Laser−Micropipet Combination for Single-Cell Analysis

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Due to its potential for exquisite mass detection limits and resolving power, capillary electrophoresis is used for biochemical measurements on single cells; however, accurate measurements of many physiological parameters require sampling strategies that are considerably faster than those presently available. We have developed a laser-based technique to lyse single, adherent, mammalian cells on millisecond time scales. The cellular contents are then introduced into a capillary where electrophoretic separation and detection are performed. Improved temporal resolution of biological measurements results from the extremely rapid lysis made possible by this method. Additionally, the cell is not perturbed by mechanical or electrical stresses prior to sampling. Such disturbances can alter cellular physiology, resulting in inaccurate measurements. The fast cell lysis, the absence of cellular stresses prior to lysis, and the application to adherent mammalian cells are significant refinements to CE-based measurements on single cells. With this laser−micropipet combination, it will be possible to measure the intracellular concentration of molecules that change on subsecond to second time scales, for example, substrates of many cellular enzymes.

In the past decade, capillary electrophoresis technologies have been established as important tools for biochemical studies of living cells.[1−3] A number of groups have been at the forefront of applying this technology to the analysis of single cells. The Ewing, Jorgenson, and Sweedler groups have pioneered the use of capillary electrophoresis (CE) to study model neuronal cells from nonmammalian species.[3−7] By virtue of the large size of these neuronal ganglion cells (0.1−0.2-mm diameter), subcellular measurements have been possible. Typically the inlet of the capillary

is etched to a fine point and is used to sample cytoplasm from a cell or disrupted cellular fragment. The cytoplasmic contents are separated within the capillary and detected on- or off-line. The groups of Yeung and Ewing initially demonstrated the vast potential of capillary electrophoresis for the study of mammalian cells.[8−12] For many studies of these much smaller mammalian cells (10−50-μm diameter), an entire cell is loaded into the capillary and then the cell is lysed with a hypotonic or detergent-containing buffer. The cellular contents are separated in the capillary and detected by a variety of methods such as laser-induced fluorescence (LIF), or electrochemical detection.

For measurements on mammalian cells, important issues to consider are the temporal resolution of the measurement technique and the effects of perturbing the cell prior to complete lysis (breakup of the plasma membrane and release of cellular contents). The manner in which the cell is lysed will govern the time required to terminate biochemical reactions (the temporal resolution of the biological measurement) and will influence cellular processes occurring during the period of sampling. Many biological events take place on time scales of seconds or less. Enzymes typically have turnover numbers on the order of $1-10^4/$s.[13] Metabolite concentrations can change by greater than 10-fold in 1 s.[14] Accurate measurements of such cellular properties require that complete cell lysis occurs rapidly in comparison to the rate of change of the measured parameter. If disruption of the cell membrane proceeds at too slow a rate, significant changes in the parameter of interest can occur during the course of cell lysis, resulting in an inaccurate view of the actual physiological state of the cell. Additionally, membrane permeabilization during lysis results in the influx of extracellular ions, such as calcium (Ca^{2+}), activating many enzymes including kinases, phosphatases, proteases, and nucleases.[14] Even after disruption of the plasma membrane, biochemical processes will proceed until reactions are terminated, i.e., by separation of reactants, usually by diffusion and electrophoresis, or by inactivation of reactants, typically by denaturation of proteins.[15] Accurate measurement of many

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cellular properties will require complete cell lysis in milliseconds or faster.

Another important consideration in the use of CE for making whole cell measurements is the effect of manipulating the cell prior to sampling. For nonadherent cells, the use of electroosmotic flow (EOF) to move the cell into the capillary inlet may impact the cellular process to be measured. A large body of literature exists on the biological effects of electric fields.[16−19] Electric fields as low as 1−15 V/cm can cause membrane lipids and proteins to migrate into highly concentrated clusters.[20−24] This clustering of molecules in cells can have profound effects on cellular physiology.[25,26] For example, many pathways involving tyrosine kinases, such as platelet-derived growth factor signaling, are activated by the dimerization or clustering of receptors.[25,26] In most instances, receptor aggregation by any mechanism appears to be sufficient for pathway activation, suggesting that electric field-induced clustering will also lead to activation. Application of high electric fields (>167 V/cm) can induce permeabilization of cell membranes which can have drastic effects on cellular physiology.[27,28] Cells exposed to 167 V/cm for as little as 5 ms have localized increases in cellular permeability with concomitant influx of Ca^{2+}.[28] As discussed above, such Ca^{2+} influx will activate numerous cellular processes and influence the results of some biochemical measurements. At very high electric fields (1−2 kV/cm), large pores are formed in cellular membranes allowing even high-molecular-weight molecules to enter or exit the cell.[27] To move a cell into the capillary, investigators have induced EOF using potential differences ranging from 10 to 300 V/cm.[11,29,30] These potential differences can perturb cellular physiology and can alter some biochemical measurements on cells.

