Chemical Analysis of Single Cells

Laura M. Borland, Sumith Kottegoda, K. Scott Phillips, and Nancy L. Allbritton

Department of Chemistry, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599; email: nlallbri@unc.edu

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Abstract

Chemical analysis of single cells requires methods for quickly and quantitatively detecting a diverse array of analytes from extremely small volumes (femtoliters to nanoliters) with very high sensitivity and selectivity. Microelectrophoretic separations, using both traditional capillary electrophoresis and emerging microfluidic methods, are well suited for handling the unique size of single cells and limited numbers of intracellular molecules. Numerous analytes, ranging from small molecules such as amino acids and neurotransmitters to large proteins and subcellular organelles, have been quantified in single cells using microelectrophoretic separation techniques. Microseparation techniques, coupled to varying detection schemes including absorbance and fluorescence detection, electrochemical detection, and mass spectrometry, have allowed researchers to examine a number of processes inside single cells. This review also touches on a promising direction in single cell cytometry: the development of microfluidics for integrated cellular manipulation, chemical processing, and separation of cellular contents.

1. INTRODUCTION

Capillary electrophoresis (CE): typically utilizes high electric fields for separation of an analyte, which may be based on its charge, size, or hydrophobicity

A plethora of technologies now exist for analyzing the chemical constituents in single cells. Among the tools used for these analyses are capillary electrophoresis (CE), mass spectrometry, electrochemistry, flow cytometry, and fluorescence microscopy. Functional reagents are frequently key components of these technologies and, in combination with the instrumentation, enable the sensitivity and specificity for single-cell measurements. Examples include the development of ion indicators for the measurement of intracellular Ca^{2+} by fluorescence microscopy and the high-specificity, antiphosphoprotein antibodies for the quantification of intracellular phosphoproteins by flow cytometry (1, 2). These single-cell technologies have also had a substantial impact on the conduct of biomedical research, for example in the use of electrochemistry and mass spectrometry for the measurement of neurotransmitters and other secreted molecules such as peptides.

Full coverage of all technologies utilized in the chemical analysis of single cells is beyond the scope of this review. Rather, this work focuses on the analysis of single cells using microelectrophoretic separations. Both microfluidic and capillary-based electrophoresis are well suited for handling the femtoliter-to-nanoliter volumes of cells and their organelles. The methods are fast and quantitative, provide multicomponent analysis, and are compatible with high-sensitivity detection. Separation of the cellular components also enables the quantification of large numbers of analytes simultaneously without the interference of the background cellular matrix. All of these attributes are required for measurements on single cells and their components.

The separation of single cells using microelectrophoretic methods was pioneered by the labs of Jorgenson, Ewing, and Yeung (3–5). The early contributions of these labs are summarized in an excellent review by Dovichi and Hu (6). Since this time, much of the instrumentation has changed, although the central goal of introducing a cell's contents into a small-diameter tube and inducing separation in an electric field remains unchanged. Innovations in cell sampling have expanded microelectrophoretic assays to a wide range of cell types as well as to a variety of subcellular fractions (7). Most commonly, the entirety of a cell is introduced by hydrodynamic or electroosmotic fluid flow into a channel. The cell may be lysed either before or after entry into the microchannel (7, 15) by one of several methods: chemical lysis, typically by a detergent (8, 10); hypotonic lysis by a dilute aqueous solution yielding cell swelling and rupture (9, 11); mechanical lysis by a shock wave and cavitation bubble of a focused, pulsed laser beam (14, 16); or electrical lysis due to membrane breakdown by an applied voltage (12, 13). Various methods for subcellular sampling have also been developed. These include direct insertion of a capillary into a cell followed by hydrodynamic or electrokinetic withdrawal of cytoplasm into a tube (17, 18), and use of a laser to detach a cellular process for analysis (19). Permeabilization of cellular membranes by electroporation or chemical reagents followed by limited withdrawal of cellular contents has been mated with microelectrophoresis (20–22). Organelles are also appropriate for analysis when injected into a capillary either by optical trapping or by direct loading of the purified fraction (23–27). These sample loading methods have greatly broadened the range of analytes accessible for quantitation and have also increased the diversity of cell types suitable for analysis by microelectrophoretic methods (**Table 1**).

Table 1 Cell types used in single-cell analysis[a]

Cell Type	Analyte	Reference(s)
Synechococcus sp. (cyanobacteria) (PCC 7942)	phycobiliprotein	138
Deinococcus radiodurans (bacteria)	protein-GFP	86
Saccharomyces cerevisiae (yeast, baker's)	trisaccharide metabolism	48
	α-glucosidase I activity	48
Arabidopsis embryo (plant, rockcress)	DNA (LFY and AP2)	89
	α-glucosidase I	51, 52
	α-glucosidase II	52
	GTA	52
Spodoptera frugiperda (Fall armyworm)	diglucoside metabolism	53
	α-glucosidase I	52
	α-glucosidase II	52, 53
	native proteins	135
	GFP	134
	YFP	133
	β-adrenergic receptors	138
	GTA	52
Helix aspersa neuron (common garden snail)	amino acids	4
Planorbis corneus neuron (red ramshorn snail)	amino acids	39
	catechols	18, 37
	dopamine	18, 37–39
	serotonin	18, 37
Lymnaea stagnalis (great pond snail)	NOS metabolites	106
	NO_2^- and NO_3^-	106
	NO	107
Pleurobranchaea californica neuron (sea slug)	serotonin	44
	NO_2^- and NO_3^-	104
	NOS metabolites	105
Aplysia californica (California sea slug)		
neuron	NO	107
	NOS metabolites	105
	ascorbic acid	141
	D-amino acid peptide	56
	D-/L-glutamate	30
	serotonin	44
atrial gland	secretory vesicles	23
Xenopus laevis oocyte (African clawed frog)	IP_3	95, 96
	β-galactosidase activity	46
	kinases: phosphorylation of peptide substrate	17

(*Continued*)

Table 1 (*Continued*)

Cell Type	Analyte	Reference(s)
Mus musculus (mouse)		
Peritoneal macrophages	amino acids	34
	GSH	62
β-TC3 (mouse pancreatic β cell)	insulin	57, 58
3T3 (mouse fibroblast)	kinases: phosphorylation of peptide substrate	100
	farnseyltransferase: farnesylation of substrate	102
4T1 (mouse mammary tumor)	farnseyltransferase: farnesylation of substrate	102
NG 108–15 (mouse neuroblastoma)	GSH	61
NS1 (mouse myeloma)	mitochondria	24, 116
	cardiolipin from mitochondria	118
Embryo (mouse)	proteins	80
Cricetulus griseus (Chinese hamster)		
CHO (oocytes)	calcein-AM	139
	RNA	51, 87, 91
	DNA	87
	mitochondria	116
Mesocricetus Auratus (Syrian hamster)		
BHK-21 (baby kidney)	Ca^{2+}	95, 96
Rattus norvegicus (rat)		
PC12 (pheochromocytoma)	Ca^{2+}	95, 96
	amino acids	8, 15, 20, 39, 142
	dopamine	8, 15, 20, 142
Hippocampi cells	glutamate	36
Cardiomyocyte	DNA fragmentation	140
Islet of Langerhans	insulin	59, 60
C2C12 (myoblast)	kinases: phosphorylation of endogenous GFP substrate	101
peritoneal mast cells (RPMCs)	histamine	33
	serotonin	42, 43
R2C (testicle)	steroids: progesterone	94
RBL (basophilic leukemia)	kinases: phosphorylation of peptide substrate	100
Hepatocytes	Tryptophan	35
	GSH	35
Canis familiaris (dog)		
MDCK (Madin Darby canine kidney)	protein-GFP	143
Bos taurus (cow)		
Chromaffin cells (adrenal gland)	norepinephrine and epinephrine	40, 41

(*Continued*)

Table 1 (*Continued*)

Cell Type	Analyte	Reference(s)
Homo sapiens (human)		
Erythrocyte	reactive oxygen species	144
	glutamate	36
	reduced GSH	3, 63, 131
	hemoglobin	11, 66–69
	carbonic anhydrase	29
	glucose 6-phosphate dehydrogenase	97
K562 (erythroleukemia)	glycoprotein	83
	proteins	145
HEK (embryonic kidney)	protein-GFP	84
Jurkat (T cell leukemia)	fluorescein	12
	Oregon green	12
	NDA-derivatized amino acids	137
AML-5 (acute myloid leukemia)	calcein-AM	136
CEM-C2 (leukemia)	single nuclei: doxorubicin content	123
	acid organelles	112
CCRF-CEM (lymphoblast leukemia)	single nuclei: doxorubicin content	123
	acid organelles	112
Lymphocyte	amino acids	32
CSF lymphocytes	dopamine	38
	DOPAC	38
	uric acid	38
lymphoblasts	DNA (β-actin)	90
Neutrophils	ascorbic acid	146
AtT20 (pituitary adenoma)	gangliosides	54, 55
LNCaP (prostate cancer)	DNA (β-actin)	87
MCF-7 (breast cancer)	DNA (β-actin and ERα)	88
	protein	78
HT 29 (colon adenocarcinoma)	protein	64, 65, 72, 73, 75
	α-glucosidase I	52
	α-glucosidase II	52
	GTA	52
NK	IFN-γ	98
ΔH2–1 (osteosarcoma)	nuclei: via eGFP conjugated nuclear-tracking proteins	25, 122

[a]Abbreviations: DOPAC, dihydroxyphenylacetic acid; eGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; GSH, glutathione; GTA, α-1,3-*N*-acetylgalactosaminyltransferase; NDA, naphthalene-2,3-dicarboxaldehyde; NK, natural killer; YFP, yellow fluorescent protein.

