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Design and Application of Sensors for Chemical Cytometry

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Abstract

The bulk cell population response to a stimulus, be it a growth factor or a cytotoxic agent, neglects the cell-to-cell variability that can serve as a friend or as a foe in human biology. Biochemical variations amongst closely related cells furnish the basis for the adaptability of the immune system, but also acts as the root cause of resistance to chemotherapy by tumors. Consequently, the ability to probe for the presence of key biochemical variables at the single-cell level is now recognized to be of significant biological and biomedical impact. Chemical cytometry has emerged as an ultrasensitive single-cell platform with the flexibility to measure an array of cellular components, ranging from metabolite concentrations to enzyme activities. We briefly review the various chemical cytometry strategies including recent advances in reporter design, probe and metabolite separation, and detection instrumentation. We also describe strategies to improve intracellular delivery, biochemical specificity, metabolic stability, and detection sensitivity of probes. Recent applications of these strategies to small molecules, lipids, proteins, and other analytes are discussed. Finally, we assess the current scope and limitations of chemical cytometry and discuss areas for future development to meet the needs of single-cell research.

OVERVIEW OF CHEMICAL CYTOMETRY

Heterogeneity is a fundamental aspect of normal and diseased cell biology. Genetically identical cells respond differentially to an identical stimulus due to local environment conditions, varying cell states, and noise from biomolecular processes.¹ Phenotype variation is not well understood, but has far reaching implications ranging from transient drug resistance in cancer to the distribution of green and red cones in a developing retina.^{2–4}

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Notes

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Experiments measuring a bulk population response report the cell-averaged outcome, thereby missing variations from subpopulations, asynchronous behavior, or rare cell responses.⁵ Cells within a tumor are an example of cellular heterogeneity arising from both genetic and microenvironmental factors, all of which influences a cell's response to therapeutics.⁵ Single-cell assays enable identification of drug-resistant cell subpopulations and even permit lineage tracing to a parental cell providing information on both clonal dynamics and drug-resistance evolution.^{5, 6} Immune cells likewise possess significant cell-to-cell heterogeneity, which furnishes the adaptability to protect against past and future unknown microorganisms.⁷ Single cell analysis, with its precision to resolve heterogeneity in cell populations, provides the means to decipher the processes behind developmental, stem, and cancer biology.

Chemical cytometry is defined as the use of analytical tools to measure the composition of single cells.^{8, 9} This review focuses on the methods of chemical cytometry that employ chemical probes, in conjunction with a separation step, to detect analytes from single cells (Figure 1). Capillary electrophoresis (CE) is the most common separation method since the columns are well suited to handle the small volume of most cells. CE also has excellent resolving power, capable of distinguishing nearly identical species, as well as high peak capacity, enabling the simultaneous separation of many probes. CE has also been used to separate a wide variety of entities including ions, amino acids, peptides, sugars, lipids, proteins, and polynucleotides. Yoctomole detection limits (hundreds of molecules) are routinely achieved when fluorescence is used as a detection method.¹⁰ The latter requires that the target molecule is inherently fluorescent, can be derivatized with a fluorophore, or can be paired with a fluorescent reporter. Whereas image and flow cytometry require probes to be spectrally resolved, multiple probes with the same emission spectra are spatially resolved during the separation step of chemical cytometry.^{11, 12} Chemical cytometry as a physical process has poor intracellular spatial resolution since the entire cell must be lysed for separation and detection of targeted analytes. However, it is possible to design probes that are spatially targeted to specific intracellular sites (*vide infra*). By contrast, the temporal resolution of chemical cytometry is excellent, ranging from microseconds to seconds depending upon the cell-sampling method employed.^{13, 14}

CHEMICAL CYTOMETRY, SEPARATION AND DETECTION OF REPORTERS AND ANALYTES

The small amount of material in a typical mammalian cell requires an extraordinarily low limit of detection (LOD) for single-cell analysis.¹⁵ Reporter design and detection strategies must enable measurements sensitive enough to discriminate between individual cells that have small differences in analyte quantities, but robust enough to quantify large differences in concentrations within a highly heterogeneous cell population.^{15–17} Examples include the swings in intracellular calcium concentration observed amongst single neurons¹⁶ and the differential Akt activity between single tumor cells.¹⁷ To achieve these goals, the components of separation-based, single-cell analysis include, a cell station or holder, a separation region, and a detection system (Figure 1). The cell holder serves as the final storage space for the cell or its contents prior to analysis. Cell holders range from the very

