Chemical Cytometry: Fluorescence-Based Single-Cell Analysis

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Abstract

Cytometry deals with the analysis of the composition of single cells. Flow and image cytometry employ antibody-based stains to characterize a handful of components in single cells. Chemical cytometry, in contrast, employs a suite of powerful analytical tools to characterize a large number of components. Tools have been developed to characterize nucleic acids, proteins, and metabolites in single cells. Whereas nucleic acid analysis employs powerful polymerase chain reaction–based amplification techniques, protein and metabolite analysis tends to employ capillary electrophoresis separation and ultrasensitive laser-induced fluorescence detection. It is now possible to detect yoctomole amounts of many analytes in single cells.

1. INTRODUCTION

1.1. Cellular Heterogeneity

The cell is the fundamental unit of life, and individual cells often differ significantly from their neighbors. As an example, **Figure 1** presents a photomicrograph of a population of the bacterium *Deinococcus radiodurans*; the fusion protein shown plays a key role in the organism's response to genetic damage. Only a small fraction of the population shows green fluorescence, and these cells have superior survival when challenged with a genotoxin. Classic analytical methods would report the average composition of cells, which would conceal the cell-to-cell differences that determine each cell's survival under stress. Other methods are required to characterize the composition of individual cells in order to understand the behavior of a cellular population.

Although the study of microbes is important, more exciting opportunities may be found in the characterization of cells from complex organisms, which can provide insights into the function of the cell and its responses to changes in its environment. We provide three examples that motivate this work. First, a zygote undergoes astonishing changes as it develops from a single cell into a fully functional organism; cataloging the changes in cellular composition at each stage of an embryo's development provides powerful insights into ontogeny. Second, the brain is the most complex organ in a vertebrate, and characterization of differences in neuronal composition not only yields an opportunity to systematically classify cells but also provides some guidance as to the function of each cell. Third, many tumors are highly heterogeneous, and pathologists grade tumors based on the subjective interpretation of the heterogeneous appearance of the tissue. We hypothesize that heterogeneity in cellular composition increases as the disease progresses, which may serve as a prognostic indicator to guide individualized treatment. In essence, we are supplementing the subjective prognosis provided by the pathologist with an objective catalog of the components that define that heterogeneity.

Analysis of the composition of a cell is important, but cells are usually small, and their analysis presents technological challenges. **Table 1** summarizes the approximate composition of a single bacterium, yeast cell, mammalian cell, giant neuron, and

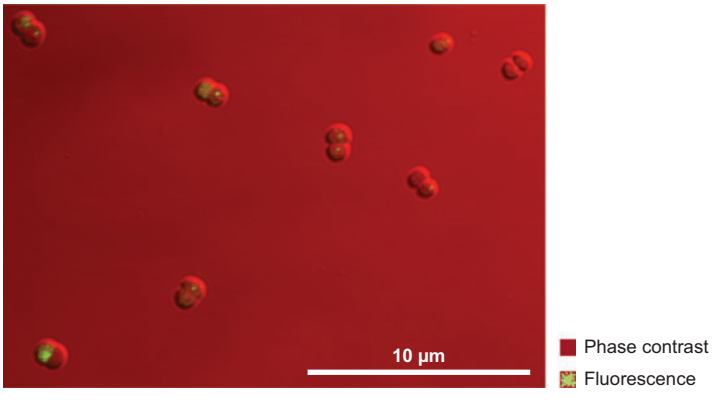


Figure 1

Combined phase contrast and fluorescence image of *Deinococcus radiodurans* that expresses the fusion between green fluorescent protein and RecA protein. Only a subset of cells expresses high levels of the repair protein, and these cells are primed to survive exposure to DNA-damaging agents.

Table 1 Approximate composition of a single cell

Component	Bacterium or mitochondrion	Yeast cell	Human somatic cell	Giant neuron	*Drosophila* egg
Diameter	1 μm	5 μm	10 μm	100 μm	500 μm
Mass	500 fg	50 pg	0.5 ng	0.5 μg	50 μg
Protein (~15%)	100 fg	10 pg	100 pg	100 ng	10 μg
Nucleic acids (~7%)	40 fg	4 pg	40 pg	40 ng	4 μg
Carbohydrates (~3%)	15 fg	1.5 pg	15 pg	15 ng	1.5 μg
Lipids (~2%)	10 fg	1 pg	10 pg	10 ng	1 μg
Small ions and molecules (~3%)	15 fg	1.5 pg	15 pg	15 ng	1.5 μg

Drosophila egg. Even in large cells, such as giant neurons, only a few nanograms of material are available for analysis. Microbes and organelles contain mere femtograms of material, presenting a formidable analytical challenge to the investigator.

1.2. Classic Cytometry

Cytometry employs instrumental methods to determine the amount of selected components in single cells; Shapiro's classic text provides outstanding background on the technology (1). Early work employed microspectrophotometers to obtain rough estimates of the nucleic acid and protein content of single cells based on absorbance at 260 nm and 280 nm, respectively (2). These initial studies laboriously measured absorbance from cells one at a time.

Two important technological advances arose from this early work. The first was the incorporation of a flow system to pass single cells sequentially through the microspectrophotometer and to record signals electronically; this system is the basis of flow cytometry. This technology benefited greatly from electronics developed by the atomic physics community for pulse height analysis of events generated by radioactive decay; these electronics were easily modified for use in recording the signals generated by single cells. The second advance was to incorporate a scanning stage to the microscope so that an entire field of view could be automatically passed through the microspectrophotometer, thereby allowing analysis of large numbers of cells; this technology is the basis of image cytometry. The incorporation of a confocal aperture improved resolution significantly, and the incorporation of digital cameras allowed rapid capture across the field of view. Image cytometry developed more slowly than flow cytometry because appropriate optical and data manipulation tools were not widely available until late in the twentieth century (1).

Flow and image cytometry are used to measure both the physical properties and the chemical composition of single cells, and are very important in both clinical and fundamental research. Physical measurements are usually related to cell size and include forward- and side-light scatter and the electrical resistance associated with a cell obscuring a narrow aperture in a flowing system, which is the basis of the venerable Coulter counter.

The chemical composition of a cell is characterized by use of affinity reagents in both image and flow cytometry. For example, intercalating dyes, such as Hoechst 33342 and propidium iodine, are used to quantitate the DNA content of a cell. These dyes are weakly fluorescent in an aqueous environment, but are highly fluorescent when intercalated between base pairs in double-stranded DNA. Recently divided cells are in the G1 phase of the cell cycle and have one pair of each chromosome. Cells that are about to divide are in the G2 or M phase of the cell cycle and have four copies of each chromosome. The fluorescence from intercalating dyes is twice as large for tetraploid cells compared to diploid cells. Cancer cells often have abnormal chromosomal composition due to the presence of additional chromosomal copies. These aneuploid cells generate a fluorescence signal that is larger than that generated by normal cells progressing through the cell cycle; aneuploid fluorescence often does not correspond to an integral number of chromosomes due to deletions and duplications of a portion of the genome. An increase in the heterogeneity of the chromosomal content and an increase in the fraction of aneuploid cells are often taken as negative prognostic indicators for cancers.