Adherent cells are not amenable to manipulation by EOF or hydrodynamic flow without first being removed from their substrate. Mechanical stresses applied to cells, such as scraping them from a surface, can trigger a variety of cellular responses. Mechanical deformation of the plasma membrane can lead to opening of stretch-activated ion channels, stimulation of the phosphoinositide cascade, and initiation of other cellular pathways.[31,32] Additionally, nearly all cells express abundant adhesion proteins which provide a structural link between the cytoskeleton and extracellular surfaces, i.e., the extracellular matrix in the intact organism and glass or plastic in tissue culture.[33] Forces applied to remove a cell from a surface are transmitted by these adhesion proteins into the cell interior leading to activation of numerous enzymes including tyrosine kinases, serine/threonine kinases, G proteins, proteases, and others.[34,35] Activation of these pathways triggers immediate and long-term changes in cellular physiology. For adherent cells, mechanical manipulation prior to the time of sampling may interfere with measurements of cellular biochemistry.

We have developed a method to completely lyse a single cell in milliseconds (possibly much faster) and load the cellular contents into a capillary for analyte separation and detection. This laser−micropipet combination takes advantage of a shock wave produced by a highly focused, pulsed laser microbeam.[36−38] The laser pulse is focused at the glass/buffer interface of a cell chamber in which the cell to be lysed is cultured. The 5-ns light pulse is believed to cause localized plasma formation at its focal point producing a cavitation bubble. The expansion and collapse of this cavitation bubble generates a shock wave traveling at up to 2000 m/s.[36] The interaction of the shock wave with the cell rapidly disrupts the outer cell membrane, releasing cellular contents.[39,40] By positioning the inlet of a standard capillary directly above the cell prior to lysis, the cellular contents are loaded into the capillary by a combination of gravity flow and/or electrophoresis upon lysis. We have demonstrated the successful application of this rapid method of cell lysis and capillary loading using single mammalian tumor cells containing intracellular fluorophores that are electrophoretically separated by capillary electrophoresis and detected by LIF. The absence of manipulation prior to lysis and the extreme rapidity of the lysis technique result in minimal effects on the cell up to the time of sampling. The swift cellular lysis will make more accurate "snapshots" of intracellular composition possible.

EXPERIMENTAL SECTION

Reagents. Fluorescein diacetate mixed isomers (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 purchased from Molecular Probes, Eugene, OR. Tissue culture materials were obtained from Gibco BRL, Gaithersburg, MD. All other reagents were purchased from Fisher Scientific, Pittsburgh, PA. Buffer A, a physiologic extracellular buffer, was composed of 135 mM NaCl, 5 mM KCl, 10 mM HEPES, 2 mM $MgCl_2$, and 2 mM $CaCl_2$ and adjusted to pH 7.4 with NaOH.

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Cell Culture. Rat basophilic leukemia (RBL) cells, a tumor mast cell line, were used as the model cell for this study. These cells were grown at 37 °C and 5% CO_2 in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum, and L-glutamine (584 mg/L). Penicillin (100 units/mL) and streptomycin (100 μg/mL) were added to the media to inhibit bacterial growth. The cells were grown in a cell chamber made by using Sylgard (Dow Corning, Midland, MI) to attach a Teflon "O" ring ($^{15}/_{16}$-in. outer diameter) to a 25-mm, round, no. 1, glass cover slip. Cell attachment to the glass surface was enhanced by coating the cover slip with Cell-Tak (Becton Dickinson, Bedford, MA) prior to adding the cells to the cell chamber. Prior to use, the cells were allowed to grow in supplemented media for 12−24 h after plating in the cell chamber. Cells were plated at concentrations determined empirically to produce approximately one cell per 63× field of view on the day of the experiment.

Loading Cells with Fluorescent Compounds. Cells were loaded with fluorescein diacetate and/or Oregon Green diacetate for these experiments. These cell permeant compounds are not fluorescent until they are hydrolyzed by ubiquitous intracellular esterases after the compound(s) passes into the cell. Once the fluorescent, free acid is formed; it is no longer membrane permeant and remains trapped within the cell, although cells do transport the dye out over time via anionic transporters.[41] For the single-cell experiments, cells grown in DMEM in a cell chamber were washed once in buffer A. Solutions of fluorescein (20 nM) and/or Oregon Green (500 nM) diacetates were made in buffer A with glucose (10 mM) immediately prior to use and added to the cell chamber after removing the wash buffer. The cells were incubated at room temperature in the dark for 30 min to load the fluorescent compounds. The cells were then washed five times in buffer A and used within 10−15 min. The amount of dye loaded into individual cells by this method varies to some degree due to cell-to-cell differences in hydrolysis of the diacetate and loss of the free acid from the intracellular space. Estimates of the moles of fluorescent marker contained in each cell used in the experiments were made by comparison of the fluorescence peak areas from the electropherograms to standards. Fresh buffer A (10 mL/min) was continually exchanged in the cell chamber during the course of the experiments to remove fluorescent dye transported from the cells into the surrounding buffer. In the absence of the flow system, dye accumulated in the extracellular buffer over time increasing the fluorescence baseline.

