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Technical Notes

Instrumentation for Chemical Cytometry

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Capillary electrophoresis is ideally suited to chemical analysis of individual cells. Small mammalian somatic cells (∼15 μm in diameter) can be analyzed by injecting the intact cell into a capillary, lysing the cell, separating and detecting the cellular components, and reconditioning the capillary prior to the next injection. In this paper, we report on technical improvements to single-cell analysis. We designed an inexpensive multipurpose single-cell injector that facilitates the following: (i) monitoring of injection, (ii) reproducible pressure- or electrokinetic-driven injection of the cell, (iii) complete cell lysis by SDS within 30 s of injection, and (iv) pressure-driven capillary reconditioning. Furthermore, we report on the analysis of glycosylation and glycolysis in single human carcinoma cells (HT29 cell line). The reliability and quality of the analysis is confirmed by comparing electropherograms from single cells and those from purified cell extracts.

Cytometry studies the characteristics of single cells. Flow and image cytometry are used to monitor a few components from a large number of cells. Typically, three or fewer components are monitored simultaneously. Thousands of cells are analyzed within 1 min using flow cytometry.

Much more information, at a much lower speed, can be obtained by performing chemical analysis of single cells. These analyses are carried out with microscale separation tools, such as capillary electrophoresis (CE), combined with highly sensitive detection techniques, such as laser-induced fluorescence (LIF).[1−2] To distinguish this approach from classical cytometries, we introduced the term *chemical cytometry*,[3] which refers to the use of chemical instrumentation for qualitative and quantitative analysis of the chemical contents in single cells.

Several techniques have been reported for manipulation of cells for chemical cytometry.[4] Relatively large cells, for example oocytes or neurons, can be homogenized in a microvial and the homogenate can then be prepurified and assayed using microcolumn chromatography. This type of assay was first demonstrated by Jorgenson and co-workers.[5] Ewing's group showed that, for large cells, subcellular sampling was also possible.[6,7] Allbritton and co-workers used a laser-induced shock wave to lyse a cell within 30 ms and immediately inject the cellular lysate into a capillary.[8] Yeung's group showed that intact small cells, like most mammalian somatic cells, can be injected into and lysed within the capillary before analysis.[9] No matter the size of the cell, however, reproducible sampling, cell lysis, electrophoretic separation, and capillary reconditioning are essential prior to subsequent analyses.

For the injection of intact cells, the capillary is immersed into the cell suspension so that the capillary's lumen is in close proximity to the cell chosen for analysis. The cell is then drawn into the capillary, along with a plug of cell medium, by a flow induced by either electroosmosis or siphoning.[4,10] Since the cell medium can affect the migration of different chemical species, a small, reproducible volume of cell medium is desired during each single-cell injection to minimize variations in migration profiles.

Once injected into the capillary, the intact cell must be lysed. Four techniques of cell lysis inside the capillary have been described: (i) ultrasonic treatment, (ii) strong electric field generated with a Tesla coil, (iii) low-ionic-strength running buffer, and (iv) injection of a surfactant following cell injection.[11−14]

In many applications, a surfactant is present in the running buffer to improve separation, for example, in micellar electrokinetic capillary chromatography. This buffer can diffuse into contact with the cell, and most somatic cells are lysed by contact with the surfactant.[3,15,16]

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When a cell is lysed inside a capillary, cellular debris, created from insoluble cellular components, can accumulate on the capillary walls, which can affect subsequent CE separations.[12] Therefore, thorough cleaning and reconditioning of the capillary are essential when capillaries are being reused during multiple analyses of single cells by CE.

Here we have optimized conditions for cell injection by siphoning and electroosmosis, cell lysis by a surfactant present in the running buffer, and removal of cellular debris from the capillary by pressure-driven flow of cleaning solutions. This technology was applied to the analysis of glycosylation and glycolysis in single human carcinoma (HT29) cells.

EXPERIMENTAL SECTION

Electrophoresis. The electrophoresis system with LIF detection used in this study has been described in detail elsewhere.[17] Separations were carried out at an electric field of 400 V/cm in a 40-cm-long, 20-μm-i.d., and 140-μm-o.d. fused-silica capillary (Polymicro, Phoenix, AZ).

Multipurpose Single-Cell Injector. The injector allows injection of intact cells into the capillary while facilitating lysis within the capillary, separation of cellular components, and reconditioning of the capillary. Once installed, the capillary was not handled. The major components of the injector are a capillary Plexiglas holder and a vial holder, Figure 1. The transparent holder was mounted between the condenser and the lens of an inverted microscope without blocking light transmission for bright-field observation, which is essential for injection of an intact cell into a vertically oriented capillary. See Supporting Information for a detailed description and a sketch of the capillary and vial holders.