simple *i.e.* Eppendorf tubes, to the more sophisticated microfabricated cell traps or microfluidic flow channels.^{18–22} Single living cells, fixed cells and cell lysates have all been used as inputs for chemical cytometry. Living cells can be lysed chemically, electrically, sonically or by laser cavitation just prior to or during introduction into the separation channel.^{23–27} The time between the initiation of lysis and the cessation of the cell's chemical reactions with the reporter is defined as the temporal resolution of the analyte's measurement.^{13, 28}

Only a handful of separation and detection methods meet the stringent analytical requirements of separation based single cell analysis (Figure 2).^{18–22} Nonetheless, the separation step offers the advantage, relative to other single cell methods, that the probes need not be entirely specific nor undergo a change in optical properties upon binding or reaction. Multiple products and closely related molecules such as isomers can readily be distinguished in the separation step.^{18–22} CE, microelectrophoresis chips and nano-HPLC/ultraperformance liquid chromatography (UPLC) techniques have been used to separate species ranging in size from small molecules to macromolecules. Common detection methods for chemical cytometry include fluorescence, electrochemical detection (ED), and mass spectrometry (MS). For reporter-based chemical cytometry, fluorescence is often the preferred detection method due to its sensitivity, with LODs of as little as 1 to a few hundred molecules. However, this method of detection requires the design of a fluorescent reporter.^{29–31} Electrochemical detection also achieves limits approaching single molecule detection.³² However, this technique can only be used for electroactive species, and is prone to interference from electrode fouling by sample components. MS offers label free analysis since any ionizable molecule within a cell is measured. Importantly, the comparatively modest sensitivity of MS remains a significant limitation for single-cell applications, and currently limits analyses to high abundance proteins and secondary metabolites.^{33–36}

MICROELECTROPHORESIS

Electrophoretic separation of pL to μ L-sized cells has been accomplished in capillaries and in channels of microdevices. These microseparation methods have been used to quantify organelle pH, proteins, peptides, lipids, gasotransmitters, small molecules and metal ions in single cells.^{24–27, 29, 37–48} The method employs electrokinetic separation within a small-diameter, fused-silica capillary taking advantage of the differential mobility of analyte molecules under an applied electric field.^{5, 21} An asset of this method is that any modification to an analyte or reporter almost always results in a new mobility under appropriate separation conditions. Fluorescence detection is typically performed on-column by interrogating the capillary with a focused light source, generally a laser, and detecting emitted light with a photomultiplier tube (PMT) or photodiode.^{18, 21, 26, 45} ED coupled to CE offers a multitude of advantages for single cell analysis due to its high sensitivity, low LOD, and low cost.^{23, 25, 49, 50} MS coupled to CE has led to impressive advances in elucidation of the proteome and metabolome of single cells.^{33–36, 51} CE-MS has been successfully used to characterize metabolite, and protein biomarkers within individual neurons, and single *Xenopus laevis* embryos respectively. In these studies protein quantitation was restricted to higher abundance species, precluding investigations of low abundance proteins which are critical in the etiology of many diseases.^{36, 52} In an impressive

advance, Smits and colleagues have used this approach to characterize more than 5800 proteins from a single *Xenopus laevis* embryo, the largest single cell proteomics investigation to date.⁵² Almost 10% of the characterized proteins underwent a change in abundance during early embryogenesis.

Microelectrophoresis chips employing solution or gel-based separations have emerged as a viable alternative for single cell analysis.^{11, 27, 51, 53–61} The microchips offer significant advantages over traditional CE due to their rapid separations (ms to s), and are more amenable to parallelization due to their small footprint, and very short separation distances.²² These devices can also be interfaced with detection strategies similar to that used for CE. Herr's group has pioneered the development of microelectrophoresis chips for gel-based separations of proteins from single cells for immunoblotting and western blotting-based assays. This type of analysis supports multiplexing of up to 12 proteins.⁶² Numerous opportunities remain for the development of microelectrophoresis devices for single cell assays since other components such as valves, pumps, electrodes, fiber optics, and other functionalities can be fully integrated into the devices.²²