For cell population experiments, trypsin was used to detach the cells from the culture flasks. The cells were washed once in buffer A and resuspended (5×10^6 cells/mL) in buffer A with fluorescein diacetate (1 μM), Oregon Green diacetate (1 μM), or both reagents. After a 30-min incubation period in the dark at room temperature, the cells were washed twice with buffer A. The cells were lysed by flash-freezing in liquid nitrogen followed by rapid thawing in a 37 °C water bath. The lysate was centrifuged ($16000g$, 5 min), and the supernate was frozen until used.

Capillary Electrophoresis. A high-voltage power supply (CZE1000R, Spellman, Plainview, NY) was used to drive the electrophoresis. Fused-silica capillaries [50-μm inner diameter (i.d.), 360-μm outer diameter (o.d.)] coated with a proprietary neutral coating (Supelco, Phoenix, AZ) were used for the separations to minimize EOF and thus shorten the migration times for the negatively charged fluorophores used in these experiments. The capillary length was 90−100 cm, and electrophoresis was performed in buffer A. The cell chamber served as the inlet reservoir and was held at ground potential. The outlet reservoir was held at a positive voltage of 15−18 kV. In some experiments, electrophoresis was initiated by the operator upon seeing cell lysis. In these experiments, the fluid level around the capillary outlet was 5 cm lower than that around the inlet so that gravitational fluid flow also played a role in loading the capillary with the cellular contents. In other experiments, electrophoresis was initiated simultaneously with lysis by electrically triggering electrophoresis off the laser pulse. In this case, the inlet and outlet were at equal heights. For the experiments reported in this paper, similar results were obtained for both methods. The inlet of the capillary was used as a micropipet for introducing the cellular contents into the capillary after cell lysis. After removing 5 mm of the polyimide coating from the inlet of the capillary, the inlet was mounted perpendicularly to the cover slip of the cell chamber on a micromanipulator (World Precision Instruments, Sarasota, FL) (Figure 1).[42] The micromanipulator enabled precise positioning of the capillary lumen over the cell selected for analysis. Free acid standards of fluorescein and Oregon Green and the supernate from the cell population experiments were loaded into the capillary by gravitational fluid flow, and the loaded volumes were calculated from Poiseulle's equation.[43] Typically buffer solutions were loaded into the capillary by placing the capillary inlet into the sample solution and elevating the level of the inlet solution 8 cm above the level of the outlet solution for 5 s.

Fluorescence Detection. An optical window in the polyimide coating of the capillary was created 75−85 cm from the inlet. The capillary lumen was interrogated by the focused laser beam of an argon ion laser (488 nm, Uniphase, San Jose, CA). The focusing lens possessed a focal length of 3.8 cm (CVI, Albuquerque, NM). Fluorescence was collected at a right angle to the capillary and laser beam with a microscope objective (40×, 0.75 n.a., Plan Fluor, Nikon, Melville, NY) and the light measured with a photomultiplier tube (PMT, R928, Hammamatsu, Bridgewater, NJ) after spectral filtering with a 488 notch filter (Kaiser Optical Systems, Ann Arbor, MI) and a band-pass filter (535DF55, Omega Optical, Brattleboro, VT). The PMT current was amplified and converted to a voltage with a preamplifier (model 1212, DL Instruments, Dryden NY). The signal was digitized by a data acquisition board (DAS-1800, Keithly Metrabyte, Taunton, MA) in a personal computer (Gateway, Sioux City, SD). The data were plotted and peak areas calculated using Origin (Microcal, Northhampton, MA).

Laser-Based Cell Lysis. The microscopy system used for cell lysing experiments was similar to that described previously.[44] A frequency-doubled Q-switched Nd:YAG laser (Surelite I, Continuum, Santa Clara, CA) was used to generate a single laser pulse (10−100 μJ, 5-ns pulse width, 532 nm) directed into a fluorescence

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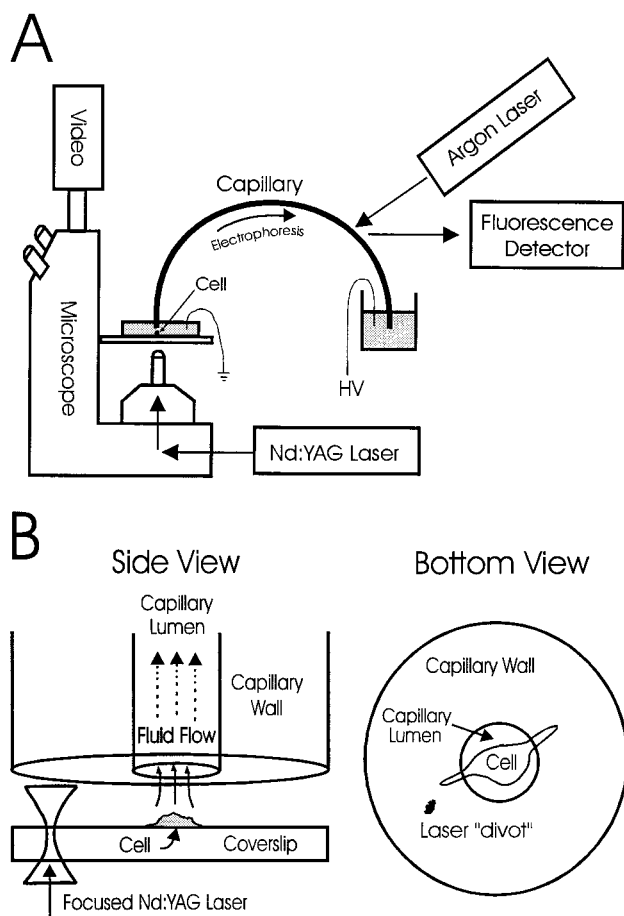
Figure 1. (A) Schematic of the system used for cell lysis, CE, and LIF. (B) Magnified view of the inlet of the capillary. The cell is positioned immediately below the capillary and at a distance from the focused laser pulse.