The injector also employs a three-way solenoid valve (General Valve, Florham Park, NJ) that is connected to a water-filled tube that terminates in a water-filled beaker 112 cm below the injection reservoir. When engaged for siphoning injection, the valve connects the outlet of the capillary through the sheath flow cuvette to the water column, which generates a partial vacuum to draw the cell into the capillary. An electronic timer controlling the valve provides a very stable injection time (RSD $\approx$ 1%). In the standby position, the three-way solenoid valve is connected to a reservoir that allows for a reverse flow of the buffer (from detection to injection end) inside the capillary. This condition is required for effective cell lysis as described below.

Running buffer for the separation is supplied from the Teflon vial holder, Figure 1. The holder has a compartment for a small vial. A Viton O-ring around the compartment guarantees a gastight seal when the vial holder is fastened to the capillary holder. The vial holder has three pins that fit into three complementary holes of the capillary holder. These pins and holes align the vial and capillary holders while they are connected. The pins also trigger a safety interlock situated inside the body of the capillary holder to prevent accidental contact with high voltage.

The capillary holder has a gas channel with an opening in close proximity to the capillary and the electrode. The gas channel is used to pressurize the solutions during cleaning and reconditioning of the capillary and during alignment of the optical detection system (see below). The gas channel is connected via a two-way solenoid valve to a cylinder of helium gas, equipped with a low-pressure model R04-200-RNKA gas regulator (IMI Norgen, Littleton, CO). The valve is controlled by an electronic timer.

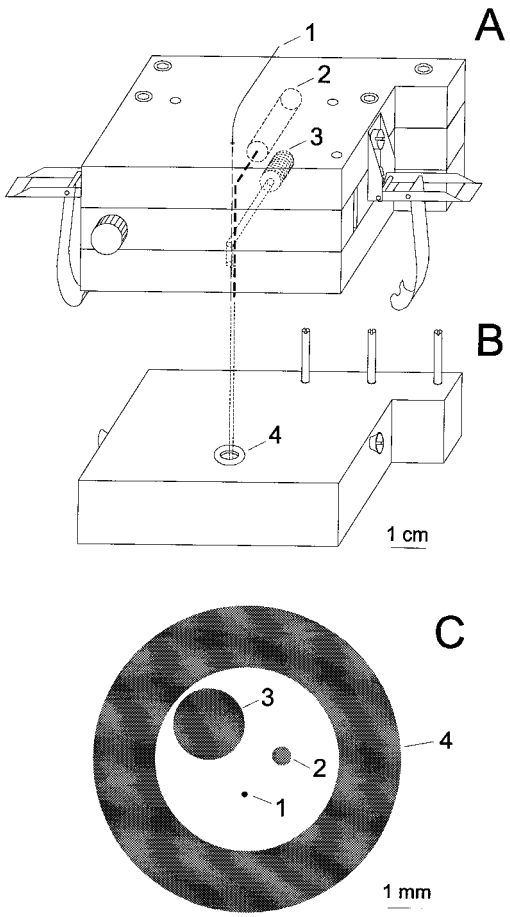

Figure 1. Key elements of the interface for the chemical analysis of single cells by capillary electrophoresis: (A) capillary holder, (B) vial holder, and (C) relative positions and sizes of (1) the capillary, (2) the electrode, (3) the gas channel opening, and (4) the Viton O-ring.

Alignment. A vial containing $\sim 10^{-9}$ M rhodamine 6G was placed in the vial holder, which was fastened to the capillary holder. The dye solution was pressurized to 480 kPa to fill the capillary with the dye. Once the capillary was filled, the pressure was set to 25 kPa to provide flow of the dye that was similar to electroosmotic flow under standard separation conditions. The alignment of the optical system was adjusted while the dye was flowing to maximize the fluorescence signal. After alignment, the dye was flushed from the capillary, using the cleaning procedures described below, and the capillary was reconditioned with the running buffer.

Stable flow is required for alignment. The sample stream diameter in a sheath-flow detector, and hence the fluorescence signal, is dependent on the sample flow rate.[18] We used pressure-driven flow for alignment because it is not effected by adsorption of impurities to the capillary wall, which can modify the electroosmotic mobility during electrokinetically driven pumping of the dye.