Fluorescently labeled antibodies are commonly used to determine the distribution of the expression of a particular protein in a cellular population. For instance, CD4-expressing lymphocytes are the target for HIV, and the determination of $CD4^+$ lymphocytes is a common tool for determining the efficacy of treatment of AIDS patients. Cells are treated with the labeled antibody, washed, and then analyzed by flow cytometry.

Multiparameter methods can be employed to simultaneously measure two or more signals from a single cell. For example, blood can be treated with both an intercalating dye and a fluorescently labeled anti-CD4 antibody. Only nucleated cells that express the CD4 antigen will generate fluorescence from both reagents, thereby allowing discrimination against non-nucleated cells that happen to express the CD4 antigen (3).

Conventional cytometry methods are common tools in biomedical research and clinical practice. They are able to measure five or more properties from a single cell, and they can process thousands of cells per second (4). Cytometers can be modified to select cells with a specific fluorescence signature and deliver them to wells on a microtiter plate; these cell sorters are powerful, nondestructive tools that are used to isolate rare cells for further study. However, conventional cytometry has two important limitations. First, only a limited number of components can be analyzed from a single cell; it is difficult to resolve the fluorescence signal from more than five components. Second, conventional cytometry employs affinity probes against known targets; the unexpected is therefore invisible.

2. CHEMICAL CYTOMETRY

2.1. Overview

Chemical cytometry employs powerful analytical methods to characterize the composition of a lysed cell. The analysis is destructive and slow, but provides the

opportunity to characterize hundreds of components from a single cell. Examples of chemical cytometry considered in this review employ a capillary-based separation technique, such as microbore liquid chromatography or capillary electrophoresis, to separate components from a single lysed cell. Ultrasensitive detection methods, typically laser-induced fluorescence, are used to measure the amount of the separated components.

2.2. History of Chemical Cytometry before Ultrasensitive Detection

Microscale electrophoresis methods were developed in the 1950s and 1960s to separate components from small amounts of sample, including single cells. The earliest study considered rRNA analysis of single cells based on electrophoresis on a silk fiber and detection by UV absorbance (5). A second study employed an acrylamide fiber to study hemoglobin from single erythrocytes (6).

A series of papers published in the 1960s and early 1970s reported using more reproducible separation methods for the study of single cells. For example, Hyden and colleagues performed sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 200-μm-diameter capillaries to separate nanogram amounts of proteins from single cells (7), Marchalonis & Nossal separated antibodies from single cells (8), and Wilson and Ruchel separated proteins from single giant neurons of *Aplaysia* (9–10). Repin and colleagues monitored lactate dehydrogenase from single oocytes (11). As a particularly interesting example, Neukirchen and colleagues utilized a miniaturized two-dimensional gel electrophoresis separation method and silver staining to characterize proteins separated from a single *Drosophila* egg (12). Several hundred proteins were resolved from the micrograms of protein contained in the egg.

2.3. History of Chemical Cytometry with Ultrasensitive Detection

Jorgenson inaugurated the modern era of single-cell analysis by combining capillary chromatography and electrophoresis with ultrasensitive detection methods for analysis of single giant neurons (13). A single giant neuron was isolated, homogenized, and centrifuged. The supernatant was injected directly onto the chromatography column and detected with either amperometry or fluorescence of naphthalene-2,3-dicarboxyaldehyde (NDA)–labeled compounds.

Ewing also performed experiments on single giant neurons, using a 10-μm-inner-diameter capillary to sample the cytoplasm of a single neuron. The sample was separated electrophoretically in the capillary and detected by a carbon fiber microelectrode (14).

In Jorgenson's early experiments, an entire cell was homogenized in a relatively large volume of buffer (13). Only a portion of this homogenate was then used for subsequent analysis. Although it is acceptable for the relatively large amount of protein present in a giant neuron, dilution must occur for this approach to be useful for analysis of typical somatic cells. Rather, it is more useful to inject a cell directly into the capillary, where it can be lysed before subsequent analysis.

In an early example, Hogan & Yeung employed monobromobimane to derivatize glutathione in individual erythrocytes; the cell was lysed and its labeled contents were

separated by capillary electrophoresis and detected by laser-induced fluorescence (15). Ewing's group reported an on-column lysis and labeling scheme for chemical cytometry. In this experiment, the cell was injected into the capillary, lysed, and its contents labeled with a fluorogenic reagent. By performing the labeling chemistry within the capillary, excessive dilution was avoided and the entire reaction product was available for subsequent electrophoretic separation (16).

Rather than relying on chemical derivatization, Yeung used native fluorescence to monitor the separation of hemoglobin variants from a single erythrocyte, catecholamines from single adrenal chromaffin cells, serotonin in neurons, and proteins from neurons (17–21). Sweedler has employed native fluorescence to study neurotransmitters in single neurons (22–23). Native fluorescence eliminates the need for derivatization, but usually requires excitation in the UV portion of the spectrum using relatively expensive and temperamental lasers. Krylov and colleagues have also used native fluorescence to monitor expression of green fluorescent protein (GFP) from single eukaryotes (24), and our group has studied GFP expression in single prokaryote cells (25). GFP is excited in the blue portion of the spectrum and can be detected with very high sensitivity. GFP-fusion proteins can be prepared by genetic engineering, which represents a highly precise means of fluorescently labeling a specific protein.

Most work has used surfactant or other reagents to lyse cells. Allbritton and colleagues used a pulsed laser to lyse a cell before analysis of kinase activities by capillary electrophoresis. The pulsed laser allows very fast lysis of the cell, eliminating potential artifacts associated with cellular response to the stress associated with a slower lysis process (26–30). Alternatively, an electric field can be used to induce rapid cell lysis, which has the potential for high-throughput analysis (28). Use of hydrodynamic, rather than electrokinetic, injection of reagents leads to improved mixing and efficient lysis (31). For an overview of methods used in single-cell analysis, see the article by the Allbritton group in this volume (32).

Zare and colleagues reported a microfabricated device to automate cell lysis, labeling, and separation. This system has the potential to automate cell isolation with the subsequent processing necessary to characterize cell composition (33).

3. CHEMICAL CYTOMETRY OF PROTEINS BY ONE- AND TWO-DIMENSIONAL CAPILLARY ELECTROPHORESIS

3.1. Instrumentation

Our group has performed chemical cytometry based on capillary electrophoresis with ultrasensitive laser-induced fluorescence detection (**Figure 2*a***). In our experiments, a cell is aspirated into the capillary, lysed, and labeled if necessary (34–35). Components are separated electrophoretically and detected by fluorescence.