microscope (Axiovert 135, Zeiss, Thornwood, NY) (Figure 1). The pulse was focused ($\sim$0.3$-$0.4 μm at its waist) with a microscope objective (63$\times$, 1.25 n.a., Zeiss) within the cover slip adjacent to its interface with the buffer. The cell to be lysed was positioned 20$-$30 μm lateral to the focal point of the laser pulse so that no direct interaction of the laser beam with the cell occurred. A CCD video camera system (ZVS-47E, Optronics Engineering, Goleta, CA) recorded the real-time bright-field image of the cell every 33 ms. Lysis was determined from the appearance of the cell after the pulse. In most cases, the cell membrane was completely disrupted, leaving behind only a remnant of the cell membrane attached to the cover slip with the cellular contents in the immediate vicinity of the cell (see Figure 2).

Estimation of the Intracellular Concentration of Oregon Green. Adherent cells were loaded with Oregon Green diacetate as described above. The average concentration of the dye in the cells was determined using the "wedge" method (A. Miyawaki, personal communication). Briefly, a pinhole placed in front of a PMT mounted on a microscope was used to measure the fluorescence intensity from the central region (5-μm radius) of a cell. This region of the cell contains the nucleus and is nearly constant in thickness ($\sim$5 μm deep). Under identical imaging conditions (pinhole size, etc.) the fluorescence intensity at the 5-μm-deep location along a "wedge" of Oregon Green was measured. The wedge was constructed with three cover slips: a

bottom (4.5 $\times$ 5 cm), a top (2.5 $\times$ 7.5 cm), and a center piece (2.5-cm radius, thickness 0.15 mm). The edge of the center piece was placed between the ends of the top and bottom cover slips. At their opposite end, the top and bottom cover slips abutted each other. This formed a triangular or wedge-shaped region (varying in depth from 150 to 0 μm) between the top and bottom cover slips which was filled with Oregon Green. The region of the wedge, which was 5 μm deep, was determined by microscopy. To estimate the intracellular concentration of Oregon Green, the fluorescence intensity of the cell was then compared to the fluorescence intensity of "wedges" containing varying concentrations of Oregon Green.

RESULTS AND DISCUSSION

Minimizing the time required for cell lysis and for initiation of CE will enhance the temporal resolution of single-cell biochemical analyses. In the following section, we describe a new technique to perform cell lysis on millisecond time scales followed by loading of the cellular contents into a capillary for separation and detection. This technique dramatically improves the temporal resolution for CE-based biochemical measurements on single cells.

Cell Lysis by a Pulsed Laser Microbeam. To determine the characteristics of cell lysis by the production of a shock wave, a single, pulsed, laser microbeam (10$-$100 μJ) was employed to lyse RBL cells while a video camera recorded the bright-field image of the cell every 33 ms (Figure 2). Direct, localized alteration of a portion of the plasma membrane can be performed by focusing this laser pulse at lower powers within the cell;[45] however, for the experiments described here, we wished to perform complete cell lysis without direct exposure of the cell to the laser beam. In this way, neither photodestruction nor photobleaching of intracellular species would occur. To lyse a cell, the beam was positioned 20$-$30 μm to the side of the cell. A shock wave was generated by focusing the pulsed laser microbeam within the cover slip just below the glass/buffer interface.

RBL cells contain histamine and serotonin within secretory granules. These granules appear as small ($\sim$0.1$-$0.4-μm diameter), refractile particles that move by Brownian motion when released from the cell. Using lower powers (10$-$20 μJ) to generate the shock wave, the cell membrane appeared to be permeabilized by the shock wave. These cells became spherical after the wave had passed through the cell, and the cellular contents (secretory granules) were seen to "leak out" of the cell over 10$-$30 video frames. As higher energies ($>$20 μJ) were used, the membrane was completely disrupted, leaving remnants of the plasma membrane present in the first video frame (33 ms) after the shock wave. The actual time period over which lysis occurs is likely much shorter than 33 ms, but the time resolution of the video analysis is limited by the video rate (30 frames/s). At these higher energies, the secretory granules were again seen in the immediate vicinity (within $\sim$10 μm) of the remnants of the plasma membrane. The fact that the secretory granules remained localized suggests that only limited dilution of cellular contents occurred at the time of lysis. By increasing the beam energy over the range of 20$-$100 μJ, cells could be lysed at greater distances from the focal point of the pulsed beam. This laser-