Analytical Procedures. Cell injection was carried out from 50 μL of cell suspension deposited on an ultralow attachment plate (Corning Costar, Acton, MA). The injection process was observed

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under an inverted fluorescence microscope (model IMT-2, Olympus, Melville, NY), equipped with a CCD camera. A hydraulic model MX630R micromanipulator (Newport, Nepean, ON, Canada) was used to position the capillary.

The capillary holder's position was first adjusted so that the capillary was centered in the microscope's field of view. The capillary holder was lowered until the capillary contacted the cell suspension. A cell was superimposed with the inner bore of the capillary by moving the microscope stage. The capillary was lowered to ~10 μm from the cell support surface. The cell was then injected by siphoning or by electroosmosis. Injection conditions were 1-s injection time, 11-kPa negative pressure (for siphoning injection), or 10-kV high voltage (for electroosmotic injection). With these conditions, the cell was introduced into the capillary along with a ~370-μm-long plug of cell medium.

After cell injection, the capillary holder was raised several centimeters above the microscope slide. The vial holder was attached to the capillary holder to immerse both the capillary and the electrode in the separation buffer. High voltage was then applied to the electrode to effect separation.

The capillary was reconditioned after analysis of each cell. To recondition the capillary, 0.1 M NaOH, water, and running buffer were placed in the vial holder and pumped through the capillary by helium gas at 480 kPa for 1 min each.

Reagents. Unless otherwise stated, all reagents used in this work were commercial chemicals of analytical grade used without additional purification. Aqueous solutions were prepared using distilled deionized water and filtered with 0.22-μm pore size disposable filters (Millipore, Bedford, MA). The same buffer (10 mM phosphate, 10 mM phenylboronic acid, 10 mM disodium tetraborate, and 10 mM sodium dodecyl sulfate (SDS) at pH 9.0) was used as separation buffer and sheath-flow fluid.

Cell Culture Procedures. The HT29 cell line was grown to 80% confluence in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum and 40 μg/mL gentamicin at 37 °C in a 5% CO_2 atmosphere. For the analysis of glycosylation and glycolysis, the cells were incubated for 18 h with 25 μM LacNAc-TMR.[19] After incubation, the cells were washed eight times with phosphate-buffered saline (PBS) to remove the residual substrate from the medium. For single-cell experiments, the cell suspension was diluted with PBS to a final density of 10^4 cells/mL. Cellular extract of TMR-labeled sugars was prepared as described earlier.[3]

To visualize the cellular membrane in fluorescence microscopy, the cells were stained with DiI (Molecular Probes, Eugene, OR).[20] A working solution of DiI was prepared immediately before the experiment by adding 5 μL of DiI stock solution (3 mg/mL in DMSO) to 100 μL of diluent C (Sigma, St. Louis, MO). Then, 105 μL of the DiI working solution was added to 0.5 mL of cell suspension (total 2×10^6 cells) in PBS. The cells were incubated for 5 min at room temperature, washed 5 times with PBS to remove unreacted DiI, resuspended in PBS, and analyzed.

RESULTS AND DISCUSSION

Cell Injection. Injection of intact cells into the capillary is usually performed on an inverted microscope with the capillary at an inclination. The advantage of such a design is that a capillary holder can be mounted to the side of the light pathway. The main disadvantage of the inclined position is that the capillary tip hasto be tapered, so that the orifice can be placed close enough to the cell for injection.[4,9] The use of tapered capillaries is not ideal because extra time is required to etch the capillary and the tapered capillaries are fragile.

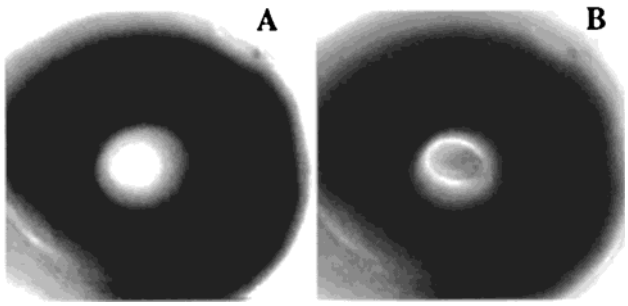

Figure 2. Microphotograph of the vertically aligned capillary tip: (A) without the cell, (B) with the cell superimposed with the inner bore of the capillary. The cell can be seen very well since the inner bore of the capillary appears very bright under phase contrast.