Laser-induced fluorescence (LIF): method in which the absorption of laser radiation excites a molecule or atom; the system then relaxes by emission of radiation

Electrochemical detection (EC): uses an applied potential to oxidize or reduce analytes at an electrode surface; the resulting electron current is proportional to the concentration of the analyte

NDA: naphthalene-2,3-dicarboxaldehyde

A key need in the analysis of single cells by microelectrophoresis is increased throughput. Most electrophoretic separations on single cells have been low in throughput, with fewer than 35 cells analyzed per day. Many biological studies, however, require data from large numbers ($\geq$1000) of cells. Some key biological states, for example those of stem cells, are occupied by low numbers of cells, and many cells must be examined to identify these rare cell types. In other instances, understanding the diversity present within a large population of cells, for example cancer cells, is critical to understanding the dynamics of the disease and requires assessing a multitude of cells. Lastly, because cells are highly heterogeneous, analyzing only a few cells may yield misleading results, especially if the cells are extreme outliers in the population.

Both parallel and serial measurements have been explored as means of increasing the throughput of cellular analyses by microelectrophoretic methods. Dovichi and colleagues have recently constructed an array of five capillaries for parallel cellular analyses (28). Impressively, the Dovichi group also developed a juncture between two five-capillary arrays so that this system could be utilized for two-dimensional capillary electrophoresis of cellular proteins. Although this work analyzed cell homogenates, taking the next step to array-based analyses of single cells appears readily attainable. Rapid, serial analysis of single cells has been performed in both capillaries and microfluidic devices. Chen and Lillard analyzed hemoglobin and carbonic anhydrase in red blood cells at a rate of ~0.3 cells/min using one capillary to supply a stream of red blood cells to a second separation capillary (29). However, the highest-throughput measurements on single cells (10 cells/min) in which a separation of analytes was demonstrated are those achieved by Ramsey and colleagues in a microfluidic device (12). In this study, leukemic cells were lysed at a fluidic junction by a combination of an electric field and a detergent, followed by the electrophoretic separation of two intracellular fluorescent dyes.

Despite these advances, there is a continued need to develop strategies for high-throughput analysis of cells using microelectrophoresis. Microfluidic devices will likely be the best option for high-throughput measurements on cells not adherent to a surface, as the cells are easily moved to the separation channel; however, capillary-based measurements may be optimal for cells that are attached to a surface because the capillary can be readily transported to the immobilized cell.

2. ANALYTE DETECTION AND MEASUREMENT IN SINGLE CELLS

2.1. Amino Acids

Amino acids play important roles in cellular signaling and act as building blocks or intermediates in the synthesis of bioamines, nucleic acids, and proteins. Thus there is widespread interest in the quantitation of intracellular and intraorganelle amino acids. Currently, laser-induced fluorescence (LIF) and electrochemical detection (EC) are the most sensitive detection methods associated with CE for single-cell analysis. However, most amino acids are neither electroactive nor fluorescent. Therefore, covalent labeling with fluorescent or electroactive tags is necessary to achieve sufficient sensitivity for single-cell analyses. Naphthalene-2,3-dicarboxaldehyde (NDA) is the

most common derivatizing agent used in the analysis of amino acids. In the presence of cyanide, NDA reacts with primary amine groups to produce cyano[*f*]benzoisoidole products that are both fluorescent and electroactive. Work by the Jorgenson group (4) heralded the modern era of amino acid analysis in single cells by combining NDA-derivatization with CE and open tubular liquid chromatography (OTLC). Individual neurons of *Helix aspersa* were derivatized with NDA, then loaded into a capillary and analyzed either by chromatography with EC or by CE-LIF. A total of 17 amino acids were separated by the OTLC-EC method and concentrations were estimated at 40 pmol to 40 fmol. The separation of six amino acids, including tryptophan, serine, alanine, glycine, glutamine, and aspartic acid, was achieved using CE-LIF (4).

Micellar electrokinetic chromatography (MEKC): separation of an analyte based on the differential partitioning between a micellar phase and an aqueous phase

Most recent studies utilized on-column cell lysis and derivatization with NDA prior to chemical separation. An intact single cell was loaded into the capillary by electroosmotic migration, then injected with a plug of NDA/CN^- for derivatization and lysis buffer for cell lysis. This method was utilized by the Liu group (30), which used micellar electrokinetic chromatography (MEKC) with LIF detection to separate glutamate enantiomers, as both L- and D-amino acids play a role in neurotransmission. Individual neurons from the abdominal ganglion of *Aplysia californica* exhibited higher levels of D-glutamate than of L-glutamate (**Figure 1*a***) (30). In a separate

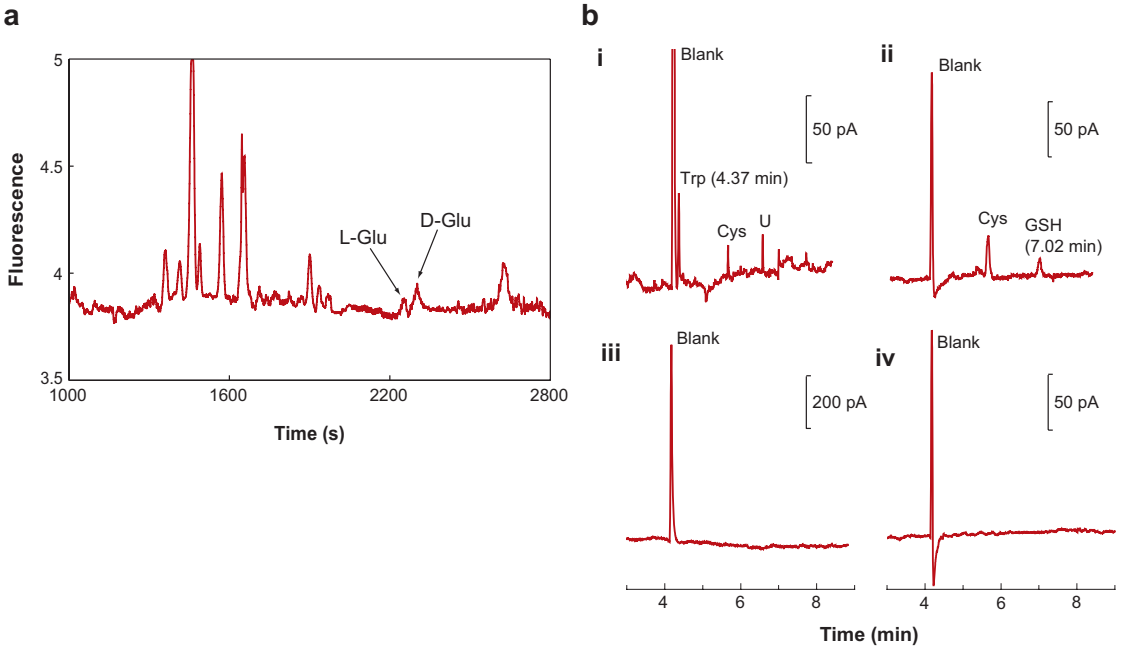


Figure 1

(*a*) Separation of D/L-Glu in a single abdominal cell from an *Aplysia* ganglion. Reprinted with permission from Reference 30. (*b*) Electropherograms of tryptophan and glutathione (GSH) from single hepatocytes. Detection of tryptophan and an unknown analyte (U) detected at the carbon fiber bundle electrode (panel *i*). Detection of GSH at the Au/Hg electrode (panel *ii*). Shown for comparison: (*iii*) Electropherograms of blank runs (no cells) at the carbon fiber bundle and (*iv*) the Au/Hg electrode. Reprinted with permission from Reference 35.

experiment, glutamate, aspartic acid, and glycine were identified in giant dopamine neurons of *Planorbis corneus* by CE-EC (31).

GSH: glutathione

Pheochromocytoma (PC12) cells, isolated from the adrenal glands of rats, have been used extensively as a model system for the study of neuronal differentiation. CE-LIF was used to measure four amino acids—alanine, glycine, glutamate, and aspartic acid—and the cysteine derivative taurine in PC12 cells. The average quantities of amino acids in single PC12 cells ranged from 200 amol to 5 fmol (8). Swanek and colleagues (31) conducted a similar study of amino acid levels in PC12 cells by CE-EC. Because background signals from the surrounding cell media contributed to the overall electrochemically detected signal, internal standards were used to aid in the quantification of the amino acids originating inside the cell.

The Jin group (32–34) analyzed the amino acid levels in different cells of the immune system. Custom-made cell injectors were used to load cells into the capillary after which the cells were lysed and then reacted with NDA. Carbon fiber microdisk array electrodes were used for EC detection of five amino acids—alanine, aspartic acid, glycine, glutamate, and serine—plus the derivative taurine in mouse peritoneal macrophages. The amino acid levels in each cell ranged from 0.3 to 6.5 fmol (34). In related work, serine, alanine, glycine, and taurine were identified in human lymphocytes, with the amounts per cell ranging from femtomoles to attomoles (32). Histamine, a chemical mediator implicated in allergic reactions derived from the amino acid histadine, was monitored in single rat peritoneal mast cells by CE. The average histamine level was 96 fmol/cell (33).

Tryptophan and glutathione (GSH) are important indicators of liver activity and can reflect liver function when measured in single liver cells. Although CE-EC is usually a very sensitive method for the detection of electroactive species such as tryptophan and GSH, these two species cannot be detected simultaneously. Because tryptophan and GSH are uniquely sensitive to different electrode materials and thus cannot be detected simultaneously using traditional methods, Jin and colleagues constructed a carbon fiber bundle–Au/Hg dual-electrode detection system. Tryptophan and GSH were identified and quantified from individual rat hepatocytes by the dual electrode (**Figure 1*b***) (35). Other compounds derivatized with NDA (e.g., cysteine, tyrosine, histamine, L-dopa, dopamine, serotonin, epinephrine, and norepinephrine) were measured and monitored simultaneously with tryptophan and GSH using this dual-electrode system.