LIQUID CHROMATOGRAPHY

High performance liquid chromatography (HPLC) utilizes an analyte's differential partitioning in a liquid mobile phase and a stationary phase to accomplish separation.⁶³ In ground breaking work, Kennedy and Jorgenson quantified neurotransmitters within *Helix aspersa* neurons using open tubular dimethyloctadecylsilane reverse phase-HPLC with electrochemical detection in the 1980's.⁶³ Since then, the majority of more recent LC-based, single-cell assays have used MS for detection. While a powerful method, LC-MS assays of single cells are largely restricted to very large-sized cells such as *Xenopus laevis* embryo cells and/or high-concentration analytes including, abundant proteins (basal transcription factors), toxic secondary metabolites (diarrhetic shellfish toxins produced by the *Dinophysis* genus), and plant pigments (anthocyanins).^{52, 64, 65} Hen *et al.* recently loaded 100 living cells into a sample loop for simultaneous lysis, protein digestion and assay by LC-MS lowering detection limits to identify 600 distinct proteins.⁶⁶ Thus while LC-MS still does not reach the needed sensitivity for cytometry of most analytes in single mammalian cells, progress is exciting and will undoubtedly enable new biologic insights.

REPORTER DESIGN ELEMENTS

Effective reporters of intracellular biochemical processes must display a multitude of traits, including the ability to (1) penetrate the plasma membrane, (2) distinguish between closely related biochemical events, (3) retain structural integrity in the metabolically active intracellular environment, (4) control when (and/or where) the measurements are acquired, and (5) provide an exquisitely sensitive readout. Furthermore, the design strategies must be functionally adaptable to accommodate the diverse array of analytes that have attracted biological and biomedical interest, including metal ions, gases, metabolites, hormones, and enzymes.

Cell Permeability

There are a variety of physical and chemical strategies to introduce membrane impermeable agents into the cytoplasm of target cells. However, physical methods, such as microinjection or electroporation damage the plasma membrane and activate cell repair pathways. By contrast, cytoplasmic access via passive diffusion is the ideal form of cellular entry. Probes that are small molecules or peptides have a better chance at crossing the lipid bilayer if they are lipophilic, relatively small in size, and do not have significantly polar groups.⁶⁷ However, the requisite traits of an effective intracellular reporter may preclude ready membrane permeability. Under these circumstances, various covalent accessories can be appended to the reporter such as cell penetrating peptides (CPPs)^{68, 69} or lipids^{70, 71}. A disulfide bridge is commonly inserted between the reporter and the lipophilic auxiliary group. Upon entry into the cell, the high intracellular glutathione content subsequently reduces the disulfide, thereby separating the reporter by its membrane-permeant accessory. Alternatively, hydrophobic light-cleavable “caging” groups have been used to assist in transporting membrane-impermeant reporters into the cell.⁷² Unfortunately, none of these methods is universally applicable. The efficacy of any of these agents can be difficult to predict as it depends on the unique structural characteristics of cell impermeable agent.

Selectivity and Stability

Reporter selectivity for the analyte can be an extremely challenging problem, whether it's distinguishing between structurally similar small molecules or catalytically similar enzymes. For example, the substrate specificity of some protein kinases is virtually identical. On the other hand, in many disorders, the activity of certain protein kinases is dramatically upregulated, enabling selective detection of the target kinase even though the reporter displays less than absolute selectivity. In short, the quest for reporter selectivity for a given target, while daunting, depends upon the circumstances and is not insurmountable. An equally important issue, however, is validation of probe selectivity within the complex intracellular milieu. Methods that attenuate or accentuate probe readout are the most direct approach, and include inhibitors or activators for target enzymes, chelators for or delivery of target ions, and knock-downs or knock-ins of proteins in biochemical pathways that generate the target analyte.