We designed a light-transparent capillary holder that can be mounted between the light source and the lens of a microscope without significantly influencing the image quality. The capillary can then be mounted vertically. The vertical alignment of the capillary has three advantages: the capillary need not be tapered, the capillary can be brought very close to the cell, and the cell can be seen while the inner bore of the capillary is placed on it, Figure 2.

Siphoning and electrokinetic flow have been used to inject intact cells into a capillary.[4,10] In both cases, the cell is injected by the drag force created by the fluid flow over the cell and toward the capillary. The drag force includes two components: frictional and pressure drag. Frictional drag occurs due to the interaction of the boundary layer of fluid with the surface of the cell. Pressure drag occurs due to the positive pressure difference between the upstream and downstream sides of the cell. Both frictional and pressure drags are proportional to the velocity, v, of the fluid flow washing the cell. Therefore, total drag force is also proportional to v:

$$F_{\text{drug}} = av \tag{1}$$

where a is a constant for identical cells and fluids. Before being injected, the cell is washed by the fluid flow outside the capillary. The fluid flow converges into the capillary. The flow continuity condition and the convergent nature of the flow require that the velocity increase with decreasing distance, R, from the capillary entrance:

$$v \cong (r^2/(R + r)^2)v_0 \tag{2}$$

where v_0 is the velocity of the fluid flow inside the capillary and r is the capillary inner radius. The velocity of flow inside the capillary is described by the Poiseulle and the EOF velocity

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equations for siphoning and electroosmosis, respectively:

$$v_0^{\text{siph}} = r^2P/8\eta L \tag{3a}$$

$$v_0^{\text{EOF}} = \epsilon\zeta U/4\pi\eta L \tag{3b}$$

where P is the pressure drop across the capillary, η is the viscosity of the buffer, L is the length of the capillary, ϵ is the dielectric constant of the buffer, ζ is the zeta potential, and U is the voltage applied to the capillary.

The drag forces on the cell in the case of siphoning and electroosmotic injections are

$$F_{\text{drag}}^{\text{siph}} \cong \frac{a}{8}\frac{r^2}{(R+r)^2}\frac{1}{\eta}\frac{r^2}{L}P \tag{4a}$$

$$F_{\text{drag}}^{\text{EOF}} \cong \frac{a}{4\pi}\frac{r^2}{(R+r)^2}\frac{\epsilon\zeta}{\eta}\frac{1}{L}U \tag{4b}$$

Thus, the drag force caused by siphoning can be increased by (i) reducing the distance, R, between the cell and the entrance to the capillary, (ii) decreasing the viscosity, η, of the buffer, (iii) increasing the capillary inner radius, r, (iv) reducing the length, L, of the capillary, and (v) increasing the pressure drop, P, across the capillary. The drag force caused by electroosmotic flow is affected by all the above factors except for capillary inner radius and pressure. Instead, it depends on zeta potential, ζ, which is a function of the density of negative charge on the inner capillary wall, and applied voltage, U.

The force of cell adhesion to the surface, F_{adh}, can prevent cell injection. Most cell types, including the tissue culture cells used in this study, tend to adhere to the surface of a plate or microscope slide. This adhesion is caused mainly by interactions between cell surface proteins and the surface of plastic or glass material. Live cells are usually negatively charged; therefore, electrostatic interaction can also influence adhesion if the surface is charged. That is, F_{adh} is determined by both cellular status and surface parameters such as the chemical nature, charge, and hydrophobicity of the surface. Obviously, the cell can be injected only if $F_{\text{drag}} > F_{\text{adh}}$.

We maximized $F_{\text{drag}}^{\text{siph}}$ using the three parameters, R, L, and P. First, aligning the capillary vertically allowed us to center it over the cell and thus reduce R to zero and consequently maximize the second term in eq 4a. Second, we chose the capillary length, $L = 40$ cm, close to the minimum possible for our instrument. Finally, we set up the maximum value of negative pressure $P =$ 11 kPa with a 112-cm water column driving the siphoning. Two other parameters, r and η, were predetermined by the average cell size of ~15 μm and by the choice of the optimal separation buffer for electrophoresis of TMR-labeled sugars, respectively.[19,21]

Using maximum $F_{\text{drag}}^{\text{siph}}$, we tried cell injection from ordinary microscope slides and from polystyrene microplates used for cell growth. We found that injection could not be achieved because the cell adhesion to the chosen surfaces was too strong. Cell adhesion force, F_{adh}, can be minimized by modifying the physical−chemical properties of the cell support surface.[22,23] We found that commercially available ultralow attachment plates reduced F_{adh} enough for cell injection at maximum achievable $F_{\text{drag}}^{\text{siph}}$. Therefore, these plates were used for all further studies.