Wang and Yeung (36) measured glutamate in human erythrocytes and single neurons without the need for labeling with NDA. A dual-enzyme on-column reaction method combined with CE-LIF was used to detect the formation of reduced nicotinamide adenine dinucleotide (NADH), the product of the reaction of glutamate dehydrogenase and glutamic pyruvic transaminase with cellular glutamate. Sub-femtomole levels of glutamate were detected in both cell types (36).

2.2. Neurotransmitters

Dopamine is an important neurotransmitter implicated in drug abuse, schizophrenia and Parkinson's disease. Determining how dopamine is synthesized, packaged, and

released from single cells is essential to understanding how the brain as a whole works. Neuroscientists are particularly interested in the concentrations of dopamine in different compartments of the cell.

Early studies by Ewing's group used tapered capillaries, etched to 2–5 μm in diameter, to sample neurons for quantification of their intracellular dopamine content. These tapered capillaries were inserted into single *Planorbis corneus* neurons in order to sample ultrasmall intracellular volumes (~270 fL). Neurotransmitters, including dopamine and serotonin, were electrophoretically injected and separated by CE-EC (18, 37). Even smaller tapered capillaries, with 770-nm diameter tips, have been used to sample even smaller cell lines, such as PC12 cells. An ultrasmall capillary can sample 8% of the total volume of a PC12 cell, which can then be separated and detected by CE-EC. This sampling and detection method was used to determine the dopamine content of a typical PC12 cell (240 ± 60 μM) (15). Subsequently, electroporation was coupled to the use of the 770-nm-diameter sampling capillaries as a nondestructive method of sampling single cells (20).

Ewing and colleagues identified dopamine in human lymphocytes by performing CE-EC on whole, single-cell human lymphocytes (38). Dopamine synthesis inhibitors decreased dopamine levels in the lymphocytes, whereas the lymphocytes also showed evidence of cellular uptake as extracellular dopamine increased intracellular dopamine (38). Whole *Planorbis corneus* neurons sampled by CE-EC led to the discovery of two compartments of dopamine: readily available cytoplasmic dopamine and dopamine in storage vesicles (9). *Planorbis corneus* cells were also used with a new CE detection technique, scanning electrochemical detection. This technique allows for secondary verification of the identity of the analyte (39). Although electrochemical detection is the preferred method for dopamine detection, dopamine has also been derivatized with NDA for use in CE-LIF (8).

Norepinephrine and epinephrine from single bovine chromaffin cells have been separated by Yeung and colleagues using CE. When illuminated in UV, these analytes fluoresced in the 320–350 nm wavelength range (40). In an interesting experiment, CE was used to monitor the native fluorescence of norepinephrine and epinephrine released from single chromaffin cells following application of acetylcholine. Immediately following the sampling of extracellular norepinephrine and epinephrine, the cells were lysed and the intracellular neurotransmitter content was analyzed, revealing the relative amounts of secreted and nonsecreted neurotransmitter (41).

Another neurotransmitter, serotonin, has been examined in single cells using CE by both Yeung's and Sweedler's groups. CE with native fluorescence detection was used to quantify serotonin from rat peritoneal mast cells (RPMCs). On-capillary monitoring of the release of serotonin following the application of polymyxin B sulfate showed a 28% release of serotonin from RPMCs (42, 43). CE with native fluorescence detection was also used to separate and detect serotonin in *Aplysia californica* and *Pleurobranchaea californica* neurons following homogenization of single neurons in small vials. In these experiments, wavelength-resolved fluorescence detection, which compares fluorescence emission spectra, was used to better identify the analyte being separated (44). Recently, the Sweedler group designed a novel CE-LIF system that used a hollow cathode metal vapor laser emitting at 224 nm for fluorescence

excitation. The emitted fluorescence was spectrally distributed into three wavelength channels, 250–310 nm, 310–400 nm, and >400 nm, and was collected by separate photomultiplier tubes. This new system more accurately detects, quantifies, and identifies serotonin, dopamine, and related neurotransmitters in single *Lymnaea stagnalis* neurons based on the neurons' multichannel spectral signatures (45).

TMR: 5-carboxytetramethyl rhodamine

2.3. Oligosaccharides

Oligosaccharide metabolism has been extensively examined in single cells by capillary electrophoresis. Precedence for oligosaccharide work in single cells was established by the development of in vitro CE-based assays for examining the enzymes responsible for sugar metabolism, such as β-galactosidase and the family of α-glucosidases (46, 48). The commercially available fluorescent probe, fluorescein di-β-D-galactopyranoside, was used to examine β-galactosidase activity in *Xenopus* oocytes microinjected with RNA encoding for β-galactosidase. CE-LIF was used to monitor fluorescein, released as a result of β-galactosidase-induced hydrolysis, from 100-pL samples microaspirated from a single oocyte (46).

Palcic and colleagues synthesized oligosaccharide substrates labeled with 5-carboxytetramethyl rhodamine (TMR) for use in examining α-glucosidase activity in vitro and in vivo (47). TMR has been extensively used to label oligosaccharides as it is pH-insensitive, easy to attach, photostable, inexpensive, easily matches with the He-Ne laser line, and has superior detection limits over other fluorophores. Yeast cells, loaded with a TMR-labeled trigluocoside, were isolated and sampled whole by CE-LIF to investigate α-glucosidase I activity (48). Confocal microscopy confirmed loading of the TMR-labeled oligosaccharide probe over several hours. Pharmacological inhibition of hydrolysis by castanospermine was used to confirm α-glucosidase I activity towards the oligosaccharide probe (48).

The lactose-derived disaccharide probe LacNAc-TMR was used by Dovichi's group to monitor α-glucosidase activity in HT-29 (human colon adenocarcinoma) cell lines. The group used CE-LIF to examine the metabolism of the LacNAc-TMR disaccharide in single HT-29 cells. Metabolism of the fluorescent oligosaccharide probe was correlated with the cell cycle by confocally imaging the cells loaded with a DNA-intercalating dye prior to lysis (49). Work with LacNAc-TMR in HT-29 cells revealed not only hydrolyzed monosaccharide products, but also the formation of monofucosylated trisaccharides, Le^x-TMR and Le^y-TMR. This discovery demonstrated the activity of Golgi-located fucosyltransferases within this cell line (47). Studies of LacNAc-TMR in HT-29 cells also showed the importance of single-cell assays as means of avoiding or eliminating sample bias. LacNAc-TMR metabolism in cellular extracts yielded an array of enzymatic reactions not seen in the single cell, suggesting that the time (1.5 h) and procedures required to prepare cellular extracts upregulated a cascade of enzymatic activity not produced or observed in whole, single cells, which are quickly lysed before CE-LIF examination (50).

More recent work by Palcic and colleagues employing micromanipulators and nanoscale reaction vessels has advanced the study of single cell oligosaccharide metabolism to new levels. The activity of three enzymes, α-glucosidase I, α-glucosidase II, and α-1,3-*N*-acetylgalactosaminyl-transferase (GTA), was probed

using a TMR-labeled diglucoside substrate (DG-TMR) in Sf9 cells, HT-29 cells, and *Arabidopsis* embryos (51–53). A single cell loaded with DG-TMR substrate was placed into a nanovessel and lysed with 1 nL of buffer. Low-nanoliter aliquots of the cell lysate were examined for the hydrolytic conversion of DG-TMR to monoglucoside. An advantage of this type of assay is that multiple measurements can be made from a single cell (51–53).

Gangliosides are glycosphingolipids comprising sialic acid–containing saccharide groups attached to a fatty acid–containing sphingosine tail, known as a ceramide. Ganglioside metabolites are used as disease markers in Tay-Sachs disease and related genetic disorders. Following synthesis and characterization of TMR-labeled GM1 and its metabolites, Dovichi and colleagues used CE-LIF to measure galactosidase activity in single AtT20 cells following formalin fixing, with 11 metabolic products detected at low zeptomole levels (54, 55).

2.4. Peptides

Peptides are an important class of cellular molecule that act as neuropeptides or hormones, form during the metabolism of proteins, and play a role in immune surveillance. Although most peptides and proteins present in animals are made of L-amino acids, CE-based systems have been used to identify D-amino acid–containing peptides in single cells. The D-amino acid–containing peptide, NdWFa (NH_2-Asn-D-Trp-Phe-$CONH_2$), was identified in individual peptidergic neurons in the abdominal ganglion of *Aplysia californica* mollusks by Sweedler and colleagues (56). Matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-MS) studies confirmed the mass and sequence of NWFa/NdWFa whereas CE-LIF-based chiral separation of fluorescamine-labeled individual neurons identified NdWFa as the dominant form of the peptide.

Insulin is a naturally occurring hormone secreted by the pancreas. An increase in blood glucose concentration stimulates secretion of insulin from pancreatic β cells; this process signals the peripheral tissues to consume the excess glucose. Because the insulin:glucose regulatory system is disrupted in diabetics, there is substantial interest in understanding the control of insulin secretion. Tong and Yeung (57) used CE-LIF to detect the native fluorescence of insulin in individual rat (RINm5F) and mouse (β-TC3) cells. The average insulin content of a single β cell was 1.6 ± 0.7 fmol. In another study, insulin release from single β-TC3 cells was quantified after on-capillary permeabilization with digitonin (58). The amount of insulin released and the amount remaining in the cell were measured simultaneously by CE-LIF. Similarly, the Kennedy group (15, 59) developed a competitive immunoassay based on the CE-LIF method to monitor the glucose-induced secretion of insulin from single rat islets of Langerhans. This method was later automated to measure insulin release with a temporal resolution of 3 s (60).