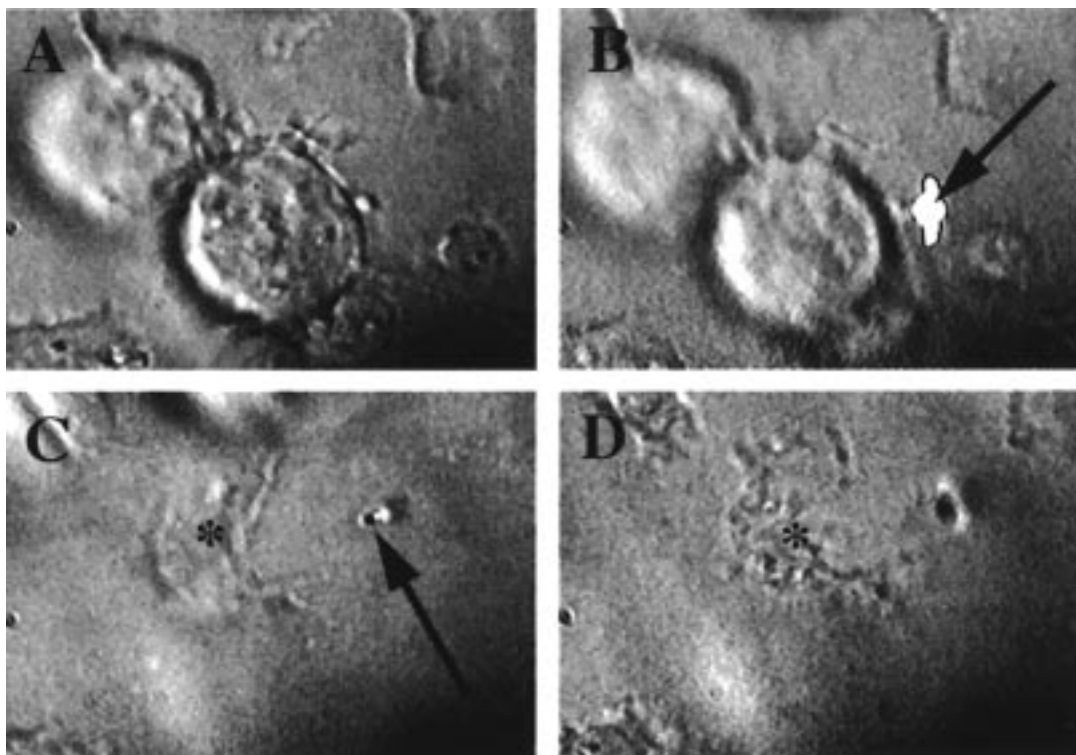(45) Tao, W. J.; Wilkinson, J.; Stanbridge, E. J.; Berns, M. W. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 4180$-$4.

Figure 2. Serial images of a cell taken before and after lysis. (A) Immediately prior to lysis. (B) A video frame capturing the laser pulse. The focal plane is just below the surface of the cover slip so that the cell is slightly out of focus. The bright ovoid image (arrow) is a reflected image, not the actual laser pulse. (C) At 33 ms after cell lysis. Cell remnants and secretory granules can be seen (asterisk). The "divot" caused by interaction of the laser with the glass substrate is present (arrow). (D) The same frame as in panel C. The cell remnants are now in the focal plane of the microscope lens (in focus).

based method of cell lysis enables the experimentalist to locally ablate the cell membrane or to vary the degree and field of cell lysis. While this technique will not achieve the spatial resolution of microscopy-based techniques, it may be possible to perform subcellular measurements on cells such as neurons in which a cellular extension could be sampled without disrupting the cell body.

In each case of cell lysis, a small "divot" appeared in the glass cover slip at the point the laser beam was focused. In some instances, a bubble with a diameter $<5-10 \ \mu m$ appeared in the frame coincident with the laser pulse at the intersection of the laser beam and the glass/buffer interface. In other experiments a circular "wave" could be seen in the video frame coincident with the laser pulse. The bubble and wave were always absent from subsequent frames. The "divot", bubble, and wave further support that a plasma is formed in the cover slip producing cavitation and a shock wave.[36-38] In all experiments, the cell was seen to be completely disrupted in the second video frame ($n = 20$) (Figure 3). Since the laser pulse occurred at a random time during the initial video frame ($0-33$ ms), and an image of the intact cell was never present in the next frame (>33 ms), the cell was lysed in 33 ms or less.