Cells are chosen and injected into a capillary with the aid of an inverted microscope (**Figure 2*b***) (35). The operator views a drop of cell suspension through the microscope. The capillary is attached to a three-axis micromanipulator, which allows the capillary to be centered over a cell of interest. To inject the cell, a 1-s-long pulse of vacuum is applied to the sheath-flow cuvette (35); the vacuum pulls the cell into

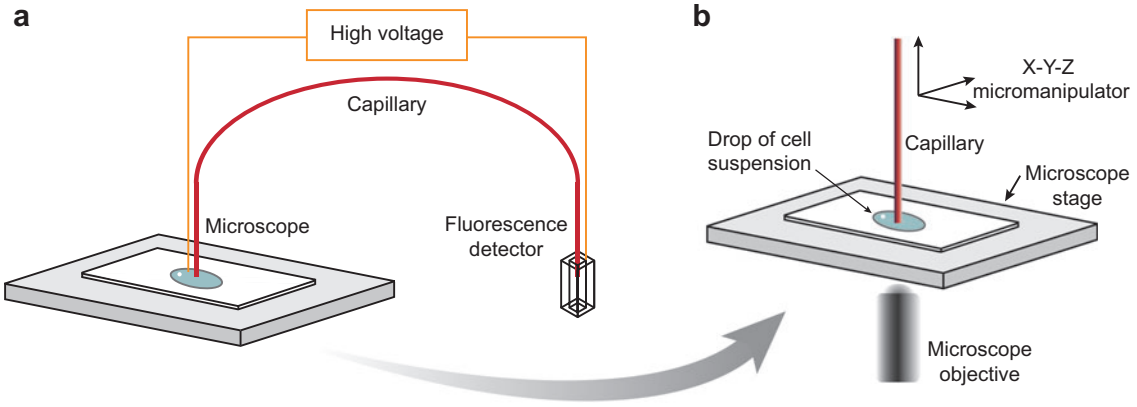


Figure 2

(*a*) Schematic of instrumentation for chemical cytometry. (*b*) Instrument for injection of a cell into the separation capillary.

the capillary for lysis, labeling if necessary, and electrophoretic analysis. The material used to coat the microscope slide influences the injection of the cell. If the cell adheres too strongly to the surface, it will not be possible to inject the cell, and if the cell does not adhere to the surface, the cell will float free in suspension, making capture of the cell difficult (36).

This group has focused on the development of extremely high sensitivity fluorescence detection systems for chemical cytometry, and we routinely obtain detection limits of a few hundred copies of a wide range of analytes. A schematic of our detector is shown in **Figure 3** (37–39). The detector design is based on a flow cytometer, where the analyte migrates from the separation capillary into a flowing stream in a sheath-flow cuvette. Fluorescence is excited with a low-power laser beam ~100 μm downstream from the capillary in the postcolumn detector (we used a 10-mW diode laser pumped solid-state laser operating at 473 nm for most experiments). Fluorescence is collected with a 60 × 0.7 NA microscope objective, spectrally filtered to reduce scattered laser light, and imaged onto a gradient-index lens, which acts as a limiting aperture and which couples fluorescence to a fiber optic. We employed a high-quantum-yield single-photon-counting avalanche photodiode to detect fluorescence (40).

3.2. Fluorescent Labeling of Proteins and Biogenic Amines

We have used chemical cytometry based on capillary electrophoresis to study proteins and biogenic amines in a wide range of cell types, including the HT29 colon cancer cell line; the MCF7 breast cancer cell line; the AtT20 adrenal gland cancer cell line; the MC3T3 osteoprecursor cell line; the hTERT and the CP18821 telomerase–expressing Barrett's esophagus cell lines; the A549 lung cancer cell line; the SupT1 T-cell line; MC3T3 osteoprecursor cells; RAW 264.7 macrophage cells; *D. radiodurans* cells; primary neurons, neural stem-cells, macrophages, and T-cells; and single-cell

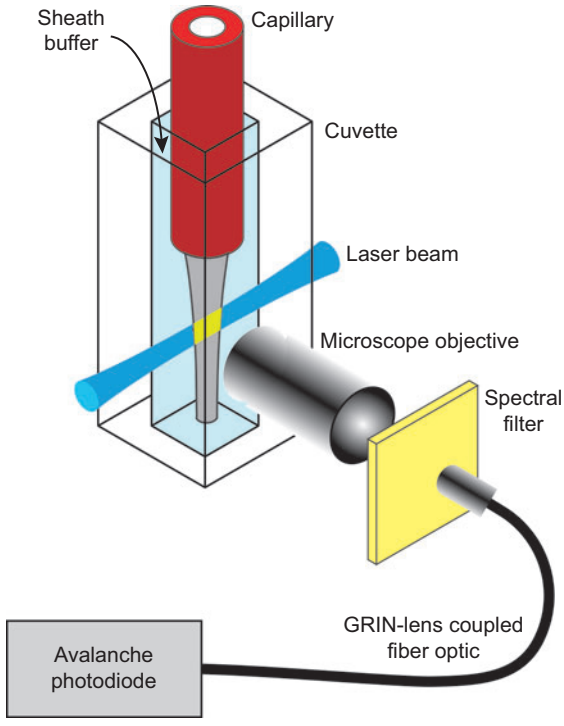


Figure 3

Sheath-flow cuvette as a postcolumn laser-induced fluorescence detector.

C. elegans and mouse embryos (41–51). We rely on covalent labeling of primary amines to fluorescently label biogenic amines and the ε-amine of lysine residues on proteins. Lysine is a common amino acid in most organisms, and the vast majority of proteins contains one or more lysine residues, at least in yeast (52).

Fluorescent labeling allows the use of low power and rugged solid-state lasers as excitation sources, which is preferable to the expensive and temperamental UV lasers required for excitation of most native fluorescence. However, labeling is not without its challenges. The presence of several labeling sites on a protein can produce an experimental artifact. Most derivatizing reagents convert the cationic lysine residue to a neutral or anionic product. For example, fluorescein isothiocyanate converts lysine residues to the fluorescein thiocarbamyl product, which corresponds to a change in the residue's charge state from +1 to –2. If every lysine residue on the protein is labeled, or if the same residues are labeled on each copy of the protein, then the derivatized products will all carry the same charge and have the same electrophoretic mobility. However, exhaustive labeling of proteins is challenging (53), and the labeling reaction inevitably produces a complex mixture of products. If there are n possible labeling sites, then there are 2^n-1 possible fluorescent products (54). This mixture forms a charge ladder for the protein, where each charge state has its own mobility, leading to complex electrophoretic profiles (55–59). Each charge state can be formed by reaction with different combinations of residues, which can lead to additional band broadening.

For example, we labeled enhanced GFP (eGFP) with 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ), which converts the cationic lysine residue to a neutral product, and we performed capillary electrophoresis on the reaction products while monitoring GFP fluorescence (57). There are 20 lysine and 6 arginine residues for a total of 26 positive charges. We observed a set of peaks appear with lower mobility than the unlabeled protein; additional peaks appeared with lower mobility as the reaction progressed, eventually forming a very broad, featureless peak. We interpreted these peaks as the reactions product for increasing numbers of labeled lysine residues. The mobility decreased linearly ($r < -0.999$) by $2.8 \pm 0.2\%$ for addition of a label, which is close to the mobility change expected simply because of charge neutralization.

