laser pulses at or near the glass/buffer interface create localized plasma formation, followed by a shockwave, and finally cavitation bubble formation, collapse, and liquid jet formation. It has been shown that bubble expansion/collapse and liquid jet formation are responsible for cell lysis, and occur in less than a millisecond (12,13). Cell-sampling is achieved by synchronizing the laser pulse and cell lysis with the application of a high voltage gradient along the capillary which loads the cell contents into the separation column. Although extremely fast, there have been some concerns raised about the efficiency at which the cell contents are injected into the capillary/channel using this method. Moreover, because of the large amount of energy involved, there is potential for partial degradation of the sample during the lysis procedure. However, the performance of laser lysis has been difficult to evaluate and optimize because of the lack of a reliable experimental method to measure cell sampling efficiency (SE).

In the present study, we describe a novel approach to estimate the SE of single-cell CE systems based on the use of fluorescent calibration microspheres and flow cytometry which does not require any knowledge or assumption regarding the cell volume. Using this method, we investigate the effects of laser focus placement and laser pulse energies on the sampling of GFP expressed in single mammalian cells.

Materials and Methods

Reagents and Buffers

Extracellular buffer (ECB) was used as running buffer in CE (2,3). ECB is a physiological buffer (pH 7.4) composed of 135 mM NaCl, 5 mM KCl, 10 mM *N*-2-hydroxyethylpiperazine-*N*-2 ethanesulfonic acid (HEPES), 2 mM $MgCl_2$, and 1 mM $CaCl_2$. Fluorescent dyes 5-carboxy-Oregon green and carboxyfluorescein succinimidyl-ester (CFSE) were obtained from Invitrogen (Carlsbad, CA). The CFSE was hydrolyzed with NaOH (pH 10) and then diluted with ECB. Oregon green was also diluted in ECB. The 0.2-μm yellow-green fluorescent carboxylated polystyrene microspheres were obtained from Invitrogen (Carlsbad, CA).

Cells and Cell Culture

We used HEK 293T cells engineered with the murine stem cell virus (MSCV) vector to express the enhanced GFP. GFP-HEK 293T cells were grown at 37°C with 5% CO_2 in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

Flow Cytometry

GFP-HEK 293T cells of homogeneous fluorescence were obtained by fluorescence-activated cell sorting (FACS). The cells were first harvested by trypsinization and spun down at 800*g* for 3 min, and resuspended in phosphate buffered saline (PBS) supplemented with 2% FBS. Cell sorting was performed on a FACS Aria (BD Biosciences); cells were selected for median fluorescence on the GFP (green) channel. Further, flow cytometric analysis was performed using a FACSCanto (BD biosciences) flow cytometer. Photomultiplier tube (PMT) voltages were kept constant at 425 V for all experiments. The results were collected on the GFP channel which had a 535/50 nm bandpass filter (Chroma Technology, Rockingham, VT) and a 2-mm thick Schott (Mainz, Germany) glass filter (OG515) to match the optics in the CE setup.

Capillary Electrophoresis

The CE system (Fig. 1) was composed of a 40-cm long 30 μm ID, 360 μm OD fused silica capillary (Polymicro Technologies, Phoenix, AZ) of which the sampling end of the capillary was fixed over the stage of an inverted microscope (IX71, Olympus Canada, Markham, ON) using a 3-axis translation stage allowing precise positioning of the capillary tip. The outlet end was immersed in buffer in the outlet vial along with the high-voltage electrode. LIF detection was performed using a single mode fiber coupled 15-mW 488-nm continuous wave solid state diode pumped laser (FTEC 488, Blue sky research, Milpitas, CA) which was focused using a 2.54-cm focal length biconvex lens (CVI laser, Albuquerque, NM) onto a small window, burned into the polyimide coating of the capillary 12 cm from the sampling end. Fluorescence emission was gathered by a 40× microscope objective (Olympus) and filtered through a 535/50 bandpass filter (Chroma Technology), a 488-nm Raman notch filter (CVI laser), focused through a spatial pinhole filter (custom made), filtered through a 2-mm thick Schott Glass filter (OG515), and directed onto a PMT (H9306-04, Hamamatsu, Japan). The amplified PMT output signal was sent to a data acquisition card and processed by a program written with Testpoint software (Keithley Instruments, Cleveland, OH). A high voltage power supply (CZE1000R, Spellman High Voltage, Hauppauge, NY) was used to create an electrical gradient along the capillary for CE. The optimal voltage was determined by plotting a Joule curve, where the optimal voltage was taken as the voltage at which the relationship between current and voltage became nonlinear. This was found to be ~220 V/cm when using ECB as a buffer. New capillaries were preconditioned with 0.1 N NaOH overnight before use, and conditioned before each run by rin-