The intracellular milieu can be an exceptionally unfriendly place for xenobiotics. A case in point is peptides, which can contain amide bonds (especially those involving Arg and Lys residues)^{31, 73} prone to intracellular proteases. Although the intracellular lifespan of peptides can be enhanced via the application of protease inhibitors, disrupting key protease targets, like the proteasome, can have unintended consequences that could compromise biochemical measurements. A more useful alternative to the application of protease inhibitors is the preparation of peptide-based probes that are resistant to protease action. A number of strategies have been applied, including the introduction of unnatural residues, such as D-amino acids or N-methyl derivatives, at proteolytically sensitive sites.^{31, 73} Alternatively, cytosolic proteases typically have a narrow tunnel or deep cleft that leads to the active site and the N-terminus of peptides enter this tunnel to be degraded. Consequently, protease resistance can be conferred by modifying the peptide's N-terminus so that it is unable to access the catalytic cleft.⁷⁴ Finally, peptide “stapling” and cyclization have been used to

promote peptide stability in cytosolic environments. The “staple” is a covalent bond between two residues that enforces the α -helicity of a peptide, which can enhance both protease resistance and cell permeability. However, the rigid secondary structure of stapled peptides can be inconsistent with many protein-processing enzymes, including protein kinases and protein phosphatases. Indeed, the introduction of structural modifications to resist proteolysis must be performed with care so as not to interfere with the intended biological measurement. An especially insidious example of the hostile intracellular environment faced by biochemical probes is the presence of protein tyrosine phosphatases (PTPases). PTPases catalyze the hydrolysis of phosphate monoesters, which can interfere with probes designed to assess protein kinase activity. This is especially troublesome with protein tyrosine kinase (PTKs). In this regard, peptide-based PTK reporters containing a constrained tyrosine (**1**) analog, 7-(*S*)-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (L-Htc; **2**), serve as effective PTK substrates (cf. **1** and **2**). In addition, the nature of the constraint dramatically reduces the susceptibility of the phosphorylated product to phosphatase-catalyzed hydrolysis while enhancing the proteolytic resistance of the peptide.⁷⁵

Temporal and Spatial Control

Loading active reporters into cells can result in varying times that the reporters are exposed to the analyte or enzyme under study. This is not necessarily a problem if the final readout is simply the measurement of an equilibrium condition (e.g. assessing the presence of a metal ion) *or* if following loading, a biochemical event is initiated by the application of triggering event (e.g. antibody binding to a receptor). However, it may be useful to probe the biochemistry of resting cells, such as in the case of diseased cells obtained from patients. Furthermore, the acquisition of well-defined kinetics requires well-defined start points. These needs can be addressed by employing light-activated reporters, which can be loaded into cells and subsequently activated with high temporal resolution when needed.⁷² Finally, we note that general measurements throughout the cytoplasm only provide an averaged assessment of biochemical activity. The biochemistry of the cell is highly compartmentalized, resulting in biochemical events at different intracellular sites. However, it is possible to localize probes at specific sites within a cell using compartment-targeted small molecules or specific amino acid sequences.

CLASSES OF BIOCHEMICAL MEASUREMENTS

A variety of analytes have been measured using chemical cytometry, including metal ions, gases, small molecule metabolites, nucleic acids, hormones, and enzymatic activity (Figure

3). These species, which vary in molecular weight from less than 50 g/mol to over 50,000 g/mol, can be detected at levels as little as 10^{-21} moles (Figure 4).

Detection of Small Molecules

Two different strategies have been utilized to assay small molecules within or secreted by single cells, reactive fluorescent probes and reversibly binding fluorescent reporters. In the first strategy, reactive probes incorporating a fluorophore convert a nonfluorescent or fluorescent analyte into a fluorescent product(s). This product is then readily identified during a separation step based on its characteristic migration time and quantified from its fluorescence intensity. An advantage of chemical cytometry is that the reactive probe does not need to be entirely specific since the various product forms are readily separated and identified.^{11, 12} This strategy has been used with great success to detect various nitrogen species as well as reactive oxygen species (ROS). Nitric oxide (NO), a second messenger participating in a range of cellular processes *e.g.* neural transmission and immune response is commonly detected using a diamino-quenched fluorophore that becomes fluorescent upon reaction with NO.^{11, 38, 45, 76, 77} Additional reducing species also react with these probes to yield fluorescent products that are separated and identified by CE. Rhodamine, BODIPY, and fluorescein are commonly used fluorophores and a comparison of the different probes and their applications have been covered by Ye *et al.*⁷⁸ The probe can also be chemically engineered to tailor it for the specific study requirements. For example, Zhang *et al.* created the water soluble diamino BODIPY probe **3** by adding sulfonate groups so that both extracellular and intracellular NO (**4**) of a trapped cell can be measured before and after lysis.⁷⁹