The tripeptide glutathione, γ-Glu-Cys-Gly, is the most abundant low-molecular-weight thiol in mammalian cells. Due to its biological and clinical significance in limiting cellular oxidative damage, numerous single-cell assays have been developed to measure and monitor GSH. Hogan and Yeung (3) demonstrated the on-capillary lysis and detection of GSH from human erythrocytes by CE-LIF. When erythrocytes were

prelabeled with monobromobimane, a sulfhydryl-reactive fluorophore, detection limits for GSH were in the attomole-to-femtomole range. Orwar and colleagues (61) used NDA as a derivatizing agent to quantitate GSH in single mouse neuroblastoma (NG 108–15) cells. Under physiological conditions, NDA reacts rapidly with GSH without the addition of CN^-, permitting the detection of low femtomole levels of GSH in single cells by CE-LIF.

The Jin group introduced a derivatization-free CE-EC system for the direct analysis of GSH in single cells. They utilized a gold-mercury amalgam microelectrode for end-column amperometric detection following CE separation. Average cellular GSH levels were 5.8 fmol for single mouse peritoneal macrophages (62), 11 fmol for single rat hepatocytes (35), and 0.1 fmol for single human erythrocytes (63). As described above, a carbon fiber bundle–Au/Hg dual electrode was used to simultaneously quantify both tryptophan and GSH in single rat hepatocytes (**Figure 1*b***) (35).

2.5. Proteins

Recently, proteins have become one of the most investigated analyte groups in single-cell CE-based analysis. The increased focus on proteins is due to the biological and clinical significance of many classes of proteins, as well as to the complexity and scope of their action in cells. A typical eukaryotic somatic cell expresses about 10,000 different proteins averaging 600 copies each (6); therefore, extremely sensitive detection techniques and advanced separation methods are necessary to analyze the minute amount of protein present in a single cell. Capillary-sieving electrophoresis and MEKC are the most successful separation methods for use with proteins and are most often used in combination with capillaries possessing covalent or dynamic surface coatings. Although this chapter cannot cover the entire scope of single-cell proteomics, excellent reviews on this topic are available in this volume and elsewhere (64, 65). This section focuses predominantly on the different fluorescence-labeling methods for protein detection.

2.5.1. Detection of proteins by their native fluorescence. Hemoglobin and carbonic anhydrase were the first proteins analyzed from individual erythrocytes (11). Because they are at extremely high concentrations in erythrocytes, they can be detected using their native fluorescence. A single erythrocyte was injected into a capillary with the aid of negative pressure. Cells were then lysed by exposure to the hypotonic electrophoretic buffer and their contents were separated and detected by CE-LIF without a sieving matrix. Lillard and colleagues (66) employed CE-LIF to study hemoglobin variants in human erythrocytes. In this study, hemoglobin from fetal, normal adult, and adult A_1 red blood cells was analyzed. Fluorocarbon-coated capillaries and fluorocarbon surfactants were used to separate the α, β, and glycated polypeptide chains from denatured hemoglobin molecules without the use of a sieving matrix. Erythrocytes from diabetics showed higher glycated hemoglobin levels than did erythrocytes from nondiabetics. In a subsequent study, Lillard and Yeung (67) coupled capillary isoelectric focusing with LIF to study hemoglobin variants in human erythrocytes. Hofstadler and colleagues (68) used CE coupled to mass spectrometry

to analyze hemoglobin from human erythrocytes. They detected both the α and β chains of hemoglobin by ESI-FTICR-MS. The Ren group (69) employed CE with chemiluminescence detection to study hemoglobin from human erythrocytes. The chemiluminescence detection was based on the catalytic effects of the iron group in hemoglobin on the luminol-hydrogen peroxide reaction.

GFP: green fluorescent protein

2.5.2. Detection of proteins by covalent labeling with fluorophores. Most proteins cannot be detected by high-sensitivity LIF because of the low abundance of tryptophan and tyrosine in cellular proteins (70). Therefore, amine-reactive fluorescent tags are commonly used to covalently attach fluorophores to both the ε-amine of lysine and the amino terminus of proteins. Lysine is one of the most abundant amino acids and at least one lysine is present in a majority of proteins. Because the covalently attached fluorophores possess substantially better absorption coefficients and quantum efficiencies than the native protein fluorophores, detection limits are improved by several orders of magnitude, permitting the detection of low copy number proteins.

The Dovichi group has extensively studied the proteins of HT-29 cells. In most of their studies, cell injection was performed with a multipurpose single-cell injector (71) followed by cell lysis and fluorescent labeling of cellular proteins. Under those conditions, the fluorophore 3-(2-furoyl)quinoline-2-carboxaldehyde reacted with all available free amino groups (72). Proteins were separated by CE with LIF detection in a postcolumn sheath flow cuvette. This one-dimensional separation with a surfactant-containing buffer yielded separation efficiencies of greater than 400,000 theoretical plates with resolution of 30 components from a single cell (73). The same single-cell approach was used to study protein expression in a *Caenorhabditis elegans* zygote (74). The protein profile of individual HT-29 cells was also generated using a similar one-dimensional CE separation with a sieving matrix (64, 65).

CE-based assays of single cells were also used to examine variations in the protein profile of individual cells resulting from changes in cell physiology. The Dovichi group observed that cell-to-cell variation in the protein profile correlated with the phase of the cell cycle. On average, 60% of the cell-to-cell variation in protein expression was due to differences in the cell cycle state (**Figure 2*a***). For single cells in the same cell cycle state, the variability in protein expression was less than 30% (75). Similar patterns were observed for green fluorescent protein (GFP) expression in 4T1 sister cells (**Figure 2*b***) (76).

Because the separation power of one-dimensional electrophoresis is insufficient to resolve the large number of proteins present in a single cell, a 2D-CE method was developed to study the protein content of single cells. The method developed by Dovichi and colleagues utilized capillary sieving electrophoresis in the first dimension and capillary-based MEKC separation in the second dimension (77, 78). This method provides rapid and reproducible separations up to a 600-spot capacity (79). In one study, different protein expression patterns were observed for normal and drug-treated MCF-7 and MC3T3-E1 cells (77). Cell cycle–dependent characterization of single MCF-7 cells in G1 and G2/M cell cycles were also studied using 2D-CE. Cells of different phases showed a 2.5x increase in variability over cells of the same phase. Over 100 components were resolved by 2D-CE with a 260-spot capacity (80).

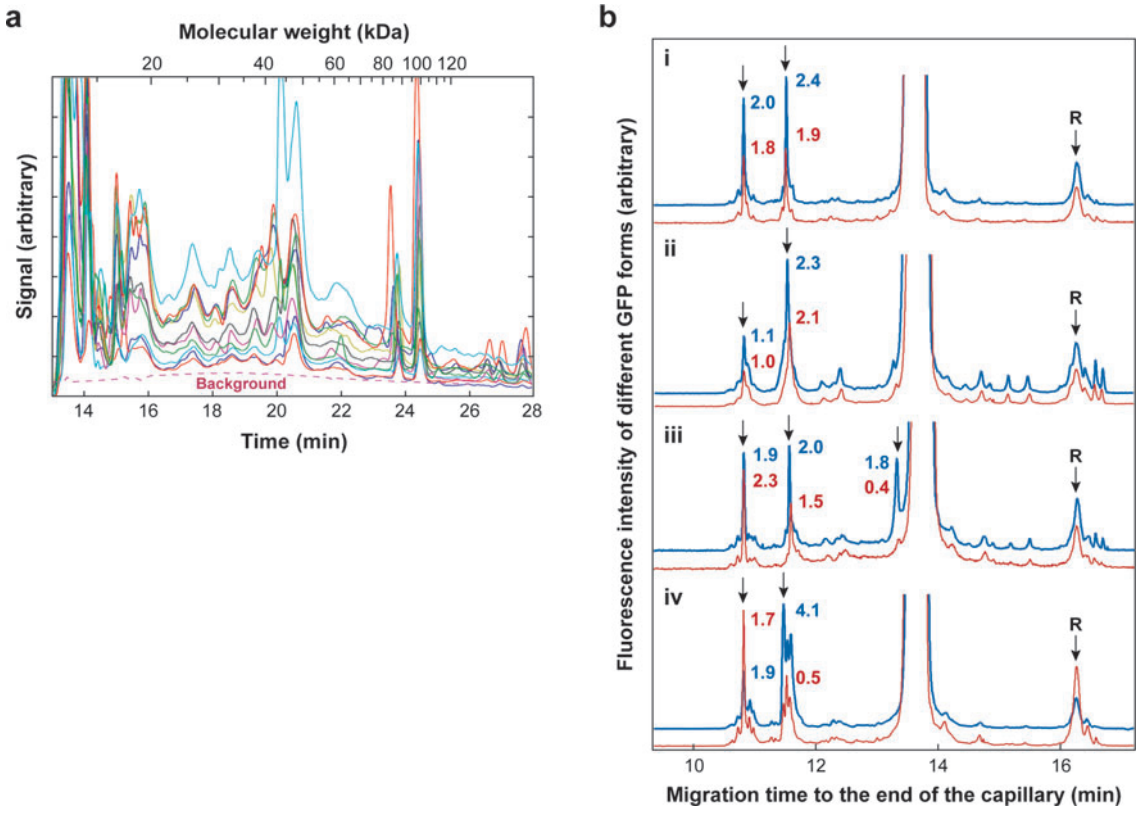


Figure 2

(*a*) Protein profile of 11 different HT-29 human cancer cells analyzed by capillary-sieving electrophoresis with laser-induced fluorescence. The dashed curve represents the background signal of the cellular supernatant. Each trace represents a different single cell. Reproduced with permission from Reference 75. (*b*) Differences in GFP (green fluorescent protein) expression patterns between 4T1 sister cells stably transfected with GFP. Each panel shows an electropherogram of a different pair of sister cells (*red*, *blue traces*). Numbers indicate relative intensities of adjacent peaks. Reproduced with permission from Reference 76.

Additionally, cell-to-cell heterogeneity was observed in a cell cycle phase study of mouse monocytic-macrophage cells (RAW 264.7) (81). See also the Dovichi group's article on chemical cytometry in this volume (82).