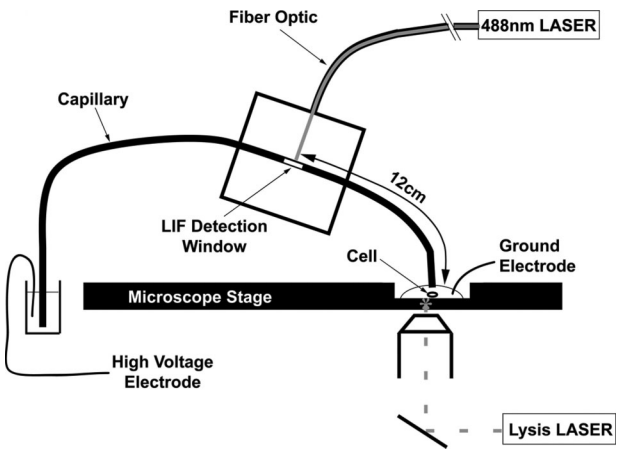

Figure 1. Schematic of the microscope stage-mounted CE setup and single-cell lysis system.

sing for 5 min with 0.1 N NaOH, 15 s with distilled water, 2 min with 0.1 N HCl followed by 1 min with ECB. Gravity loading was accomplished by lowering the outlet vial with respect to the microscope stage. Runs with fluorescent microspheres were performed without a voltage gradient using a combination of a vacuum pump and gravity induced siphoning to adjust the rate of fluid flow to match the intended average microsphere migration speed (i.e. the speed of the analyte to which the microspheres are being compared).

Determination of the Detection Threshold

Using gravity loading, we injected plugs of 1 nM Oregon green or base-hydrolyzed CFSE into a bare capillary by gravity loading and subjected it to CE with LIF detection. Because of the hydrophilic nature of uncoated silica capillaries and the large surface area present on the capillary tip compared to lumen size, significant spontaneous injection of dye occurred upon removal of the capillary from the dye solution (14). To account for this, as well as loading due to diffusion, the capillary tip was kept in the dye solution for a specific duration of time while either being subjected to gravity loading for the entire duration, half the duration or not gravity loaded at all. The peak areas were then plotted against the duration they were exposed to gravity loading and the slope of the relationship yielded the average electropherogram peak area per second of gravity loading. The amount of dye loaded per second due to gravity was then estimated using the Poiseuille equation (15) and used to determine average electropherogram peak area per molecule of dye. Next, by taking the minimum detectable peak size as 1,000 mV, based on a 10-s long peak at a height of double the height of the noise in the baseline (100 mV high; baseline noise, 50 mV), the detection threshold was determined from the number of molecules of dye needed to reach the minimum detectable peak size. Peak areas derived from microspheres and GFP expressing cells run on the CE system were integrated using Origin 7.0 software.

Capillary Coating

Capillaries were prerinsed with 0.1 M NaOH overnight, followed by a 5-min rinse of water at 60 mm Hg, a 30-min rinse with 1% w/v polybrene (PB) at 10 mm Hg, a 5-min rinse of water at 60 mm Hg, and a 30-min rinse of 1% v/v poly(vinyl sulfonate) (PVS) at 10 mm Hg. Between runs, capillaries were rinsed for 3 min with 1% PVS, and 1 min in ECB. Capillaries were stored overnight in 1% PVS.