ROS are involved in inflammation, aging and cancer, and are produced by mitochondria and intracellular enzymes (*e.g.* NADPH oxidase).⁸⁰ ROS are also used as a signaling molecule within cells, for example in EGFR tyrosine phosphorylation.⁸⁰ A strength of chemical cytometry is high efficiency separation, which enables multiple probe products to be quantified from the same single cell to track multiple steps within the ROS pathway.^{39, 76, 81} Superoxide anion ($O_2^{\cdot-}$) and NO were simultaneously detected by reaction with the 1,3-dibenzothiazolinedicyclohexene derivative (**5** $\rightarrow$ **7**) and 3-amino,4-aminomethyl-2,7-difluorescein diacetate (**6** $\rightarrow$ **8**), respectively, which helped to elucidate the role of $O_2^{\cdot-}$ in altering NO levels in neural cells exposed to stimulants.⁷⁶ The separation step also enables analytes and internal standards to be simultaneously used for greater accuracy in quantitation. Superoxide production in skeletal muscle tissues can be measured using triphenylphosphonium hydroethidine (**9** $\rightarrow$ **10**) when paired with unreactive rhodamine 123 as an internal standard. Thus accumulation of the reporter within mitochondria membranes can be tracked at varying membrane potentials.^{39, 40}

A key feature of chemical cytometry is that promiscuous probes, which are generally more easily designed than highly specific probes, are assets enabling simultaneous measurement of multiple reactions since the separation step compensates for the lack of probe specificity. Antioxidants such as glutathione and cysteine, play a role in mitigating ROS impacts and are measured using a single cyanine dye (**11**) to react with both glutathione and cysteine to form fluorescent species at 805 and 755 nm, respectively.²⁷ When combined with the sulfonated fluorescein probe **14**, hydrogen peroxide can be simultaneously measured at 525 nm.²⁷

The flexibility provided by separations in chemical cytometry has been used to measure a diverse range of molecular species, including ions (Na^+ , K^+ , Ca^{2+} , Mg^{2+}),⁴⁸ small molecules (amino acids, taurine),⁸² and small proteins⁸³. An additional advantage of chemical cytometry over other single-cell assays is the ability to implement competitive assays within the separation device. Insulin secreted from islet cells was quantified in a microfluidic

device by co-injecting the cell secretate with fluorescein-tagged insulin, and anti-insulin antibodies followed by measurement of the ratio of unbound fluorescein-insulin to antibody-bound fluorescein-insulin to quantify secreted insulin.⁸³ This competitive detection achieved a temporal resolution of 10 s to follow glucose-responsive insulin secretion over time and should be applicable to a multitude of other hormones.¹⁴

Lipids/Lipid Kinases

Lipids encompass a diverse array of compounds that perform numerous functions within mammalian cells including, serving as structural components of cell membranes, energy storage sources, and intra/inter-cellular signaling molecules. The diverse nature of lipid function is a direct consequence of their high degree of structural heterogeneity, which arises from the numerous biosynthetic transformations available to acetyl, propionyl, and isoprene lipid precursors. Eight classes of lipids are produced in eukaryotic cells, fatty acyl lipids, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, polyketides, sterol lipids, and prenol lipids.⁸⁴ Although lipids are ubiquitous chemical components of mammalian cells, numerous challenges exist for the analysis of signaling lipids at the single cell level, (1) extremely low intracellular concentrations of signaling lipids ($<10^{-18}$ mol), (2) large dynamic ranges in their concentrations, (3) the presence of multiple structural isomers, (4) low solubility in highly aqueous environments, and a (5) lack of amplification and affinity capture reagents.^{84,85} These aspects represent unique opportunities for chemical cytometry, and present challenges that capillary electrophoresis with fluorescence detection (CE-F) is uniquely suited to tackle since CE-F offers exquisite separation capabilities, ultra-high sensitivity and dynamic range, and compatibility with both aqueous and organic buffer systems.^{10, 29, 30, 43, 47, 85–90}