2.5.3. Detection of single proteins in a single cell. Identification of a single specific protein from among the many thousands of proteins within a single cell requires either that the protein be at a very high concentration relative to other proteins or that the protein be tagged to distinguish it from other proteins. Proteins can be selectively tagged with an antibody or with a molecularly engineered fluorophore, i.e., GFP. These two strategies have been pursued by several different

investigators. Xiao and colleagues (83) created a CE-based immunoassay to measure P-glycoprotein (PGP) levels in normal and multi-drug-resistant K562 cells. Single cells were loaded with a goat IgG antibody against PGP and a fluorescein-tagged antibody directed against goat IgG. The cells were then were analyzed by CE-LIF to detect the PGP-antibody complex.

The genetic fusion of GFP to a protein of interest followed by expression of the GFP-protein construct in cells is used throughout biology to examine gene expression, protein mobility and localization, and other aspects of protein function. Malek and Khaledi (84) utilized this technology to examine GFP expression in HEK293 cells by CE-LIF. GFP was detected at very high sensitivity with detection limits of 50 zmol in single cells. Brown and Audet employed a novel approach to estimating the cell-sampling efficiency of the single-cell laser lysis CE-LIF system. They investigated the effects of laser focus placement and laser pulse energies on the sampling of GFP expressed in HEX 293T cells (16). Hu and colleagues combined fluorescence microscopy and chemical cytometry to measure total GFP fluorescence from single cells. In this study, total fluorescence of GFP expressed by 4T1 cells was first measured by an inverted fluorescence microscope, then the cells were analyzed by CE-LIF. This investigation demonstrated that the total fluorescence intensity depended on the number of intracellular fluorophores and was independent of the cell size, cell shape, and intracellular GFP distribution (85). The expression pattern of GFP was observed in dividing cells by two-channel CE-LIF to show that GFP was not equally distributed to the daughter cells (76). Turner and colleagues performed CE with ultrasensitive fluorescence detection of GFP from a single bacterium (*Deinococcus radiodurans*) by CE-LIF. The system had detection limits of 100 ymol (60 GFP copies) (86).

Chemical cytometry: uses high-sensitivity analytical tools, including mass spectrometry, electrochemistry, and capillary separation methods, to chemically characterize single cells

RT-PCR: reverse transcription–polymerase chain reaction

2.6. RNA

Measurement of RNA from single cells is generally performed using an amplification step known as reverse transcription–polymerase chain reaction (RT-PCR), which also converts the RNA into complementary DNA (cDNA). Single-cell (SC) RT-PCR was performed by Lillard and Zabzdyr in vials containing a single cell with low μL volumes of PCR–nucleotide mixture. The first reported use of CE-LIF and SC-RT-PCR investigated β-actin expression in human prostate carcinoma (LNCaP) cells (87). A gene-specific primer pair for β-actin was used to selectively amplify β-actin mRNA from the cell, while hydroxypropylmethylcellulose and ethidium bromide were used as a sieving matrix and a fluorescent label for DNA, respectively (87). Lillard and Zabzdyr (88) also measured the expression of two genes, β-actin and αER, in single human breast cancer (MCF-7) cells using CE-LIF and SC-RT-PCR. In similar experiments, Lu and collaborators measured APETELA2 (AP2) and LEAFY (LFY) gene expression in the shoot apical meristem, leaf, root, and stem of single *Arabidopsis* cells in response to the growth regulator gibberellic acid (89). In this instance, YO-PRO1 replaced ethidium bromide as the DNA label of choice.

The Yeung group (90) took SC-RT-PCR to a new level by developing an on-line capillary PCR method with CE-LIF, all on a single capillary. A 100-cm-long capillary,

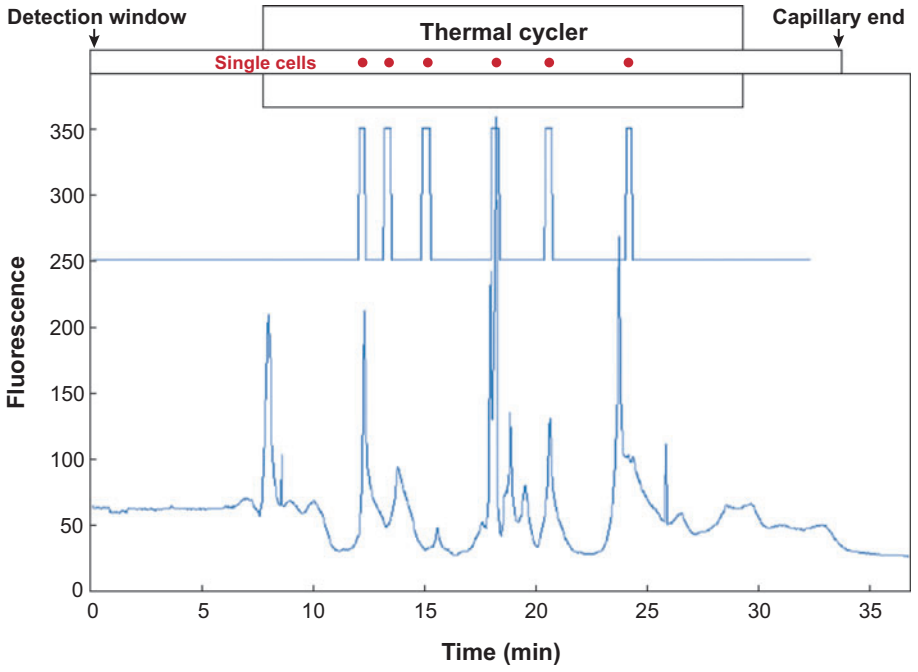


Figure 3

On-line capillary polymerase chain reaction with capillary electrophoresis–laser-induced fluorescence. An electropherogram (*bottom*) shows amplified β-actin DNA from single cells. The spacing and position of the DNA peaks in the electropherogram correspond to the position of single cells (*circles*) within the capillary, as calculated by migration velocity and starting position of the cell prior to reaction. Reproduced with permission from Reference 90.

placed into a PCR thermocycler, was loaded with human lymphoblast cells mixed with β-actin primer, SYBR Green I, and the PCR mixture. The cells were monitored by fluorescence microscopy to ensure sufficient spacing between individual cells in the capillary, after which RT-PCR was initiated. Following RT-PCR, the sieving buffer was added, an electric field was applied, and the amplified DNA encoding β-actin was measured. Discrete fluorescent peaks in the capillary corresponded to each cell originally loaded into the capillary, as shown in **Figure 3** (90).

Lillard & Han (91) performed direct measurement of RNA from single cells without conversion to DNA and the consequent amplification. Ethidium bromide–labeled RNA fragments were first detected using CE-LIF in single Chinese hamster ovary (CHO-K1) cells. Separation of an RNA ladder was used to characterize sieving performance while RNase I was used to verify that only RNA was detected. RNA degradation, following application of hydrogen peroxide, was observed in single cells. In subsequent work, Lillard's group tested alternative fluorophores (SYBR Green I and SYBR Gold) in labeling of RNA in CHO-K1 cells (92). CE-LIF was also used to examine RNA expression in CHO-K1 cells in different stages of the cell cycle (M, G1, S, G2). The total amount of RNA increased over each phase; however, the rate of increase of individual RNA sequences varied with the cell cycle state (93).

2.7. Miscellaneous

2.7.1. Steroids. Malek and Khaledi measured steroidal compounds in single R2C cells (Leydig tumor cells from rat testicle) (94). DMSO was used to permeabilize the cell membrane, allowing entry of dansylhydrazine into the cell. Within the cell, dansylhydrazine reacted with progesterone, converting it to a fluorescent compound detectable by CE-LIF. Progesterone in the R2C cell was calculated at 0.62 μM.

2.7.2. Inositol-1,4,5-triphosphate. Inositol-1,4,5-triphosphate (IP_3) is a second messenger that is generated in response to the binding of many hormones, growth factors, and neurotransmitters to their cognate receptors on the plasma membrane of cells. IP_3 binds to receptor/channels to release Ca^{2+} from the endoplasmic reticulum (ER) into the cytosol. Allbritton and colleagues developed a CE-based system to measure IP_3 in single cells (95). A permeabilized PC12 or BHK-21 cell loaded with the calcium-sensitive dye mag-fura-2 was used at the outlet of the capillary as a sensor for detection of IP_3. Cytoplasm sampled from a *Xenopus* oocyte was separated by CE and the cell components were delivered from the capillary onto the "detector" cell. When IP_3 eluted onto the detector cell, Ca^{2+} was released from the ER, altering the fluorescence of the mag-fura-2 contained in the ER. Thus, the fluorescence of the detector cell was used to quantitate the concentration of IP_3 in the cytoplasmic sample. **Figure 4** shows a schematic of this technique. IP_3 was measured in *Xenopus* oocyte both with and without the addition of the agonist lysophosphatidic acid to the oocyte. The basal concentration of IP_3 was 40 nM, rising to 650 nM after agonist addition. IP_3 production was also measured at varying times following sperm infusion with a *Xenopus laevis* egg. These measurements demonstrated that a wave of increased IP_3 accompanied the Ca^{2+} fertilization wave as it traversed the cell (96).

2.7.3. Antigen-antibody complexes. In an interesting experiment combining nanomaterials with single cells, antibody-coated latex particles were used to bind glucose 6–phosphate dehydrogenase proteins and act as intracellular protein counters. After microinjection of the antibody-coated particles into erythrocytes, CE with light scattering as a detection scheme was used to monitor the resulting antigen-antibody particle complexes. The performance of this CE-based assay was comparable to more traditional fluorescence assays, with a detection limit of 620 molecules of glucose 6–phosphate dehydrogenase (97). In a separate experiment, CE with LIF was used to detect IFN-γ in natural killer (NK) cells. Electroporation was used to permeabilize the NK cells, allowing fluorescently-labeled anti-IFN-γ antibodies entry into the cells. Zeptomole detection limits for the antigen-antibody complex were attained and new alloantigens of IFN-γ were discovered (98).