Loading a Capillary with the Contents of a Single Cell. To demonstrate that the contents of an adherent RBL cell could be loaded into the capillary after lysis, a single RBL cell loaded with fluorescein was positioned below the lumen of a capillary. For these experiments, the inlet of the capillary was positioned $15-25 \ \mu m$ above the cell, although the capillary could be positioned flush with the cover slip so that the cell sat within the entrance of

the lumen. The $15-25$-μm distance above the cell was arbitrarily chosen. The cell was lysed as described above and electrophoresis was initiated upon cell lysis. Analysis of the video images from multiple experiments showed that in every instance the cellular contents had disappeared in the video frame after the lysing pulse (Figure 3). This finding suggests that the cellular contents were rapidly loaded into the capillary. In some experiments the plasma membrane remnants remained attached to the glass cover slip while in others the remnants were also loaded into the capillary. The current and migration times of fluorophores from the cells were the same in both cases (see next paragraph).

Confirming that the cell's contents were indeed loaded into the capillary, a single peak with a migration time similar to that of fluorescein in buffer was present on the electropherogram (Figure 4A and D). Electrophoretic traces of cells not containing a fluorescent marker showed no peaks (data not shown). Likewise, when the inlet of the capillary was placed at a distance (just beyond the field of view) from a fluorescein-containing cell, the fluorescence baseline of the electropherogram remained flat after cell lysis (data not shown). In some experiments, more than one cell was present in the vicinity of the capillary inlet. By employing higher laser powers to lyse the cell immediately below the inlet, these nearby cells could be lysed as well. Peaks corresponding to these adjacent cells could be seen in the electropherogram. It is therefore important to control cell lysis or the density of the cell population so that only the contents of a single cell are loaded.

To assess the efficiency with which a cell's contents were loaded into the capillary, RBL cells were loaded with Oregon
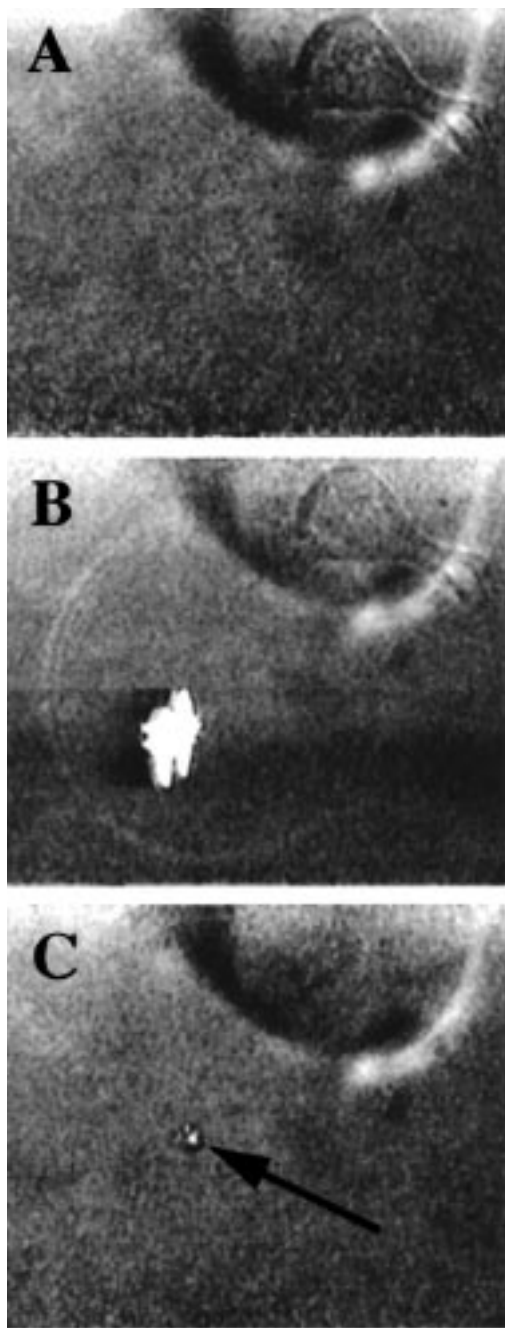
Figure 3. Serial images of a single cell positioned at the inlet of a capillary. The images in the panels were obtained sequentially, and are separated in time by 33 ms. (A) Immediately prior to the laser pulse. (B) A circular wave near the point of the laser pulse. (C) Postlysis. The cell remnants and contents have been loaded into the capillary and are no longer visible. The laser-induced defect in the cover slip is present (arrow).

Green. In this set of experiments, the average concentration of Oregon Green loaded into the cells was $0.9 \pm 0.2 \ \mu M$ (~1 amol/cell), measured using the "wedge method". When cells containing Oregon Green were lysed and loaded into a capillary as described above, on average 1 ± 0.5 amol/cell was detected on the electropherogram (Figure 5). Most of the Oregon Green contained in the cell was loaded into the capillary. Lowering the capillary so that it physically touched the cover slip did not increase the efficiency of loading although increasing the distance between the cell and capillary did diminish the efficiency (Figure
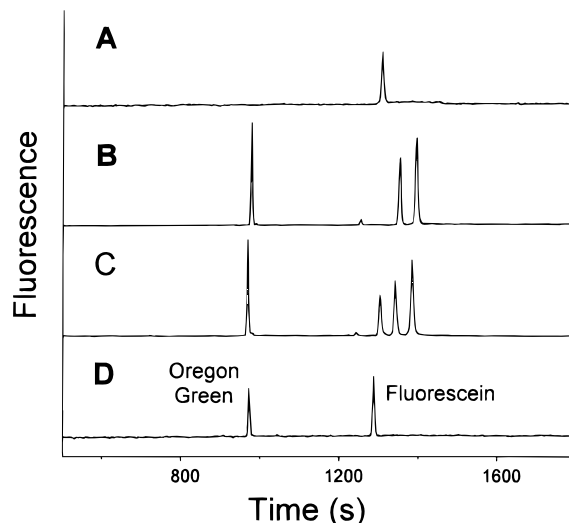