Multiple labeling can severely degrade separation efficiency. Native GFP generated over 100,000 theoretical plates in our capillary electrophoresis experiment; the labeled product barely generated 400 plates after extensive labeling. Fortunately, we discovered that peak broadening due to multiple labeling can be reduced by using FQ as the labeling reagent and by adding an anionic surfactant, such as sodium dodecyl sulfate (SDS), to the separation buffer (60). The surfactant presumably ion-pairs with unreacted lysine residues, neutralizing the charge just as reaction with FQ does.

FQ has another fortuitous property. It is a fluorogenic reagent that is nonfluorescent when excited at 473 nm and generates a very low reagent blank. In contrast, conventional labeling reagents, such as fluorescein, the rhodamines, BODIPYs, and Cy dyes, generate unacceptable reagent blanks due to unreacted dye, hydrolysis products, and impurities. FQ allows detection of picomolar concentrations of proteins with a very low background signal, which is required for chemical cytometry of proteins from somatic cells.

3.3. One-Dimensional Capillary Electrophoresis for Chemical Cytometry

Figure 4*a* presents a one-dimensional analysis of the proteins from single AtT20 cells. In this separation, a cell is injected into the capillary and lysed, and primary amines are labeled with FQ. Components are separated by capillary sieving electrophoresis (CSE). Data from two cells are shown. With the exception of a peak at 7 min, the electropherograms are quite similar to each other, each generating perhaps 20 features differing only in intensity. The integrated intensity for the top cell is twice the integrated intensity of the bottom cell. The phase of these cells in the cell cycle was not synchronized, and it is likely that the former cell is in the G2/M phase of the cell cycle while the latter cell is in the G1 phase. We have observed that cells taken from most cell cultures tend to generate single-cell electropherograms that strongly resemble one another.

In contrast, primary cells tend to generate much more heterogeneous electropherograms. **Figure 4*b*** presents three electropherograms generated from single mouse neural stem cells. Although some components appear in each electropherogram, there are dramatic differences in the data, particularly in the 10–12 min region. These differences presumably reflect the different ways in which these cells would develop.

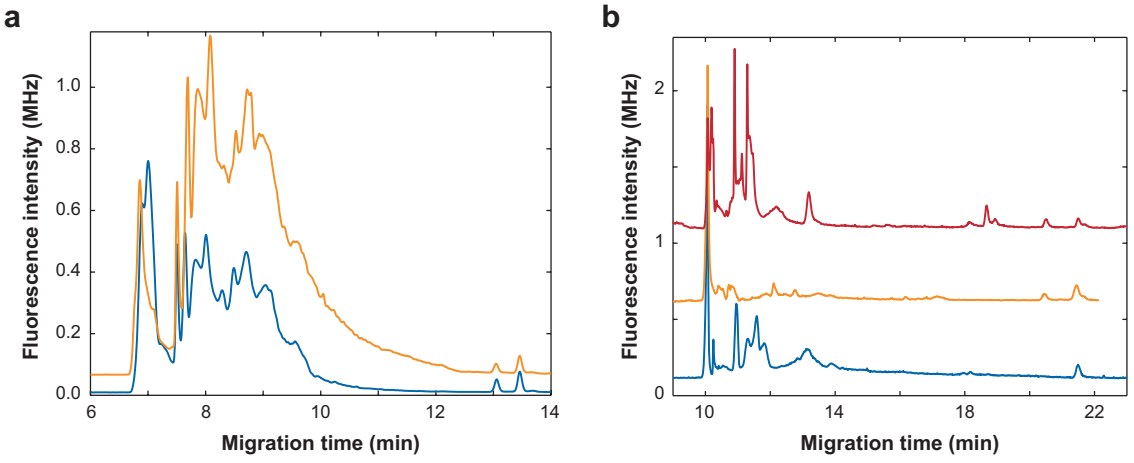


Figure 4

(*a*) Capillary sieving electrophoresis (CSE) single-cell electropherograms generated from two AtT20 cells. Electropherograms were aligned using a two-point method (61). (*b*) One-dimensional CSE analysis of three mouse neural stem cells. Traces offset for clarity. Electropherograms were aligned using a two-point method (61).

3.4. Two-Dimensional Capillary Electrophoresis for Chemical Cytometry

One-dimensional electrophoresis provides a relatively rapid measure of the composition of a single cell. However, one-dimensional separation is insufficient to resolve the complex composition of a single cell. Two-dimensional electrophoresis provides a powerful tool for increasing the number of components resolved in a separation.

Two-dimensional electrophoresis is traditionally performed first by separating proteins by isoelectric focusing in a thin tube or strip. The strip is then placed at the top of a large gel, where the components are further separated by SDS-PAGE. Silver staining, or other visualization chemistry, is used to observe the separated proteins as a set of spots distributed across the gel, where the position of each protein corresponds to its isoelectric point and molecular weight. As noted above, 25 years ago Neukirchen used a miniaturized two-dimensional electrophoresis system to analyze ~1 μg of protein from a single *Drosophila* egg (12). Unfortunately, the amount of protein in a typical human somatic cell is five orders of magnitude smaller than that contained in a *Drosophila* egg. The proteins from a single somatic cell are diluted to invisibility when separated in a classic two-dimensional gel.

We have coupled two capillary electrophoresis capillaries to generate a separation method that is compatible with the minute amount of sample contained in a single cell (47, 62–63). In this method, labeled components are subjected sequentially to two stages of separation. This method is related to a method developed by Jorgenson to couple capillary liquid chromatography with capillary electrophoresis for the comprehensive separation of complex mixtures (64). In our system (**Figure 5*a***), both dimensions of the separation are based on capillary electrophoresis, which is

much simpler than the use of chromatography for chemical cytometry. A cell is aspirated into the first capillary, the cell is lysed, components are labeled, and the labeled components are separated by CSE. Once components approach the end of the first capillary, a voltage program is used to sequentially transfer components across an interface into a second capillary. Once a fraction is transferred, the two power supplies are adjusted so that there is no voltage drop across the first capillary, where components remain stationary. High voltage is then applied across the second capillary, where components undergo separation by micellar electrokinetic capillary chromatography (MEKC), a form of capillary electrophoresis where separation is based on interaction with SDS-micelles. This series of fraction transfers and second dimension separation is typically performed 200 times to comprehensively separate the cell's components (**Figure 5*b***).

The data are continually recorded, producing a long one-dimensional data vector (**Figure 6*a***). The data consist of a set of ~200 separate MEKC separations, concatenated as a long data stream. Each MEKC separation lasted 14 s, and the entire experiment required just over 1 h to complete.