3. MONITORING ENZYMATIC ACTIVITY IN SINGLE CELLS

3.1. Kinases

Kinases play an important role in numerous cell signaling pathways and have been implicated in numerous diseases, including cancer. Examination of kinase activity is best

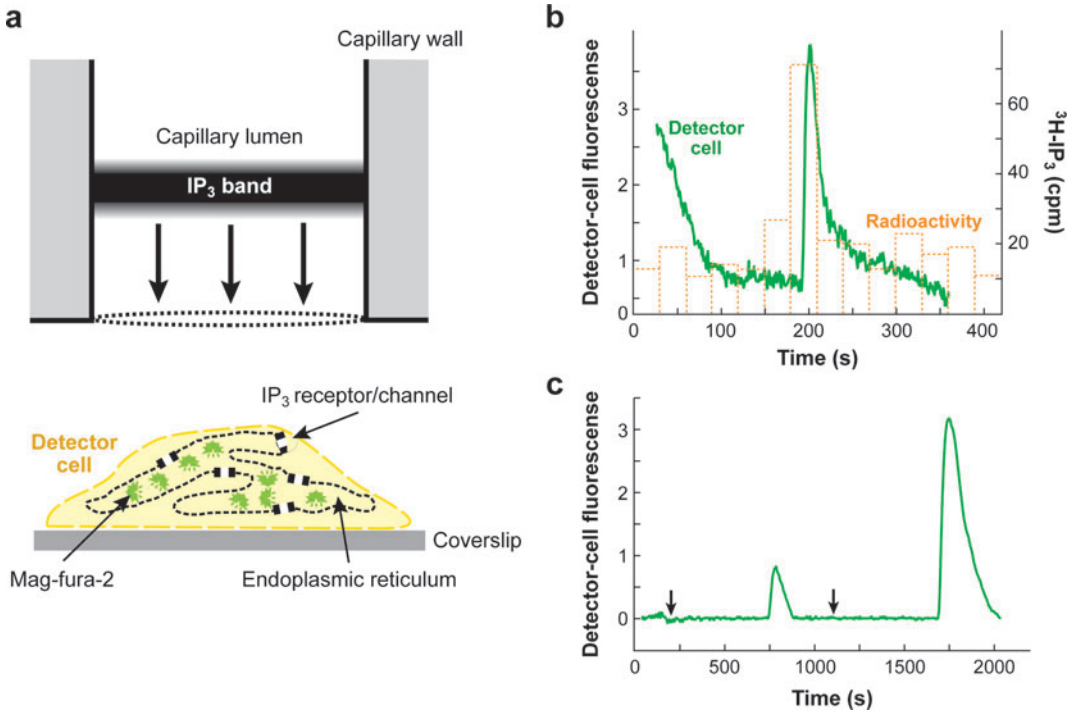


Figure 4

Inositol-1,4,5-triphosphate (IP_3) detection using capillary electrophoresis (CE) and a detector cell. (*a*) Schematic of detector cell–CE system. IP_3 eluted onto a detector cell causes Ca^{2+} release from the endoplasmic reticulum of the detector cell, triggering an increase in mag-fura-2 fluorescence. (*b*) Measurement of IP_3 elution time by a detector cell (mag-fura-2 fluorescence) (*line graph*) and radioactivity (*dotted bar graph*). (*c*) Detection of nanomolar IP_3 by a detector cell. At the first arrow, 10 nL of 100 nM IP_3 is eluted onto a detector cell. At the second arrow, 10 nL of 2.5 μM IP_3 is eluted onto the same detector cell. Reproduced with permission from Reference 95.

performed when these enzymes are in their native state inside cells; hence a capillary electrophoresis–based assay relying on designer substrate peptides was developed to monitor kinase activity in single cells (17). Fluorescein-labeled peptides were selected and synthesized as substrates for a number of different kinases, including protein kinase C, protein kinase A, CamKII, and cdc2 kinase. After microinjection and incubation of these substrates into *Xenopus* oocytes, CE-LIF was used to separate and measure the phosphorylation of the peptide substrates. Using this method, kinase activity was assessed based on the rate and percentage of phosphorylation in response to different stimuli (17). Protein kinase C activity was assessed using CE-LIF following stimulation with ATP or IgE-antigen in both rat basophilic leukemia (RBL) and mouse embryonic fibroblast (3T3) cells. The effect of the kinase inhibitor staurosporin on overall kinase activity was evaluated using CE-LIF with numerous fluorescently labeled peptide substrates loaded simultaneously into single RBL cells (99, 100).

The Krylov group used similar CE-LIF methodology to examine the phosphorylation of a molecularly engineered substrate in C2C12 rat myoblast cells (101). The substrate was composed of GFP coupled to a series of protein kinase A phosphorylation sites. When the cells were stimulated with 8 bromo–cAMP, which activates protein kinase A, only the fully phosphorylated form of the substrate was detected. Although this method eliminated the substrate-loading step, it was complicated somewhat by the nearly complete phosphorylation of the substrate in unstimulated cells (101).

3.2. Farnesyl Transferases

Farnesylation, the transfer of a 15-carbon farnesyl moiety to the sulfhydryl group of a cysteine, is a posttranslational modification that increases the hydrophobicity of proteins. Protein farnesyltransferase (PFTase) catalyzes this irreversible modification. Ras protein, which is implicated in 30% of cancers, is farnesylated by PFTase. Drugs targeting the farnesylation of ras are currently being screened for their effectiveness in treating cancer, necessitating the development of new PFTase assays. CE-LIF was used to investigate the PFTase activity in mouse mammary tumor (4T1) cells and 3T3 cells loaded with a ras-derived fluorescently labeled pentapeptide substrate. Confocal microscopy was used to confirm substrate loading, while CE-LIF was used to monitor fluorescent metabolic products of the peptide. The pentapeptide substrate was converted to products; however, none of the products matched the products expected after farnesylation, proteolysis, or methylation (102, 103).

3.3. Nitric Oxide Synthase via Metabolite Detection

Nitric oxide synthase (NOS) is responsible for producing nitric oxide (NO), a highly reactive biological molecule responsible for vasodilation, neurotransmission, and the inhibition of platelet aggregation. Because NO is quickly consumed and metabolized due to its high reactivity, NOS activity is monitored by measuring the metabolites NO_2^- and NO_3^-. Capillary electrophoresis with direct UV absorbance was used to detect NO_2^- and NO_3^- in *Pleurobranchaea californica* neurons. CE studies confirmed that high levels (2 and 12 mM, respectively) of both NO_2^- and NO_3^- existed in neurons histochemically positive for NADH-diaphorase, a signature of NOS activity. There were no detectable levels of NO_2^- or NO_3^- in neurons lacking NADH-diaphorase activity (104). CE-LIF was also used to monitor the metabolites from the arginine-citrate cycle, which is modulated by NO. L-citrulline, L-arginine, L-argininosuccinate, L-ornithine, and L-arginine phosphate were measured in *Pleurobranchaea californica* and *Aplysia californica* neurons, using fluorescamine labeling (105). *Lymnaea stagnalis* neurons were used to correlate NOS expression with metabolite presence (L-citrulline, L-arginine, L-argininosuccinate, NO_2^- and NO_3^-), using CE with LIF (106).

In a novel experiment, the fluorescent NO indicator DAF-2 was used to monitor NO in single *Aplysia californica* and *Lymnaea stagnalis* neurons (107). Because DAF-2–based measurements can be altered by ascorbic acid, which occurs at high levels in neurons, ascorbic acid oxidase was added to remove endogenous ascorbic acid.

CE-LIF was then used to separate the DAF-2-NO adjunct from DAF-2 bound to dihydroxyascorbic acid (product of the reaction of ascorbic acid oxidase with ascorbic acid) (107).

4. ANALYSIS OF SUBCELLULAR MACROCOMPLEXES

Capillary electrophoresis is suitable for the analysis of samples ranging from attoliter to nanoliter volumes. Most eukaryotic cells measure several picoliters in volume, with larger cell types such as eggs and oocytes in the nanoliter range. Within cells, however, the volume of many organelles can be measured in attoliters or femtoliters; these compartments are therefore suitable for analysis by CE. The use of CE in examining acidic organelles, mitochondria, nuclei, and secretory vesicles is described in this section.

4.1. Acidic Organelles

The effect of subcellular drug distribution on cytotoxicity and drug efficacy is the driving force behind the Arriaga group's pioneering methods for the separation and detection of acidic organelles from single cells (108–113). The group's early work perfected the detection of the antitumor drug doxorubicin (DOX) and its metabolites in single NS-1 cells by MEKC with LIF (108). The use of MEKC-LIF and on-capillary lysis of human leukemic cells (CEM-C2 and CCRF-CEM) treated with DOX revealed a unique metabolic profile for each cell line (109). The ultimate goal, to interrogate individual organelles, was achieved when acidic organelles, identified by their accumulation of fluorescent nanospheres, were separated from crude subcellular fractions. Thereafter, their DOX content was quantified by CE-LIF (110). CE-LIF was also used to detect DOX and a fluorescent reporter of reactive oxygen species within acidic organelles (111). Arriaga and colleagues have also utilized the ratiometric dye fluorescein tetramethylrhodium dextran to monitor the pH of acidic organelles from individual CEM-C2 and CCRF-CEM cells (112). Recently, this group measured the maturation and DOX content of acidic organelles from individual CEM-C2 and CCRF-CEM cells using Alexa Fluor 488 Dextran (113).