Laser Lysis

Cells were placed onto a round glass #1 coverslip (Fisher) and allowed to settle for 1–2 min at low density (1 cell per field of view with a 100× objective). The microscope stage would then be translated to target a single cell and the lumen of the capillary inlet would be centered over the cell and lowered around the cell until capillary tip touched the slide. A single 3-ns pulse from a 532 nm Nd:YVO_4 frequency doubled Q-switched laser (Claire Lasers, Kitchener, ON) was directed into the right side port of the microscope. The laser pulse was focused through a 100× 1.35 n.a. objective onto the top surface of the glass cover slip (in the *z* direction) and either approximately 10 μm from the cell (offset position) or directly under the cell (centered position) in the *xy* plane. The laser pulse was triggered simultaneously with the high voltage power supply (negative polarity) such that the single cell lysate is injected via infusion by electroosmotic flow. The energy per laser pulse (2, 5, or 9 μJ) was measured using a laser meter (Molectron) at the microscope stage as measured with the 100× objective removed. Laser and high voltage power supply timing was controlled by Testpoint software.

Mechanical—Electrophoretic Lysis

To maximize the collection of the cell contents, in some experiments, cells were lysed using a combination of mechanical forces and electrophoration. In this case, lysis was performed without laser pulse, by crushing the cells between the capillary and the slide after application of the voltage gradient.

Results

System Design and Detection Limits

For this study, we have used a single-cell CE system coupled with laser lysis. The CE system was assembled using a compact PMT and fiber optic delivery of the excitation laser source allowing the LIF detection system to be mounted on the stage of a microscope (Fig. 1) resulting in a significant reduction in the length of capillary required to reach the center of the sample holder on the microscope stage. The length of capillary from the cell sample to the detection window was 12 cm compared with the length greater than 45 cm in other similar systems (2–7); this represents a greater than 3-fold reduction in electrophoretic runtime. We estimated the detection sensitivity of the system using decreasing concentrations of fluorescent dyes. Two experiments using CFSE indicated that the detection limit was 11,400 ± 600 and 14,000 ± 2,000 molecules. The detection threshold measured with Oregon green was 13,600 ± 2,000 molecules.

SE Determination

The SE is an important parameter in single-cell CE since it is directly proportional to the strength of the signal captured (i.e. it represents the fraction of analyte that reaches the detection window). Therefore, the SE must be known before absolute quantitative measurements can be made from cells. We evaluated the SE of the system for GFP-HEK 293T cells by comparing the average fluorescence of whole cells using flow cytometry to the average fluorescence detected after cell lysis with CE–LIF. One of the greatest challenges in the determination of the SE is the large variability in fluorescence from cell to cell. We have observed this heterogeneity for GFP-HEK 293T cells (Fig. 2A) and we have reduced this inherent variability by obtaining cell samples of homogeneous fluorescence (Figs. 2B and 2C) by using FACS.

To evaluate the SE, the fluorescence signals from the FACS-sorted GFP-expressing cells and fluorescent calibration microspheres were first measured with an analytical flow cytometer (Figs. 2B and 2D, respectively). The fluorescence sig-