Next, we studied the influence of the injection time on the efficiency of injection. Reliable injection could be achieved with maximum $F_{\text{drag}}^{\text{siph}}$ and injection times as short as 0.2 s. However, for short injection times, the cell was too close to the capillary opening after injection and could be pushed out by a continuous bulk flow of separation buffer from the detection to the injection end (see below). We chose to use an injection time $t = 1$ s.

Cell Lysis. The nature of siphoning or electroosmotic injection requires that the cell be injected onto the capillary with a plug of cell medium, PBS in our case. To achieve rapid lysis, the cell has to be close to the boundary between the running buffer and cell medium, so that SDS surfactant can rapidly reach the cell by diffusion. For the standard injection conditions, $P = 11$ kPa, $r =$ 10 μm, $t = 1$ s, and $L = 40$ cm, and assuming that the viscosity of the running buffer is equal to that of water (9×10^{-4} kg m^{-1} s^{-1}), the length of injected plug can be calculated using eq 3a to be ~370 μm. A microphotograph shows that after injection the cell is ~350 μm from the capillary entrance and is thus ~20 μm from the PBS−running buffer boundary, Figure 3A. Such a short distance between the cell and the running buffer ensured that SDS rapidly reached and lysed the cell within 30 s of injection, Figure 3B. Indeed, Einstein's formula for the diffusion of a 20-nm-diameter micelle predicts that the diffusion time over a 20-μm distance is less than 10 s. When an extra 370-μm plug of PBS was injected before the cell, the distance between the cell and the PBS−running buffer boundary was ~400 μm. In this case, the cell was not lysed within 5 min.

Reproducible cell lysis also required that the heights of the sheath fluid, waste, and the capillary tip be adjusted so that direct siphoning was prevented unless negative pressure was applied to the detection end of the capillary. Otherwise, cell medium would siphon into the capillary while preparing for cell injection and thus increase the time required for complete cell lysis. We adjusted the heights of fluid levels so that slow reverse siphoning was present when the capillary was immersed in the cell suspension. The linear rate of this reverse siphoning was chosen to be ~1 μm/s. The slow reverse siphoning ensured that PBS did not enter the capillary unless negative pressure or injection voltage was applied.

Once we optimized injection by siphoning and ensured that complete cell lysis occurred within 30 s of injection, we evaluated electrokinetic injection. Live cells have a negative charge at physiological pH.[24] The major contribution to this charge is believed to be sialic acid moieties associated with membrane proteins.[25] When positive voltage is applied to the cell suspension, the cells' negative charges cause an electrophoretic force opposite to the drag force, $F_{\text{drag}}^{\text{EOF}}$. The electrophoretic force can, in principle, influence proper cell injection and thus cell lysis. We found, however, that electroosmosis at 10 kV was as effective for

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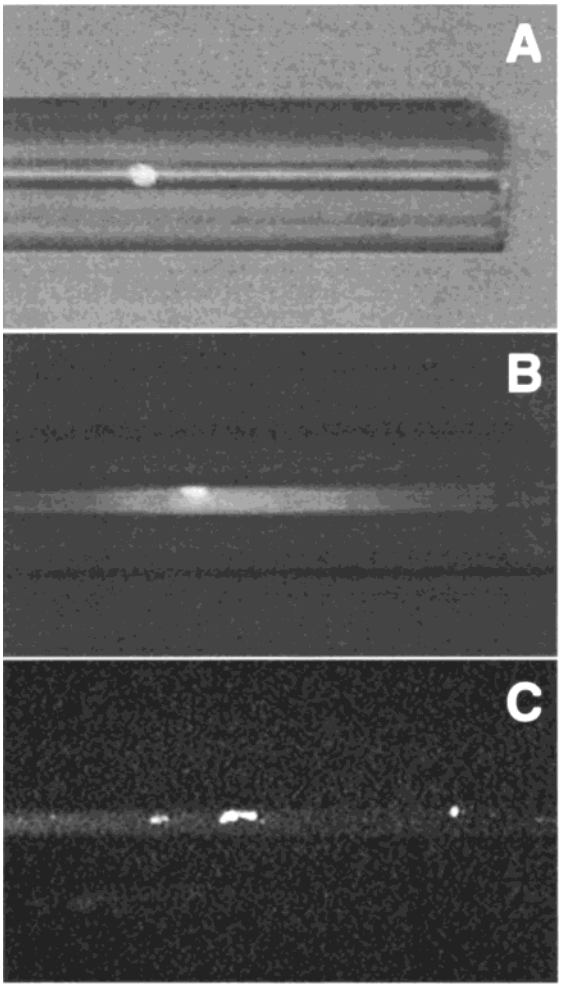