Lipid-based probes with a fluorescein or BODIPY moiety conjugated to an acyl chain often show similar physical and substrate properties to that of the native lipid enabling their successful use in chemical cytometry.^{10, 91} For example, the sphingosine-fluorescein derivative **16** exhibits a similar K_m and k_{cat} to that of native sphingosine for sphingosine kinase and has been used to track sphingosine metabolism in single primary and cultured tumor cells.^{30, 43, 89} Since sphingolipid metabolism plays an important role in regulating cellular apoptotic and survival pathways, sphingosine-pathway reporters offer an excellent opportunity for the investigation of single-cell signaling pathways that drive cancer and the immune response.^{30, 43, 84, 89} In addition to sphingolipids, reporters have been developed for both glycosphingolipids (GSL) and phospholipids. GSLs are ubiquitous on the surface of neuronal membranes and include a panoply of molecules which mediate biochemical (intracellular signaling) and disease (pathogen binding and oncogenesis) events.^{29, 86, 88, 90} Impressive mass limits of detection (MLODs) of 10^{-21} moles have been achieved for BODIPY-conjugated lactosylceramide (**17**) with a dynamic range for detection spanning 6 orders of magnitude within single primary rat cerebellar neurons.⁸⁷ In a demonstration of the multiplexing capabilities of CE, the metabolism of three different GSL reporters was tracked within single cells by using distinct BODIPY fluorophores and multi-channel fluorescence detection.⁹²

The metabolism of phospholipids, utilized as substrates and second messengers for a vast array of hormone and growth-factor-mediated signaling, has also been assayed with fluorophore conjugated lipids. BODIPY-FL conjugated phosphoinositol 4, 5-bisphosphate (**18**) was used to quantify the activity of phosphatidylinositol 3-phosphate (PI3K), phospholipase C (PLC) and multiple other enzymes, within the PIP₂ metabolic pathway. PI3K is mutated in numerous cancers, and exhibits upstream regulation of Akt, an effector of cell growth and survivability while PLC is a critical component in many G-protein-linked pathways transmitting extracellular signals into the cell interior.⁸⁵ Activation of phosphatase tensin homolog (PTEN), a tumor suppressor, was monitored by loading a BODIPY-phosphoinositol 3,4,5-trisphosphate (**19**) into single cells. MLOD's for **18** and **19** and other tagged lipids are as low as 10⁻²⁰ moles, and intracellular production of these lipids is readily measured over a greater than four-fold concentration range.⁸⁵ In addition to impressive analytical performance characteristics, single cell analysis of lipids offers many unique advantages relative to other methods, for example, a tolerance towards fluorophore coupling and acyl chain modifications, improved reporter membrane permeability due to inherent hydrophobicity, and facile extraction from single cells using organic solvents.^{30, 43, 85, 91} Novel strategies using chemical fixation reagents, such as formaldehyde and glutaraldehyde, have the power to permit user-controlled cellular reaction times followed by lipid reporter extraction on demand from individual cells for analysis. This approach has been successfully used to quantify phosphoinositol 4, 5-bisphosphate metabolism as well GSL metabolism within individual cells.^{47, 87, 93} While the past decade has been witness to great strides towards the facile and rapid analysis of lipid metabolism at the single cell level, significant opportunities remain for investigations into other important aspects of lipid metabolism.

Kinases and Phosphatases

The advent of effective pharmacologic protein kinase inhibitors for clinical applications has created a critical need for assessing kinase activity, and therefore inhibitor efficacy, in disease models and in patient samples. Biochemical analyses of aberrant signaling pathways are informative in terms of identifying the best treatment option and assessing therapeutic effectiveness in individual patients. One of the most compelling issues in preclinical and clinical drug discovery is the ability to accurately monitor drug action and patient

responsiveness. For example, as noted in a recent review, “As the age of precision medicine evolves, the heterogeneity of breast cancers continues to challenge the research community, emphasizing the need for robust patient selection strategies to guide the future clinical development of RTK (receptor tyrosine kinase) inhibitors.”⁹⁴ Others have pointed out that “understanding tumor heterogeneity - the differences between individual cells in the same tumor - is one of the biggest challenges in cancer research today. The ability to describe tumors at the resolution of single cells will enhance our ability to determine the best treatment options and to anticipate disease outcome.”⁹⁵