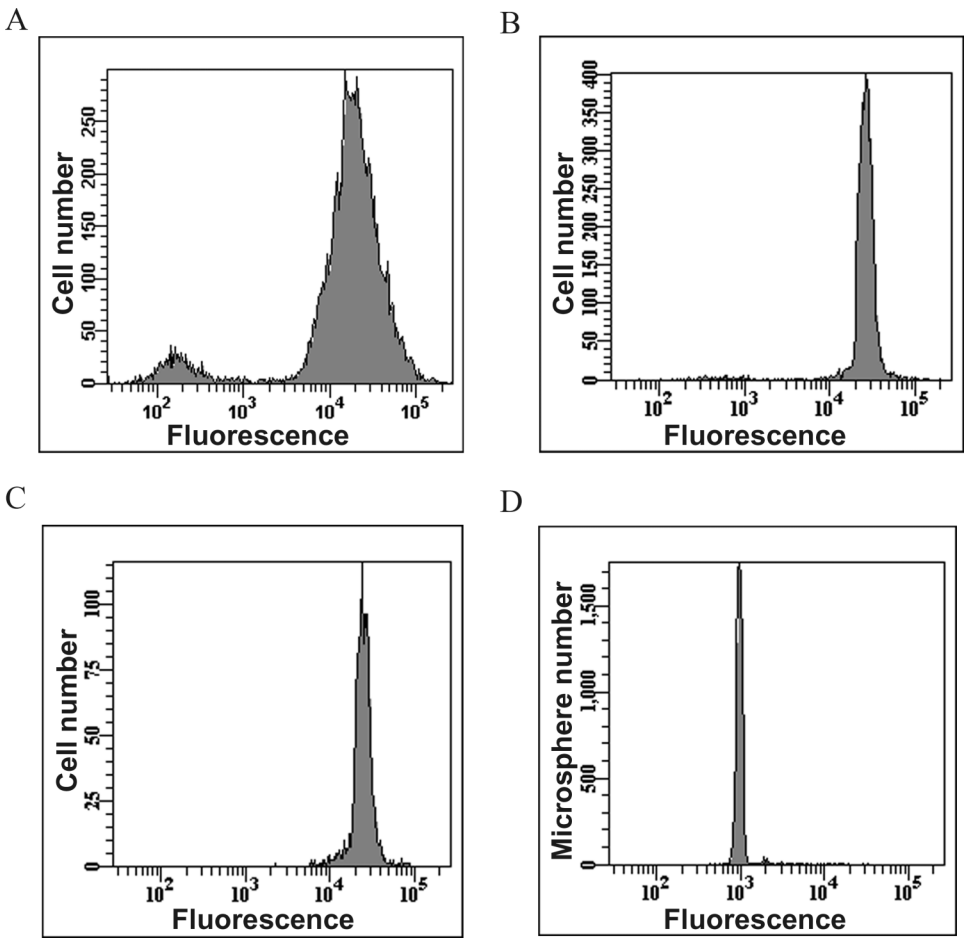

Figure 2. Flow cytometry histograms depicting the fluorescence distribution of (**A**) GFP-HEK 293T cells of heterogeneous fluorescence prior to FACS; (**B**) GFP-HEK 293T cells of homogeneous fluorescence immediately after FACS, which corresponds to the cell distribution at the beginning of an experiment; (**C**) GFP-HEK 293T cells 14 h after FACS, which corresponds to the cell distribution at the end of an experiment; (**D**) the calibration microspheres. Histograms A—C depict events gated as high forward scatter and low side scatter (the gating strategy is presented as supplementary material). For the fluorescence intensity measurements, the bandwidth of the emission filter was 535/50 (510—560 nm).

nals from single cells and single microspheres were next measured with the CE–LIF system (Figs. 3A and 3B, respectively). Since the average peak areas of single cells from these two measurements cannot be directly compared because of differences in excitation laser powers, PMT gain, and fluorescence capture efficiency between the CE system and the flow cytometer, the signals from the microspheres were used to normalize the relative fluorescence obtained from the two instruments. The microspheres were run on the CE system using pressure driven flow such that their velocity would match the average velocity of GFP for the runs performed on that day. The purpose of this procedure was to ensure that residence times in the detection area of the CE system were the same for both GFP and the microspheres. This was accomplished by filling the capillary with a low concentration of hydrolyzed CFSE, placing it into an ECB reservoir and determining the level of vacuum required at the capillary outlet to cause the CFSE/ECB interface to reach the detection window at the same time that GFP was detected in previous runs. Using this level of vacuum, the fluorescent microspheres were loaded into the capillary for analysis.

The SE was then derived by normalizing the average cell fluorescence peak area for both the flow cytometric and CE analyses by dividing by the microsphere fluorescence peak areas determined by both instruments as shown below:

$$F_{CE} = \frac{C_{CE}}{M_{CE}} \quad (1)$$

$$F_{FC} = \frac{C_{FC}}{M_{FC}} \quad (2)$$

$$SE = \frac{F_{CE}}{F_{FC}} \quad (3)$$

where F_{CE} represents the normalized mean peak area of cells as determined by CE, C_{CE} represents the mean peak area of