Figure 4. Electropherograms from single cells containing the fluorophores fluorescein and/or Oregon Green. The cells were lysed and loaded into the capillary as described in the text. The amount of fluorophore loaded into the capillary was determined by comparison with electrophoresed standards. (A) Trace from a cell incubated with fluorescein diacetate. The fluorescence of the single peak is equivalent to 2.1 amol of fluorescein. The estimated intracellular fluorescein concentration is ~2 μM assuming a 1-pL RBL cell volume. (B) Trace from a single cell incubated with Oregon Green diacetate. The migration time of the initial peak is identical to that of Oregon Green. The subsequent three peaks most likely represent contaminants that are produced or concentrated within the cell. The fluorescence of the Oregon Green peak corresponds to 4.9 amol of Oregon Green. (C) Trace from a single cell incubated with both fluorescein diacetate and Oregon Green diacetate. The fluorescence of the peaks representing Oregon Green and fluorescein were equivalent to 4.8 and 3.5 amol, respectively. (D) Electropherogram of Oregon Green (2 amol) and fluorescein (2 amol) standards in buffer. All electrophoretic runs were performed on the same day using the same capillary (100 cm, 50-μm i.d., 360-μm o.d., detection window 85 cm from inlet; voltage, 18 kV; current, 50 μA).

5). In these experiments, gravity flow was present in the capillary during cell lysis. The cellular contents were loaded into the capillary by the gravitational fluid flow, electrophoresis, and upward force components of the shock wave. In other experiments, no gravitational fluid flow was present. In this latter case, capillary loading was due only to electrophoresis and upward force components of the shock wave. Similar results were obtained for both loading methods. For capillaries possessing charged lumenal walls, EOF would also load the cell's contents into the capillary.

Electrophoretic Separation of Cellular Contents. A series of experiments were conducted in which RBL cells were loaded with fluorescein or Oregon Green alone or in combination. A single cell containing the fluorophore(s) was then lysed, and its contents were loaded into the capillary and electrophoresed as described above. Figure 4 shows representative traces from these experiments. While electrophoretic traces of cells incubated with fluorescein diacetate alone demonstrated a single peak, cells incubated with Oregon Green diacetate alone consistently demonstrated three distinct peaks of nearly equal magnitude and a small fourth peak (Figure 4B). The migration time of the initial peak was identical to that of Oregon Green in buffer. The subsequent peaks were not seen in electrophoretic traces of the
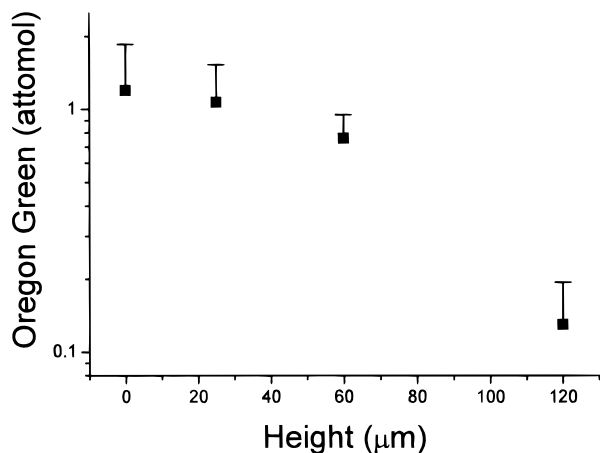
Figure 5. Dependence of the efficiency of loading on the vertical displacement between the capillary and the cell. Cells containing Oregon Green were lysed and loaded into the capillary as described in the text. The end of the capillary was placed at varying heights above the cell. The quantity of fluorophore loaded into the capillary was determined by comparison with electrophoresed standards. Each data point is the average of four measurements. The error bars represent a single standard deviation of the data points.

Figure 6. Electropherogram of two cells loaded with fluorescein and Oregon Green, then serially lysed, and loaded into the capillary. Abbreviations: Oregon Green peak from the first cell (OG1), Oregon Green peak from the second cell (OG2); fluorescein peak from the first cell (F1), fluorescein peak from the second cell (F2), initial "contaminant" peak from the first cell (C1), initial "contaminant" peak from the second cell (C2), second "contaminant" peak from the first cell (C1′), and second "contaminant" peak from the second cell (C2′).