Figure 3. Microphotograph of the capillary with a fluorescent image of (A) DiI-stained cell immediately after injection, (B) DiI-stained cell 30 s after injection, and (C) fluorescent cellular debris 10 s after high-voltage application.

cell injection as siphoning at a negative pressure of 11 kPa. Thus, the electrophoretic force with respect to the cell was much less than $F_{\text{drag}}^{\text{EOF}}$. Moreover, we found that the cells lysed completely within 30 s after electrokinetic injection via a 10 kV × 1 s pulse. We confirmed that, regardless of the nature of drag force (siphoning or electroosmosis), the net result was the same: cell lysis occurred within 30 s of injection.

When separation voltage was applied, the lysate migrated to the detector. However, a lipid part of the lysate tended to adsorb to the capillary wall. This adsorption was clearly seen when DiI fluorescent probe was used to label cellular membrane, Figure 3C. Cell debris did not influence the quality of separation in the first run. However, as cellular debris accumulated from run to run, the migration time increased (data not shown). Lee and Yeung reported similar observations.[12] Fluorescence background also increased. Therefore, we cleaned and reconditioned the capillary after every cell separation. This procedure completely recovered the capillary; no deterioration was observed in the separation quality for the lifetime of the capillary.

Separation. The mass detection limit (3σ) of the instrument was 10^{-21} mol of LacNAc-TMR. Separation of the metabolites of LacNAc-TMR in single cells was performed under optimal conditions. We found that the chemical cytometry electropherograms were similar to that for prepurified cell extract, Figure 4. In particular, the migration time for chemical cytometry was as reproducible as that for the cellular extract.

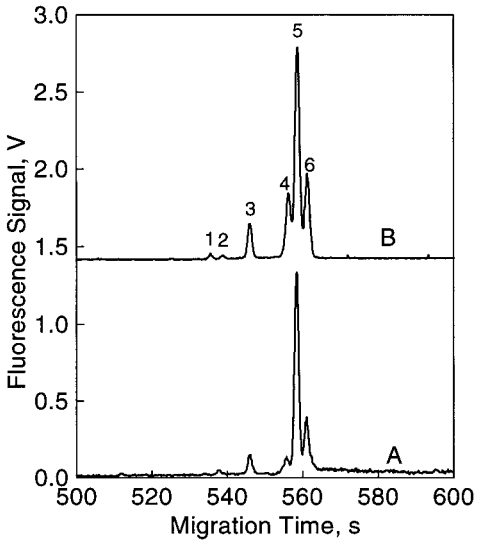

Figure 4. Electropherograms of TMR-labeled species obtained from (A) a single HT29 cell and (B) an HT29 extract. Curve B is offset for better viewing.

As we expected, the fluorescence intensity varied significantly from cell to cell. The value of total fluorescence intensity was calculated as the sum of heights for all six peaks in the electropherograms and reflected the level of substrate uptake by the cells. Relative standard deviation (RSD) of total fluorescence intensity from cell to cell was 45% for 20 cells analyzed. On the other hand, RSD of total fluorescence in CE of cellular extract using the same experimental conditions was only 10% for 18 subsequent experiments. To confirm variation in fluorescence intensity of single cells, flow cytometry was used. Flow cytometry measurement of total fluorescence of TMR-labeled products resulted in an RSD of 49%. This value is in good agreement with the RSD of 45% obtained by CE analysis. Similar variation in hemoglobin and other components' concentrations have previously been shown for erythrocytes[12,13,20]

CONCLUSIONS

Chemical cytometry using intact-cell CE is a powerful tool to study chemical contents in individual cells. To obtain high reproducibility of cell analysis we designed a multipurpose injector that allowed all of the analytical operations to be carried out with the capillary permanently mounted on an inverted microscope. Under optimal conditions, the cell and a constant volume of cell suspension are injected into the capillary and the cell is completely lysed within 30 s by the SDS-containing running buffer. The quality of electropherograms of single cells was similar to that of prepurified cellular extracts.

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SUPPORTING INFORMATION AVAILABLE

A detailed description of the capillary holder and the vial holder and an isometric exploded-view sketch emphasizing the assembly details are available. This material is available free of charge via the Internet at http://pubs.acs.org.

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