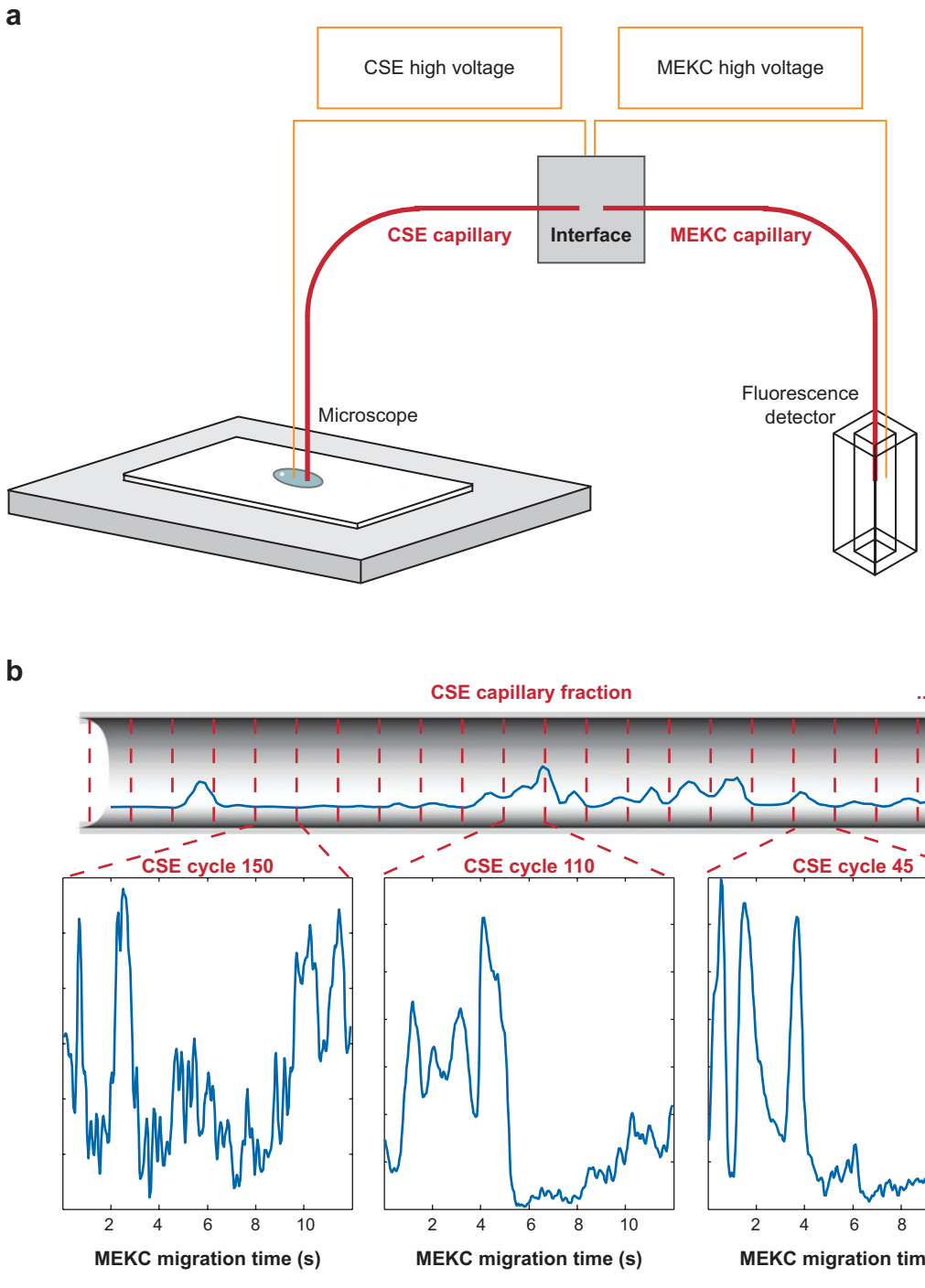
Figure 6*b* presents a close-up of the data near 24 min corresponding to the MEKC analysis of three successive fractions. The data consist of a set of peaks generated during the MEKC separation. The peaks from each MEKC separation are similar because components are sampled several times as they are transferred from the CSE capillary.

There is a dead time in the MEKC separation during the period from injection to the appearance of the first component at the detector. To speed the overall separation, we transfer fractions to the MEKC capillary before all components from the previous fraction have reached the detector. Analyte from two transfers are present in the MEKC capillary simultaneously; the fastest components from the second fraction do not quite catch up to the slowest components from the first fraction before detection.

The data can be shown as a spiral wrapped around a cylinder (**Figure 6*c***) where the axial distance corresponds to the first dimension fraction and the angle corresponds to the second dimension separation time. The image is presented as a false color related to the fluorescence intensity.

To generate an image, the cylinder is cut and flattened, creating a gel-image of the separation (**Figure 6*c***). Any component that happens to be cut will appear as a portion of a spot located on opposite sides of the image. The cut location is arbitrary and is chosen to minimize the number of components that are intersected. **Figure 6*d*** presents such an image, which resembles a silver-stained two-dimensional gel, presented here in false color. The image consists of a set of spots distributed across the surface. The positions of these spots are highly reproducible from cell to cell, with typical precision in spot position being on the order of the size of the spot itself (49–50).

The gel image is convenient for comparing spot position. However, the image, particularly when overexposed, is less useful in comparing the amplitude of spots or characterizing the dynamic range of the experiment. Instead, the data can be plotted as a surface in the form of a landscape, where height is proportional to the fluorescence intensity (**Figure 6*e***).



This two-dimensional separation is reminiscent of classic two-dimensional gel electrophoresis, where proteins are separated first by isoelectric focusing and second by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). In our case, we employ CSE in the first dimension of the separation; this form of separation is the capillary version of SDS-PAGE, which separates proteins based on their molecular weight. Unfortunately, isoelectric focusing is not compatible with our labeling chemistry (58). We employ MEKC as the second dimension in this separation. MEKC separates components based on their interaction with surfactant, and is based, in part, on the hydrophobicity of the protein.

The spot capacity of a two-dimensional separation is analogous to the peak capacity of a one-dimensional separation. If the separation mechanisms are uncorrelated and if the separation is not degraded by coupling the two separation mechanisms, then the spot capacity of the two-dimensional separation is given by the product of the peak capacities of the one-dimensional separations. In our case, we are able to achieve reasonable peak capacity in the CSE dimension. However, our MEKC separation tends to produce mediocre peak capacity, and improvements are certainly desirable. Our overall spot capacity typically approaches 500 (49–50), which is roughly one order of magnitude poorer than classic two-dimensional gels, but with six orders of magnitude higher sensitivity.