4.2. Mitochondria

Arriaga and colleagues have also used CE-LIF to monitor mitochondria from single cells. CE-LIF was used to measure DOX in mitochondria labeled with MitoTracker Green (114, 115) and to study the electrophoretic mobility of individual mitochondria from mouse hybridoma (NS1) and CHO cells (116, 117). Interestingly, these studies showed that there are many different types, shapes, and sizes of mitochondria, which may have different mobilities within a single cell (116). A schematic of cell lysis and release of mitochondria from single cells is shown in **Figure 5**. In an extension of this experiment, the Arriaga group examined the chemical composition of mitochondria, particularly the concentration of cardiolipin (diphosphatidylglycerol) therein. CE-LIF was used to quantify the amount of cardiolipin in mitochondria

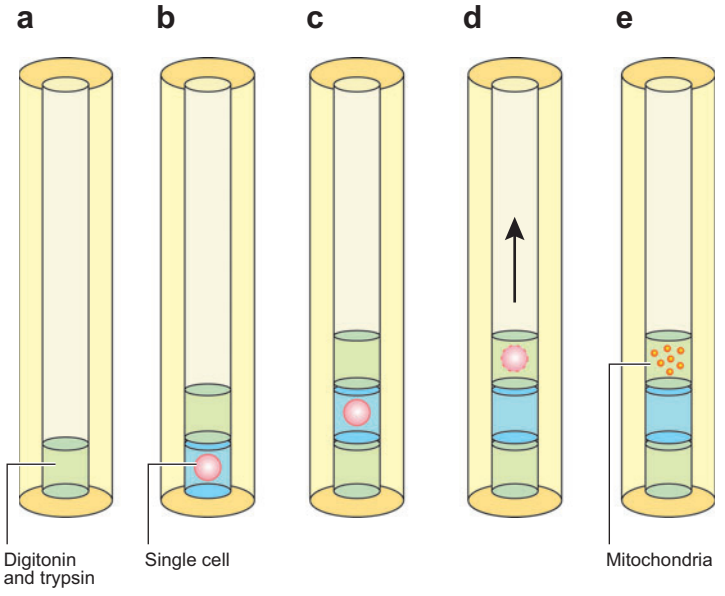


Figure 5

Cell lysis and release of mitochondria from single cells. (*a*) Digitonin and trypsin are hydrodynamically injected into the capillary. (*b*) A plug containing a single cell is loaded into the capillary, followed by (*c*) another plug of digitonin and trypsin. (*d*) Electrophoretic flow is used to move the cell into the first plug, resulting in permeation and proteolysis of the cell. (*e*) The mitochondria are released from the cell. Reproduced with permission from Reference 120.

from NS1 cells, using 10-nonyl acridine orange (NAO), a fluorophore which binds cardiolipin (24, 118, 119). Recently, a mitochondria-targeted form of DsRed2, a fluorescent protein, was used to label mitochondria in single osteosarcoma 143B cells. This targeted DsRed2 localized to the mitochondrial matrix; however, the protein leaked from the mitochondria in the presence of microchondrial disruption. When used in conjunction with NAO, intact DsRed2-labeled mitochondria were separated from damaged NAO-labeled mitochondria and mitochondrial fragments by CE-LIF (120). In a separate experiment, MEKC-LIF was used to detect the superoxide probe hydroethidine. Superoxide generation was measured in mitochondria isolated from rat skeletal tissue (121).

4.3. Nuclei

The nuclear-targeted protein nuDsRed2 and the plasma membrane–bound farnesylated enhanced green fluorescent protein (EGFP) were used as fluorescent probes to monitor the interaction of the nucleus and the plasma membrane when ΔH2–1 cells were treated with digitonin. CE-LIF of the nuclei demonstrated the absence of EGFP, demonstrating that the plasma membrane was separated from the nuclei (25). Subsequent work used two different EGFP-labeled human aminoacyl–tRNA synthetases (aaRSs), expressed in human-derived ΔH2–1 osteosarcoma cells, to examine subcellular localization of proteins within the cell, particularly the nucleus. CE-LIF of the cytosolic and nuclear fractions revealed that only one EGFP-aaRS localized in the nucleus, along with the nuclear marker nuDsRed (122).

In a separate experiment, CE-LIF was used to quantify DOX in single nuclei sampled from human leukemia CEM-C2 cells and CCRF-CEM cells. Cells were

lysed with SDS and the isolated nuclei were loaded into the capillary, eliminating the problem of detecting DOX in other cell compartments (123).

4.4. Secretory Vesicles

Secretory vesicles are used by cells to store biological molecules, such as neurotransmitters, for immediate delivery after a particular signal or trigger. Capillary electrophoresis was used to examine the contents of secretory vesicles obtained from the atrial gland of *Aplysia californica*. Single attoliter-sized secretory vesicles were optically trapped and loaded into a capillary. The vesicles were lysed and fluorescently labeled in the capillary with NDA prior to separation. CE-LIF revealed the presence of numerous low-mass compounds, including the dominant component, taurine, inside single secretory vesicles (23).

5. MICROCHIP CAPILLARY ELECTROPHORESIS

Implementation of chemical cytometry in the microfluidic format confers several advantages associated with lab-on-a-chip devices. The small footprint of microdevices and their ability to control cell movement enables high-throughput designs with scores of separation channels. With further refinement of these technologies, it should be possible to analyze thousands of cells per hour using multiplexed continuous flow methods like those described below. Because the size and shape of microchannels can be engineered for a specific task, cells can be lysed with minimal dispersion of pL content volumes, producing high-efficiency separations. Automation of the sample-handling steps inside a microdevice has great potential for eliminating many of the labor-intensive procedures associated with analyzing the contents of single cells. The successful realization of single-cell CE on-chip is tantalizing, and a number of reviews have already touched on the subject of single cells in microchips (124–129), with two devoted specifically to progress in chemical cytometry (13, 130). This work is highly interdisciplinary, requiring expertise in engineering, optics, chemistry, and cell biology; and there is a need for significant improvement in the manufacture of working devices. One of the most challenging aspects of performing single-cell cytometry in a microfluidic format is the strategy used for positioning a cell at the time of lysis. The positioning method varies greatly among different groups and impacts the analytes that can be measured. In the following sections we discuss five different methods of cell positioning for lysis, summarizing the setup, method, cells and analytes, and results.

5.1. Docking on Channel Walls

Allowing a cell to settle on a solid wall, then lysing it with SDS or an electric field can serve as a quick proof of concept without sophisticated fabrication techniques. In one study by Gao and colleagues, a low electric potential was used to position a cell in a 12 $\times$ 48 μm glass separation channel. NDA-derivatized GSH and reactive oxygen species were separated from erythrocytes after lysis in 40 ms using a field strength of 280 V/cm (131). The separation efficiency for GSH was five times better than that

obtained by pinched injection. A similar strategy of immobilizing cells on a channel wall was also used by Shi and collaborators to measure neurotransmitters in a (PC12) pheochromocytoma cell line (132).

5.2. Positioning Cells in Traps

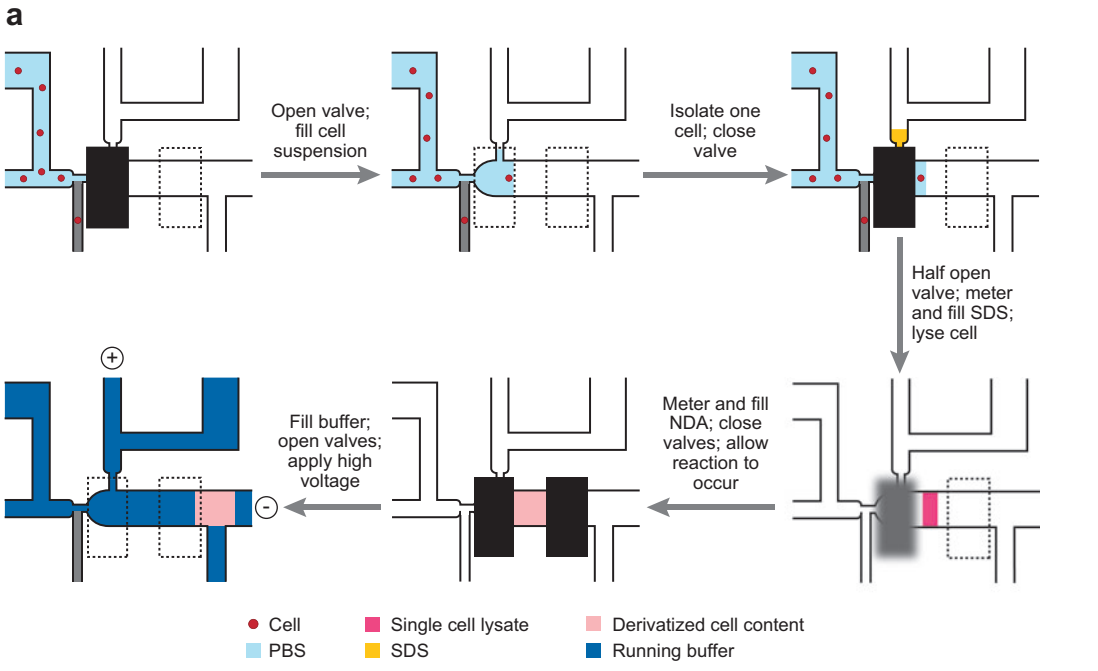
An engineered weir or obstruction at the start of a separation channel can be used to successfully integrate trapping, lysis, and separation in a single microchip design. With the cell aligned for optimal injection of its contents, the operator is free to switch buffer solutions and/or apply potentials to a separation channel to initiate the analysis process. Ros and colleagues (133) used optical tweezers to move *Spodoptera frugiperda* (Sf9) cells to an injection cross in a chip with 95 × 6 μm channels and ~7.4 × ~2.2 μm containment posts on three sides. After trapping the cell, SDS or an electrical field was used for lysis. When Sf9 cells expressing GFP and YFP proteins were lysed with 1% SDS and an electric field was applied, two broad unresolved peaks were observed using LIF. When cells expressing a single GFP protein were lysed by application of 1250 V/cm for more than 50 ms, then separated at 830 V/cm, a single sharp peak with tailing was observed, followed by an increase in the baseline fluorescence. The same group also used UV-LIF with Sf9 cells for label-free detection. Black carbon was incorporated into the PDMS microchips to increase the sensitivity (134, 135).