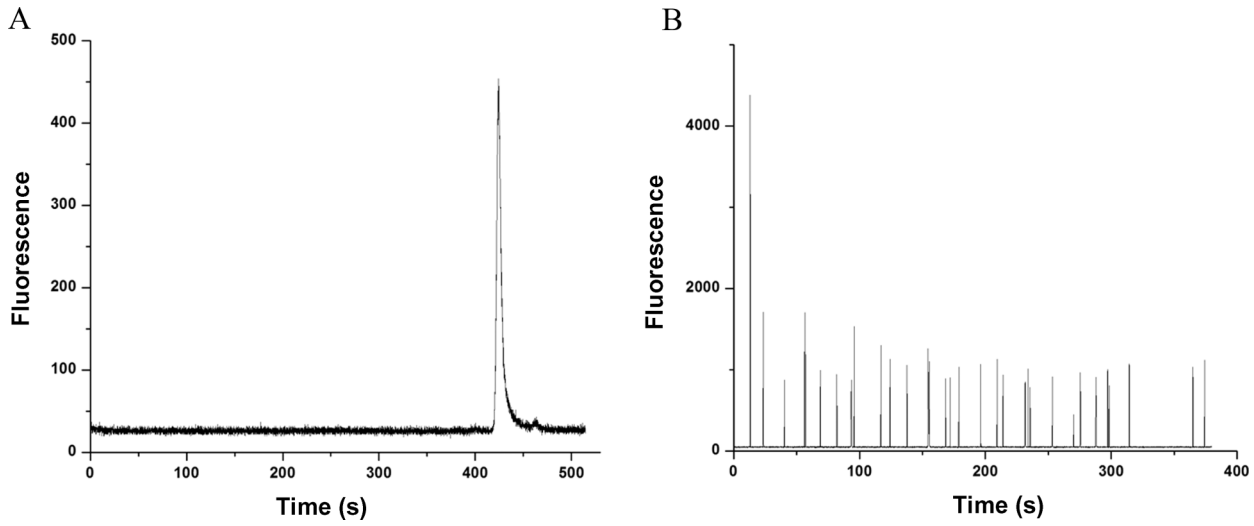

Figure 3. (**A**) Representative capillary electropherogram of a single GFP-HEK 293T cell sampled using laser lysis. (**B**) Representative graph of microspheres migrating through the CE system due to pressure induced flow; flow was controlled such that the speed of the buffer matched the speed at which GFP was calculated to migrate through the capillary due to electrophoresis. For the fluorescence intensity measurements, the bandwidth of the emission filter was 535/50 (510—560 nm).

GFP cells, and M_{CE} represents the mean peak area of fluorescent microspheres determined using the CE system. Likewise, F_{FC} represents the normalized mean peak area of cells as determined by flow cytometry, C_{FC} represents the mean peak area of GFP cells, and M_{FC} represents the mean peak area of fluorescent microspheres determined using the flow cytometer. Assuming no changes in fluorescent quantum yield of GFP in the cell versus in ECB, the normalized fluorescence is the same between the flow cytometer and the CE system at 100% SE.

Effects of Laser Pulse Energy and Pulse Placement on SE

Using the approach described in the previous section, we determined the SE of GFP-HEK 293T cells using different laser pulse energies and pulse positions relative to the cell in the *xy* plane for four different capillaries, including three coated capillaries. Because of inherent variations between individual capillaries, the SE results are presented separately (Figs. 4A–4D). First, we measured the SE using an uncoated capillary (Fig. 4A) with a laser pulse energy of 2 μJ and the pulse positioned 10 μm lateral to the cell in the *xy* plane (offset). We also lysed cells using a combination of electrophoration and mechanical lysis (Mech) to compare the efficiency of laser cell-sampling with that of another method. In this first experiment, we found that the SE using laser lysis was lower than using electrophoration/mechanical lysis ($P \leq 0.043$, one-tailed *t*-test). The electropherograms of the cells lysed with a combination of electrophoration and mechanical lysis appeared similar in shape to those obtained with laser lysis (data not shown); however, some additional peaks were apparent after the initial peak because of the slow nature of the mechanical perturbations (data not shown).

To reduce runtimes further and improve GFP sampling by reducing capillary wall adsorption of cellular proteins, we next tested PB/PVS coated capillaries which have been shown to reduce protein interaction by providing a hydrophilic capillary wall surface which remains negative in charge but is not capable of forming hydrogen bonds (16). Figures 4B–4D present the SE for three different PB/PVS coated capillaries. In the first experiment performed with this coating we compared the impact of the laser pulse location on the SE. Figure 4B shows that the SE obtained with a pulse centered on the cells is higher than with the offset position ($P \leq 0.055$, one-tailed *t*-test).