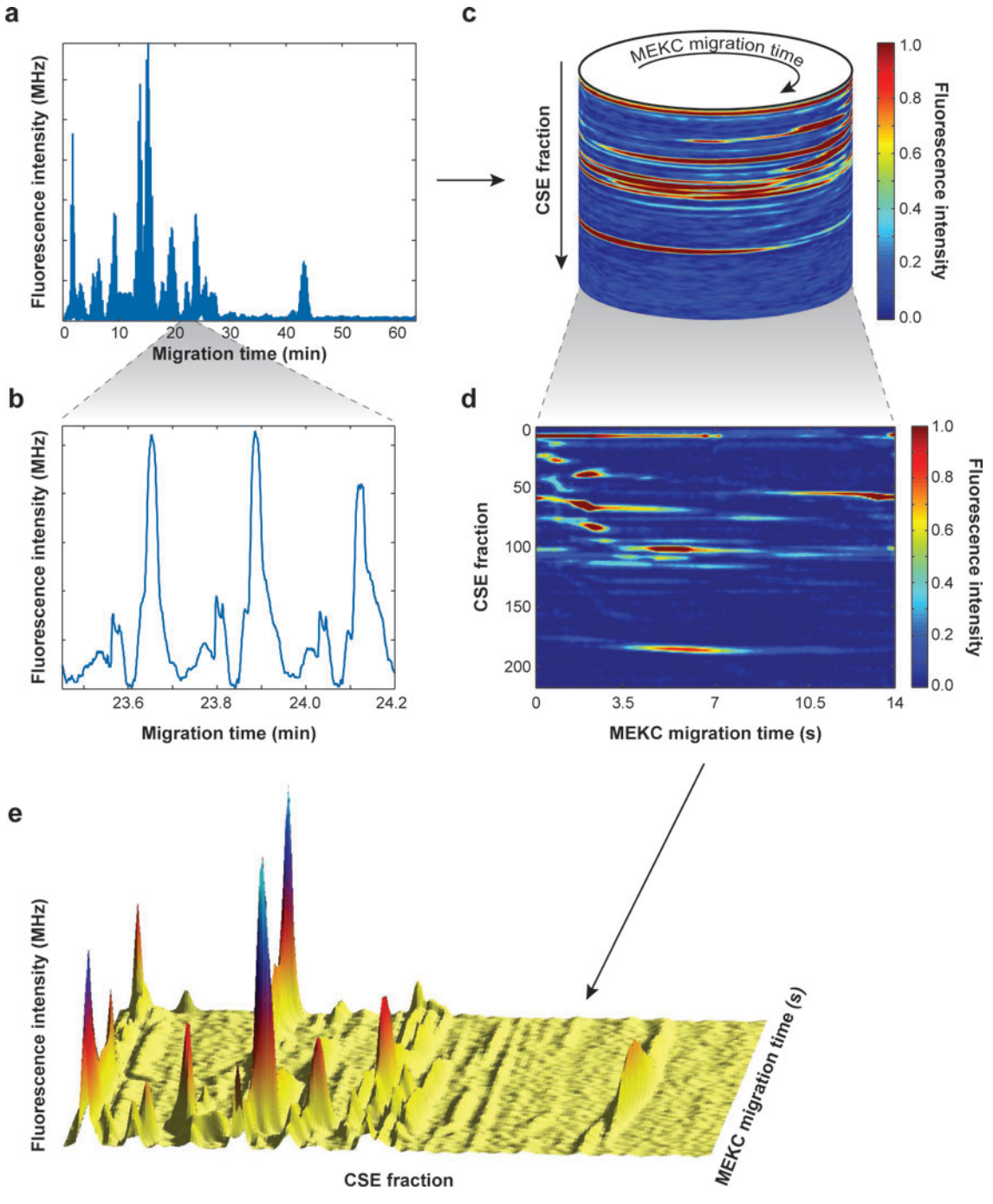
Our published work has primarily focused on cultured cells. These cells generate remarkably reproducible electropherograms, which are useful for validating the precision of the experimental protocol. The next step in the development of chemical cytometry will focus on primary cells taken from living organisms. These cells are expected to show great heterogeneity. We are particularly excited about applying this technology for analysis of cells isolated from human biopsies for early detection of cancer and for generation of prognoses with improved accuracy to guide individualized therapy.

4. CHEMICAL CYTOMETRY OF METABOLITES: METABOLIC CYTOMETRY

Characterization of a cell's protein content provides insight into the function and behavior of a cell. Greater insight can be provided by characterization of cellular metabolism, which ultimately reflects the action of those proteins. Classic cytometry can be used to monitor a single metabolic transformation; fluorogenic reagents can be used to monitor the activity of a specific hydrolytic enzyme in a single cell. Hydrolysis of a quenching group releases a fluorescent product that can be monitored by flow

Figure 5

(*a*) Two-dimensional capillary electrophoresis instrument. (*b*) Schematic of two-dimensional electrophoresis. The cell's contents undergo a preliminary separation in the first capillary by capillary sieving electrophoresis (CSE). Fractions are sequentially transferred to the second capillary, where components undergo further separation by micellar electrokinetic capillary chromatography (MEKC).



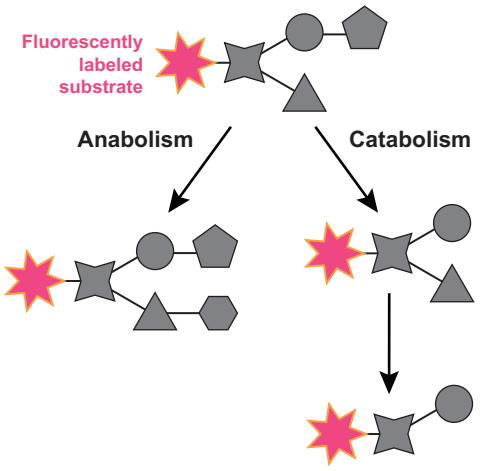


Figure 7

Metabolic cytometry. A fluorescent substrate is shown with its label denoted by the red star. This substrate can undergo anabolism to create larger structures (*left*) or catabolism to degrade the substrate to smaller metabolites (*right*). As long as the fluorescent tag remains intact, the metabolic products can be monitored by chemical cytometry.

cytometry. However, conventional enzymatic probes are unable to characterize more than one step in an enzymatic pathway and classic cytometry methods are limited in the insight that they can provide on complex metabolic cascades.

Metabolic cytometry refers to the study of a selected set of metabolic pathways in single cells (26–30, 34, 65–70). In metabolic cytometry, cells are treated with a fluorescent substrate, which is taken up and enzymatically processed to create products that, in turn, act as substrates for other enzymes (**Figure 7**). Cells are then aspirated into a capillary and lysed. Fluorescent metabolites are separated by electrophoresis and detected by laser-induced fluorescence. As long as the metabolic products retain their fluorescent tag, then the reaction products can be determined with exquisite sensitivity.