Oregon Green standard (Figure 4D). No fluorescence peaks were identified when Oregon Green diacetate was electrophoresed in buffer. To identify the origin of the extra peaks, a population of cells was incubated with Oregon Green diacetate and lysed and the supernatant isolated. Electrophoretic traces of this supernatant demonstrated the contaminant peaks, albeit in much lower amounts relative to the primary Oregon Green peak (data not shown). The two additional major peaks in the traces from cells incubated with Oregon Green diacetate may be two forms of partially hydrolyzed Oregon Green diacetate, or the additional peaks may be impurities that are concentrated within the cell. As a result of the occurrence of these additional peaks, we were able to demonstrate the ability to resolve five distinct species from a single cell (Figure 4C).

As a demonstration of the feasiblity of serial analysis of the contents of two cells using this technique, we lysed and loaded a capillary with two cells in succession. The two cells were contained in the same cell chamber and so were incubated with fluorescein and Oregon Green diacetates in a like manner. A single cell centered below the lumen of the capillary was lysed, and its contents were loaded into the capillary by gravity flow. A second cell was then centered below the capillary lumen during which time buffer continued to flow into the capillary by gravity. The second cell was lysed, and electrophoresis was initiated immediately following lysis. Thus, the contents of the second cell were loaded by a combination of gravity flow and electrophoresis. The electropherogram shows the analytes from each cell as distinct peaks (Figure 6). The fluorescence signals from the first cell are smaller than that present in the second cell. This difference in signal is most likely due to biologic diversity of cellular uptake and metabolism of the dye by the two cells.

CONCLUSIONS

We have demonstrated a new method to lyse small, adherent, mammalian cells in less than 33 ms and to subsequently load the cellular contents into a capillary for electrophoretic separation and
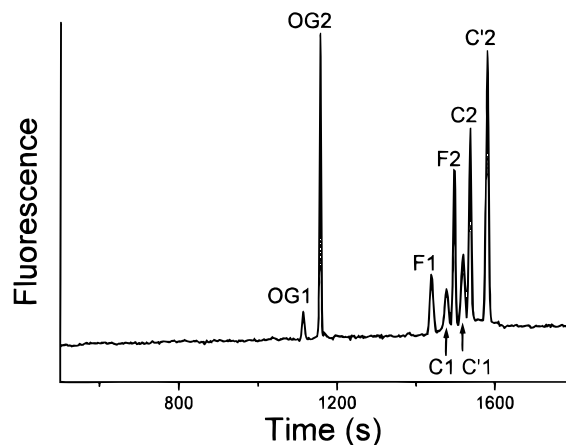
detection. Once the cell is lysed, most cellular reactions are expected to be terminated swiftly by reactant dilution from turbulence, diffusion, and electrophoresis. One disadvantage of this technique is that only one measurement can be made on each cell. An asset is that the molecules from the cell can be identified by their electrophoretic migration times. One potential use of the technique will be the measurement of intracellular enzyme activation by introduction of artificial fluorescent substrates into cells. Following cell lysis, the substrate and its metabolites can be identified by their electrophoretic migration times. The substrate and its products need not undergo an alteration in their absorbance or fluorescence properties when acted upon by cellular molecules. We suspect that it will be far easier to engineer indicator molecules that shift their electrophoretic rather than optical properties in response to cellular enzymes. The activation of enzymes such as proteases, phosphatases, nucleases, kinases, and many others could be measured by this approach. Currently, the physiologic activity in a single cell can be measured for only a few enzymes.

In the present experiments the intracellular concentration of the fluorophores was estimated to be between 0.1 and 10 μM ($\sim$0.1−10 amol). However, fluorescence detection in combination with CE can in some instances achieve sensitivities of zeptomoles and lower.[47,48] Therefore, we expect that nanomolar concentrations of fluorescent substrates loaded into cells may be detectable by combining the laser−micropipet with optimized fluorescence detection. This concentration limit is many orders of magnitude lower than that typically achievable for fluorescent indicators and substrates in cellular microscopy. For example, Ca^{2+} indicators are used at concentrations between $\sim$10 and 100 μM in fluores-

(46) Crank, J. *The Mathematics of Diffusion*, 2nd ed.; Oxford University Press: New York, 1975; p 29.
(47) Weinberger, R. *Practical Capillary Electrophoresis*; Academic Press: San Diego, 1993; Chapter 10.
(48) Pentoney, S. L.; Sweedler, J. V. In *Handbook of Capillary Electrophoresis*, 2nd ed.; Landers, J. P., Ed.; CRC Press: New York, 1997; Chapter 12.

cence microscopy. For substances that have very high cytosolic concentrations (total cytosolic Ca^{2+} concentration is ~ 100 μM), such high indicator concentrations do not greatly alter cellular physiology. However, many enzymes, substrates, and products occur at nanomolar concentrations in cells. In order not to perturb cellular physiology, indicators or substrates used to probe the activation or concentration of these molecules will most likely need to be in the nanomolar or lower concentration range. This technique in combination with fluorescent indicators or substrates may be capable of filling this huge gap in our ability to perform physiologic measurements on single cells. The laser−micropipet combination has the potential to open new domains in cellular measurements not previously accessible.