We next tested different laser pulse energies to determine whether using higher values could lead to more complete cell disruptions, or whether increased energy would lead to either degradation of GFP or excessive diffusion. Figures 4C and 4D show that, in all cases tested, low energy pulses (2 μJ) led to the highest SE whereas higher energy (9 μJ) resulted in lower SE ($P \leq 0.046$ in Fig. 4C and $P \leq 0.057$ in Fig. 4D, one-tailed *t*-tests). Pulse energies lower than 2 μJ were not examined, since the cell often remained undisrupted. In the same series of experiments, we also tested the impact of the pulse location (centered vs. offset). Similar to what was observed with the first coated capillary (Fig. 4B), focusing directly on the cell (centered) appeared to improve the collection of the cell contents for the second and third coated capillaries (Figs. 4C and 4D); however, the differences in SEs in these experiments did not reach statistical significance. For the series of experiments presented in Figure 4C, the SE obtained with a low (2 μJ) laser pulse energy combined with a centered pulse location was similar to that obtained with electrophoration/mechanical lysis ($P \geq 0.59$, two-tailed *t*-test). Interestingly, the higher absolute values of the SE obtained with the first PB/PVS

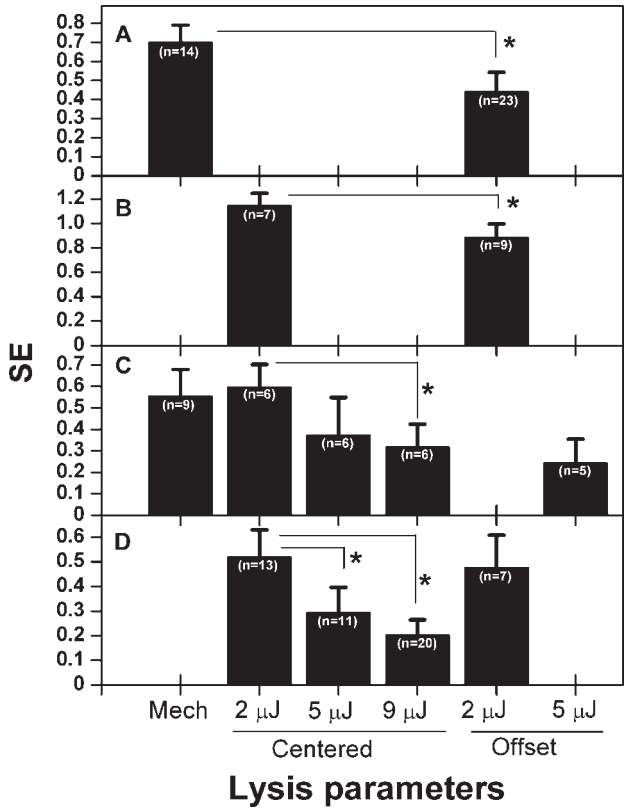

Figure 4. Effect of laser pulse energy (2, 5, 9 μJ) and pulse placement in the *xy* plane relative to the cell (centered or 10 μm offset) on the SE using a bare capillary (**A**) and three different PB/PVS coated capillaries (**B—D**). The pulse position in the direction of the *z* axis was at the glass slide/buffer interface. In some experiments, cell lysis was performed with a combination of electrophoration and mechanical lysis (Mech). The error bars represents the standard error of the mean (SEM) and "*n*" is the number of cells sampled. * indicates differences that are statistically significant ($P \leq 0.05$).

coated capillary (Fig. 4B) when compared with the bare capillary (Fig. 4A) suggest a beneficial effect of the coating. However, in the two subsequent experiments with coated capillaries (Figs. 4C and 4D), we measured SEs similar to those obtained with the uncoated capillary (Fig. 4A).

Discussion

In this study, we have used a compact microscope-mounted single-cell CE–LIF system to evaluate the impact of nanosecond laser pulse energies and pulse placement on the SE of GFP expressed in single HEK 293T cells. We have observed a significant inverse correlation between the SE and laser pulse energy, suggesting that GFP can be partially degraded during the process of plasma formation. However, a SE near ~1.0 was obtained with one of the coated capillaries using a centered laser pulse of 2 μJ. This indicates that sampling of GFP can be maximized using this lower pulse energy, without systematically compromising the efficacy of the lysis process.