Metabolic cytometry differs from chemical cytometry of proteins and biogenic amines in one important respect. In metabolic cytometry, a fluorescent substrate is prepared at high concentration in the laboratory. This substrate can be subjected to exhaustive purification, which allows the use of highly fluorescent dyes as labels. In particular, we find tetramethylrhodamine to be a very useful dye in these applications. This molecule is highly fluorescent, and compounds labeled with it appear to be taken

Figure 6

(*a*) Time trace of a two-dimensional capillary electrophoresis analysis of a single CP18821 cell. The sample underwent a preliminary separation of 5 min in the capillary sieving electrophoresis (CSE) capillary before fractions transfers began, during which time signal was not recorded. (*b*) Close-up of the three successive micellar electrokinetic capillary chromatography (MEKC) separations shown in (*a*). (*c*) The raw electrophoresis data presented in (*a*) are wrapped as around a cylinder, where the optical density is related to the fluorescence intensity. (*d*) The raw electrophoresis data presented in (*a*) as wrapped around a cylinder that is cut and flattened, creating a gel-image where the color is mapped to fluorescence intensity. The image is overexposed to highlight low-amplitude components. (*e*) Landscape view, where height is proportional to fluorescence intensity.

up efficiently by cells. In contrast, proteins and biogenic amines must be labeled after the cell is lysed, which places constraints (discussed above) on the labeling chemistry.

4.1. Metabolic Cytometry of Sphingolipids

We have been studying glycosphingolipid metabolism in single AtT20 cells. Glycosphingolipids are among the most common molecules on neural cell surfaces and consist of several components. Ceramide is the nonpolar tail that comprises a long-chain amino alcohol linked to a long-chain fatty acid. A chain of saccharide groups attached to the ceramide forms the constituent glycosphingolipids, and the presence of one to four sialic acids turns these glycosphingolipids into gangliosides. In the common naming of gangliosides, the subscript letter indicates the quantity of sialic acid groups: M for monosialo, D for disialo, et cetera. A stands for asialo, which refers to glycosphingolipids without sialic acids. The number refers to the order of peak appearance in thin-layer chromatography and thus the length of the sugar chain; the sequential removal of zero, one, and two terminal saccharides decreases retention to yield G_{M1}, G_{M2}, and G_{M3}, respectively.

These glycolipids form a large fraction of neuronal cell membranes. Defects in their metabolism lead to a series of devastating diseases, the best known of which is Tay-Sachs. The compounds are synthesized from the lipid ceramide by successive additions of monosaccharides (**Figure 8*a***). A defect in the degradation of G_{M2} to G_{M3} leads to accumulation of the former, and is the underlying cause of Tay-Sachs disease.

We have synthesized the fluorescently labeled G_{M1} (**Figure 8*b***) (70). We modified the ceramide group by incorporating the fluorescent label tetramethylrhodamine. We have enzymatically transformed the tetramethylrhodamine-labeled G_{M1} to labeled G_{M2}, G_{A1}, and G_{A2}, and have produced labeled G_{M3}, Lac-Cer, Glc-Cer, and Cer by additional chemical and enzymatic methods. These compounds are used as standards to tentatively identify metabolic cytometry products based on comigration.

Figure 9 presents metabolic cytometry data generated from single AtT20 cells. In these experiments, a cell was incubated with the substrate. The substrate was taken up, which was confirmed by fluorescence microscopy. Enzymatic transformations converted the substrate to products. To analyze the products, the cell was aspirated into a capillary and lysed, its components were separated by capillary electrophoresis, and products were detected by laser-induced fluorescence.

AtT20 cells were incubated with the substrate shown in **Figure 8*b***, aspirated into a capillary, lysed, and analyzed by capillary electrophoresis. Components were identified by comigration with standard compounds. Three unknown components were observed that did not migrate with standards and likely are more complex structures (see **Figure 8*a***).

4.2. Metabolic Cytometry of Kinase Activity

Allbritton's group has also performed a form of metabolic cytometry to assay kinase activity in single cells (26–29). In those experiments, a cell was treated with a

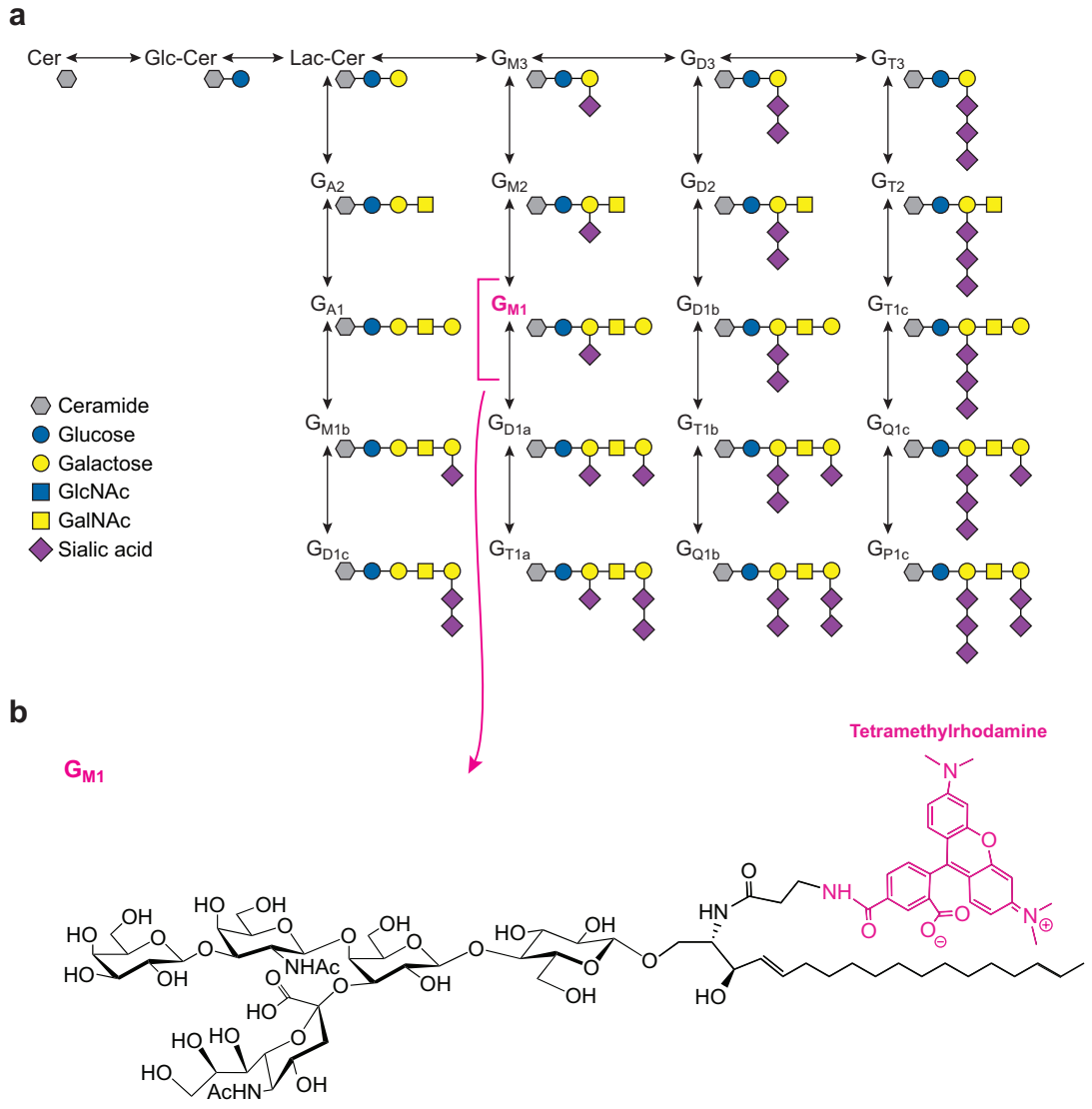


Figure 8

(*a*) Partial metabolic pathway associated with sphingolipids. Arrows denote known metabolic transformations in primates. (*b*) Structure of tetramethylrhodamine-labeled G_{M1}. The tetramethylrhodamine structure is shown.

fluorescent peptide that is a substrate for a specific kinase. Phosphorylation of the peptide led to a mobility shift for the peptide, which was easily detected by capillary electrophoresis and laser-induced fluorescence. By employing a suite of peptides, a large number of kinases can be monitored simultaneously, provided that the substrates and products generate nonoverlapping electrophoresis peaks.