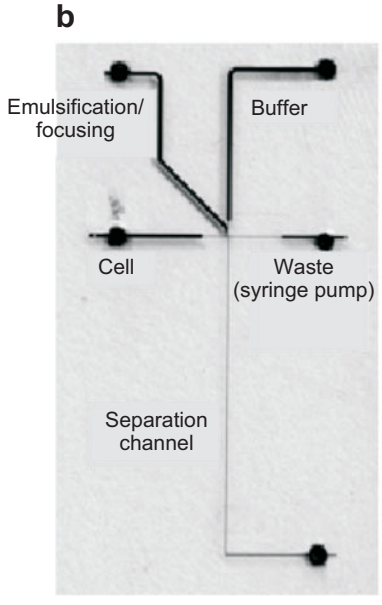
Munce and colleagues (136) used optical tweezers for selection and transportation of acute myloid leukemic (AML) cells on chip. Cells were incubated with calcein AM and Hoechst dyes in a chamber and docked in tapered 10-μm microfluidic channels. Application of a 300-V/cm electric field resulted in cell lysis in <0.3 s, followed by electrophoresis of the cell contents into a 10 × 12 μm channel. Four unidentified peaks were seen in the electropherogram, possibly due to hydrolysis of the calcein dye by the cell. With four channels, a throughput of 24 cells/h was achieved.

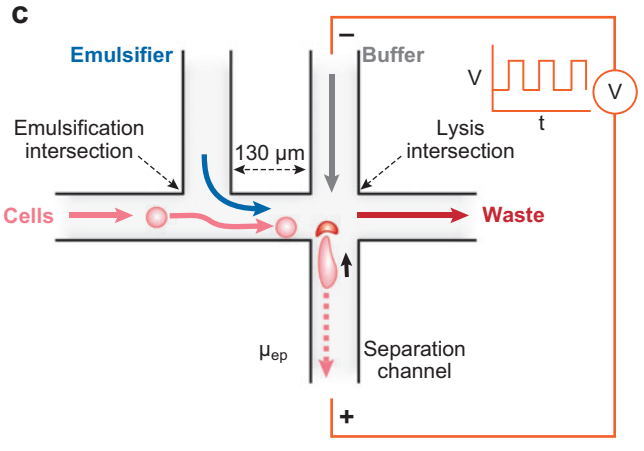
5.3. Immobilizing Cells with Valves

A technically innovative area of cell manipulation is the use of valves to control cell and reagent flows. Some of the best separations have been obtained with this technique. In 2004, the Zare group (137) revealed a three-state PDMS valve design (**Figure 6*a***) to trap an individual Jurkat T cell in a 70-pL chamber. Because the volume of a typical Jurkat T cell is 1 pL, the chamber results in a 70-fold dilution of cell contents. Lysis reagents, followed by derivatization reagents, were metered into the cell chamber through a partially closed valve acting as a "picopipette" and were allowed to react with the cell. After filling the channels with electrophoretic buffer, the valve was opened and MEKC was initiated. The separation quality for the detected amino acids was improved relative to that of the analytes detected when cell traps and docking were employed.

With a similar valve design, Huang and colleagues (138) implemented sophisticated single-molecule detection optics to increase the sensitivity for target analytes. They examined the number of β-adrenergic receptors expressed in Sf9 cells. The N-terminus of the receptors was tagged with FLAG and the Cy5-labeled monoclonal







antibody was used as a fluorescent label. In a study of five cells, they found between 2000 and 60,000 receptors per cell. The variation is likely due to the nature of virus infection in the transfection process. In another experiment, the authors studied phycobiliprotein complexes in cyanobacterial cells grown in different nitrogen environments. Up to three cells at a time were lysed and the contents separated. Cells grown in a nitrogen-rich environment had much higher levels of phycobiliprotein.

A drawback to some of the above-mentioned methods is the use of mannitol or a similar reagent as a replacement for salt in the cell buffer. Because separations must be performed partially in the cell buffer, this permits the separation buffer to be low in ionic strength and avoid excessive joule heating. However, while the isotonic conditions prevent the cells from bursting, they also induce cell death or severe damage, resulting in alteration of many analytes within the cells.

5.4. Positioning Cells in a Continuous Flow Stream

The continuous single cell lysis and separation in a modified cross channel was an exciting development by McClain and coworkers (12) in 2003. The glass chip design (shown in **Figure 6*b*,*c***) introduced cells by suction into a tapered channel perpendicular to the separation channel. At 130 μm before the cross area, a focusing channel confined the cells into a single file near the separation side of the channel and added an emulsification reagent. At the 30 × 30 μm cross, the cells were subjected to an AC field that served to rapidly lyse (<33 ms) and inject the contents downstream towards a detector. Analysis of Oregon Green and carboxyfluorescein loaded into cells demonstrated that efficient injection of the cellular contents into the separation channel was obtained when the cell velocity, driven by hydrodynamic flow, was less than the electrophoretic velocity of the injected analyte. The throughput was 7–12 cells/min for up to 45 min. The separation performance was excellent when compared with other separations of single-cell contents on-chip. In addition, a physiologic buffer was used to keep the cells in an unstressed state before the rapid lysis event.

In another twist on this strategy, Wang and coworkers (139) recently showed lysis of CHO cells in a PDMS cross channel with perpendicular hydrodynamic and electric field driven flows. Detection of calcein AM products was reported at rates up to

Figure 6

(*a*) Stepwise diagram showing the process for handling single cells with a valve system. Valves are operated to trap a single cell in the chamber. Sodium dodecyl sulfate (SDS) and a dye are metered using the "picopipette" and three-state valve system. Valves are opened and an electrical potential is applied to separate the derivatized cell contents. Reproduced with permission from Reference 137. (*b*) Image of continuous-flow separation microchip and (*c*) schematic showing its operation. The cells are carried by hydrodynamic flow (*solid arrows*) toward the waste. At the focusing junction, emulsifier is introduced. Cells are lysed by square waveform potential at the lysis intersection and the contents with high mass-to-charge ratio travel down the separation channel while larger organelles continue toward the waste. Abbreviation: NDA, naphthalene-2,3-dicarboxaldehyde. Reproduced with permission from Reference 12.

85 cells/min. Although the throughput is about 10× higher than the study mentioned above, it was accomplished using a buffer containing 250 mM sucrose and 10 mM phosphate to minimize joule heating, which may be unsuitable for the analysis of many cellular analytes.

5.5. Retarding Cell Movement with a Viscous Medium

Kleparnik and colleagues used a viscous medium to retard cell movement prior to lysis (140). Microchannels were fabricated on a CD-like plastic disc and then filled with polyacrylamide before injection of cardiomyocytes. Cells were moved into the separation cross by vacuum and movement was retarded by the polymer sieve. In each experiment, a cell was lysed in an alkaline environment for 3 min. The released DNA was separated with 60 V/cm in a 2% polyacrylamide sieving solution and the ethidium bromide–labeled contents were detected with LIF. The effect of incubation of the cells with 30 μm doxorubicin was investigated. Increased doxorubicin incubation times resulted in poorly resolved DNA fragment peaks, thought to be caused by the onset of cellular necrosis.

SUMMARY POINTS

1. Many strategies for cell lysis and loading into microchannels have been developed; these methods are suitable for all almost all conceivable cellular analytes.
2. Microelectrophoretic methods have been successfully combined with a variety of detection methods (e.g., LIF, absorbance, EC, light scatter, mass spectrometry) to quantitate analytes in single cells.
3. A large number of analytes can be measured in single cells, including neurotransmitters, amino acids, oligosaccharides, nucleotide polymers, proteins, and a variety of metabolites.
4. Macromolecular entities, such as antigen-antibody complexes, as well as organelles can be sampled from single cells and electrophoretically separated.
5. Chemical labeling of endogenous cellular analytes such as proteins, steroids, peptides, and metabolites can be performed efficiently either before or after cell lysis and yields excellent detection sensitivity.
6. Introduction of nonnative molecules such as enzyme substrates or antibodies into cells permits the measurement of many cellular attributes such as enzymatic activity and protein concentration.
7. Hundreds of components from a single cell can be resolved by 2D-CE with LIF.
8. The highest throughput for single cell analysis (10 × 80 cells/min) has been achieved on microfluidic devices.

FUTURE ISSUES

1. The vast majority of small molecule analytes within cells cannot be detected by current methods. There is tremendous opportunity for microelectrophoretic techniques to close this gap through the development of new chemical labeling and identification methods.
2. Current labeling and separation techniques make monitoring and quantifying specific proteins in cells difficult. New methods are needed to distinguish specific proteins from the thousands of other proteins within a cell.
3. Microfluidics demonstrates great promise in single-cell analysis due to the potential to integrate multiple cell- and lysate-handling steps on a single miniaturized device; however, substantial challenges must be overcome to reach these goals.
4. Strategies for high-throughput analysis of single cells need to be developed for nonadherent as well as adherent cell lines. Microfluidic devices will likely be the best option for high-throughput analysis of cells not adherent to a surface, as the cells are easily moved to the separation channel. However, capillary-based measurements may be optimal for cells that are attached to a surface, as the capillary can be readily transported to the immobilized cell.
5. Methods of quantifying large numbers of analytes from the same single cell would be of great value to cell biologists and would be complementary to many other established single-cell techniques, such as microscopy methods.
6. Although microelectrophoretic methods can reliably quantitate a large number of analytes in single cells, these techniques have not yet moved into mainstream biology. Understanding the reasons for the slow adoption by biologists will inspire chemists to design more biologically friendly applications for use in the laboratory and in related biomedical settings.

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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