Although higher laser pulse energies led to lower SEs, increasing the distance between the cell and the pulse by 10 μm did not improve the sampling. This is consistent with the observation that sampling with the laser pulse directly centered below the cell generally resulted in higher SEs than laterally offset laser pulses. This may reflect the fact that a fraction of the cell contents is directed away from the capillary lumen in the offset position. The diffusion caused by intense mixing of buffer upon the laser pulse may spread the analyte over a larger area, reducing the amount which is directly in the path of the electrical current. Since GFP is negatively charged at pH 7.4 and migrates against the electroosmotic flow, this effect would be enhanced. Alternatively, it is also possible that laterally offset laser pulses may have only provided sufficient energy to cause partial lysis. This could result in partial sampling of the cell, with the cell remnants either sticking to the capillary wall, or passing by the detector too quickly.

Besides laser parameters, an important factor that can affect the SE is the adsorption of analytes and cellular proteins (i.e. biofouling) on the surface of the capillary wall and inlet. Protein adsorption issues can be addressed with the use of specialized capillary coatings and buffers. We chose to use a PB/PVS capillary coating (16) which has been shown to reduce protein wall interactions in the analysis of numerous proteins. However, in the present study, this coating did not systematically improve the SE; the SE measured with each of the three PB/PVS coated capillaries in similar sampling conditions (centered, 2 μJ laser pulse) varied significantly (from ~0.6 to ~1.0). This suggests that capillary-to-capillary variations during the coating procedure can also have important effects. This observation is also consistent with the reported variations in electroosmotic flow and performance of different coated capillaries (6,17,18). However, since the peaks obtained even with uncoated capillaries were reasonably sharp, and exhibited a high degree of symmetry this suggests that capillary wall interactions were limited.

There are factors which may have affected the precision of our measurements of SE. First, reanalysis by flow cytometry of the distribution of the cell fluorescence at the end of the experiments revealed that a small population of cells underwent a complete loss of GFP content from the time they are collected from the FACS to the time they are analyzed by CE, up to 14 h later. These cells also shifted in size and shape, as evidenced by a reduction in forward scatter and increase in side scatter measurements from the flow cytometer. Thus, although these cells appear morphologically different when visualized with the microscope before sampling, there is a significant probability that one of these cells will be selected in a given run, leading to a decrease in the average SE measured in an experiment.

It is unlikely that the difference in the quantum efficiency of GFP in the cellular context compared with GFP in ECB affected the precision of our SE measurements. This change is probably not significant since the chromophore of GFP is protected by the rest of the GFP protein which forms a protective β-can structure around it, shielding it from quenchers such as molecular oxygen and halides (19). Furthermore, unlike for wildtype *Aequorea* GFP, the extinction coefficient of the GFP

used in this study (S65T, F64L) remains stable even at concentrations that are sufficiently high to drive dimerization (20). Although GFP is sensitive to changes in pH, the buffer used for CE was at pH 7.4, which matches the pH of the cytoplasm of cells.

Sims et al. (2) determined a SE of 100% for a similar CE–LIF system coupled with laser lysis using a fluorescence microscope-based approach. In contrast to our study, this high SE could be obtained with uncoated capillaries. This difference may be due to the fact that Sims et al. used cells loaded with fluorescent dyes which are generally stable and, unlike proteins, do not have a propensity to adsorb on the capillary wall. Furthermore, the chromophores of fluorescent dyes such as Oregon green 488 are not sheltered in GFP. Thus it is possible that differences in quantum efficiency between the fluorophore observed in the cell versus the fluorophore liberated in buffer may have superficially increased the SE they observed. Another factor that can potentially affect the precision of this fluorescence-microscope based approach to determine the SE, is the necessity to assume a specific cell volume (e.g. 1 pL).

The method that we have developed to determine the SE of this CE system is equally applicable to microfluidics-based electrophoresis systems for single-cell analysis (1) since ultimately, it reflects the fraction of analyte that reaches the detection area. This approach can be used to evaluate and optimize different strategies to lyse cells and decrease protein adsorption. Improved sampling and the ability to estimate losses will allow more sensitive and precise quantitation of fluorescent analytes in cells. This will expand our ability to measure signaling events at the single cell level.

Acknowledgments

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