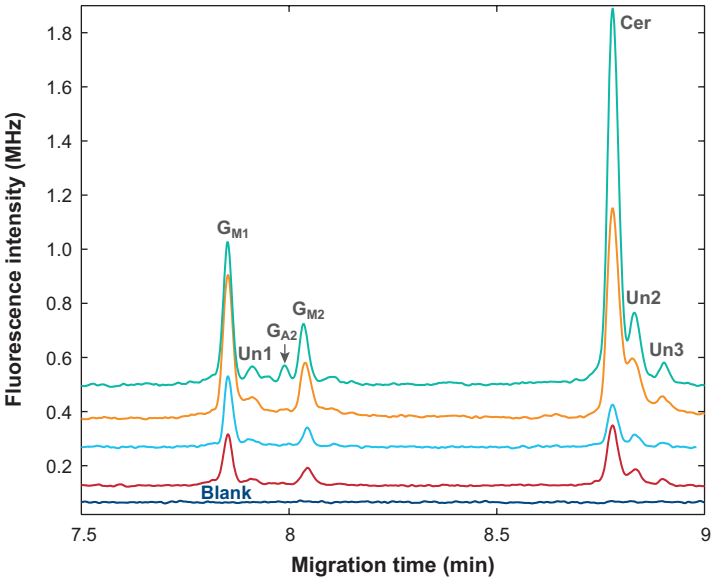


Figure 9

Metabolic cytometry analysis of four cells and the blank (*bottom trace*).

5. CHEMICAL CYTOMETRY OF DNA AND mRNA

The polymerase chain reaction (PCR) has made possible the study of DNA and mRNA expression levels in single cells. In genomic sequencing experiments, a single cell was selected and lysed in a nanoliter microfabricated well (71). The lysed cell was subjected to whole genome amplification and the products were sequenced using Pyrosequencing. For mRNA studies, the cell was placed in a cocktail containing lysis buffer and poly-dT-coated magnetic beads (72–76). The captured mRNA was converted to complemetary DNA. Hot-start PCR was performed for 40 cycles with random primers; the PCR products were studied either by Western blotting, by hybridization to an oligonucleotide array, or by direct sequencing.

This technology has been fairly widely disseminated. In perhaps its most famous application, Dulac and Axel screened single neurons from the rat vomeronasal organ and identified a family of putative pheromone receptor genes (73). Other examples include the characterization of neuronal progenitors, of cancer micrometastatic cells, and of difficult-to-culture microbes (71, 77, 78).

6. SUMMARY: CHEMICAL CYTOMETRY PROVIDES HIGH-RESOLUTION CHARACTERIZATION OF SINGLE CELLS

Although chemical cytometry has a 50-year history, the technology is nevertheless in its infancy. As might be expected, single-cell nucleic acid analysis is the most mature form of chemical cytometry; powerful nucleic acid amplification technology facilitates the study of the minute amount of material present in a single cell. Analysis of proteins, biogenic amines, and metabolites in single cells represents a much more formidable

challenge. Most work to date has dealt with cultured cells, which is necessary for method validation and for system characterization. Large-scale analyses of primary cells will be required to demonstrate the value of the technology.

Several issues remain to be addressed. First, it is becoming obvious that living cells cannot be stored for long periods before analysis; cellular response to storage likely leads to profound changes in composition. Instead, cells must be fixed with a preservative after removal from culture or from an experimental animal to eliminate artifacts associated with storage. Formalin crosslinks proteins and nucleic acids but not glycolipids, which makes it an ideal fixative for characterization of most metabolites. However, formalin is not compatible with protein analysis; we employ ethanol to fix cells for protein analysis. Although ethanol precipitates proteins, our cell lysis solution contains SDS, which efficiently solubilizes proteins.

Second, throughput of chemical cytometry is much lower than in classical cytometry. Flow cytometry can process many thousands of cells per second. We are developing multiple capillary instruments for high-throughput chemical cytometry (79). These instruments are based on the multiple-capillary electrophoresis systems that have become ubiquitous in DNA sequencing (80–83), and should allow the study of hundreds to thousands of cells per day.

Third, separation efficiency must be improved for single-cell protein analysis. Current two-dimensional electrophoresis systems are generating a spot capacity of a few hundred components; an improvement of an order of magnitude is desirable. MEKC is not an ideal separation mode for proteins, and should be replaced with a technique such as isoelectric focusing (IEF), which can provide exquisite resolution. A major hurdle deals with protein labeling. FQ, and presumably other dyes that produce an anionic or neutral reaction product, generates very complex IEF electropherograms due to multiple labeling (58). Anionic surfactants, which eliminate the peak broadening that results from multiple labeling in CSE and MEKC, are not compatible with IEF. It will be necessary to employ a labeling reagent that produces a cationic product. The recently developed chameleon dyes are interesting examples of fluorogenic reagents that produce cationic products (84–85).

Fourth, component identification must be improved. Current methods rely on comigration with standards (34, 45). Although these methods are practical for metabolic cytometry and biogenic amines due to the relatively small number of target compounds, spiking is extremely tedious for protein analysis. Ideally, an on-line mass spectrometer can be used to monitor compounds as they migrate from the capillary (86). Although the current generation of mass spectrometers does not have sufficient sensitivity to monitor the minute amount of protein present in a single cells, mass spectrometry could be used to study concentrated homogenates prepared from large numbers of cells. Based on the excellent run-to-run reproducibility of the two-dimensional electrophoresis system, component identification should be practical in fluorescence-based detection of single cells. As a complication, fluorescently labeled proteins are required for single-cell analysis. To use mass spectrometry to identify components with the same mobility, software will need to be modified to reflect the mass change induced by the fluorescent tag. Peptides are much easier to analyze than proteins by mass spectrometry, and Sweedler's group has done impressive work on

the identification of novel neuropeptides in single neurons by matrix-assisted laser desorption/ionization mass spectrometry (87–92).

Finally, improved sensitivity is required to perform chemical cytometry on a single bacterium or subcellular organelle, which contain a million-fold smaller amount of material than does a typical eukaryote cell (25, 93–101).

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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