An example of overactive protein kinase activity in human disease is the Akt serine/threonine protein kinase. This enzyme, a member of the Akt/PI3K/mTOR signaling pathway, has been implicated in a wide array of diseases, ranging from pancreatic ductal adenocarcinoma (PDAC) to rheumatoid arthritis (RA). For instance, overactive Akt activity has been associated with decreased PDAC patient survival. Unfortunately, Akt protein levels or gene copy numbers are not barometers of Akt activity, indicating that it is necessary to directly measure Akt activity. Proctor *et al.* microinjected a peptidase-resistant Akt fluorescent peptide [6FAM-GRP-(N-Me)Arg-AFTF-(N-Me)Ala-amide] into PDAC cell lines as well as into patient-derived xenografts tumor cells.¹⁷ The N-methylated Arg [(N-Me)Arg] and Ala [(N-Me)Ala] derivatives were introduced at previously identified proteolytic sensitive sites to block proteolysis.⁷³ The corresponding threonine residue (T) serves as the site of phosphorylation. 6-carboxyfluorescein (6FAM) is the fluorescent moiety used for visualization of the peptide. The phosphorylated product, the non-phosphorylated substrate, and protease induced substrate fragments were separated using CE-F and identified using synthetically acquired standards. After only 5 min of incubation inside the cells of the different PDAC cell lines, less than 50% of the intact substrate peptide remained; which highlights the robust activity of intracellular proteases. One of the advantages of CE is that, using known standards, the nature of the proteolyzed products are readily identified, in the case of the Akt reporter, 5-residue and 8-residue fragments of the parent peptide. In addition to protease activity, Akt kinase activity can be simultaneously observed. The different PDAC cell lines display different levels of Akt activity, with the implication that the cell line with the highest activity being better adapted for robust proliferation. Interestingly, although there is cell-to-cell Akt activity variability within each cell line, the cell-to-cell variability is dramatically larger in the patient-derived PDACs. This observation is consistent with the notion of tumor cell heterogeneity and the rapid emergence of resistance to chemotherapy. An analogous study using the Akt reporter was performed on fibroblast-like synoviocytes from healthy and RA donors.⁴⁶ The synoviocytes from RA patients display higher levels of both proteolytic and Akt activity, implying a role for both as a barometer of RA disease. In both studies, the Akt selectivity of the probe was validated using Akt inhibitors.

As we previously noted (see *Selectivity and Stability*), the tyrosine residue in reporters of PTK activity is especially susceptible to PTPases. By contrast, the structurally constrained tyrosine analog Htc (**2**) retains the ability to serve as a PTK substrate while resisting the action of PTPases.⁷⁵ On the other hand, the corresponding phosphotyrosine (pTyr) residue in

Glu-Glu-Leu-Glu-Asp-Asp-pTyr-Glu-Asp-Asp-Nle-Glu-Glu-amide (Nle = norleucine) can be used to readily assess PTPase activity and inhibitors in single cells.⁹⁶

Proteases

Proteases contribute to a large number of disorders, ranging from infectious diseases such as HIV/AIDS, to neurological maladies such as Alzheimer's disease. Indeed, it is likely that more assays have been developed for this enzyme family than for any other, colorimetric, fluorometric, bioluminescent, mass spectrometry-based, electrochemical, Raman scattering, surface plasmon resonance, and others.⁹⁷ Fluorometric methods, in particular, have been used to provide real time readouts in live single cells.^{98, 99} In addition, by coupling a fluorescence readout with separation, it is possible to characterize the proteolytic fragments that have been generated.

A fluorescent protease substrate [acetyl-GGVVIATVK(5FAM)rrr-amide, where r = D-Arg] derived from the β -amyloid precursor protein (β -APP) was studied in human erythroleukemia TF-1 cells using CE.¹⁰⁰ Three major fragments, plus the intact peptide, were detected, separated, and characterized. Bulk cell lysates were first used to generate the peptide fragments, which provided sufficient material for characterization by LC/MS. Subsequent single cell analysis revealed that the relative ratio of these fragments is consistent from cell-to-cell. By contrast, when the peptide was incubated in the lysates, the ratio varied as a function of the lysate preparation method, indicating that the manner by which the cell lysate is generated can alter the observed readout. Whereas the β -APP/TF-1 study demonstrated little cell-to-cell variation in proteolytic processing, Kovarik and colleagues observed a very different result in U937 acute myeloid leukemia (AML) cells.⁵⁵ Rapidly growing tumor cells commonly overexpress different proteases to help supply amino acid building blocks for growth needs. Protease inhibitors, such as the aminopeptidase inhibitor Tosedostat, have been developed to limit this amino acid supply. The protease substrate YSYQMALTPVV(K-5FAM)TL was prepared in order to assess the effect of Tosedostat at the single cell level. In the absence of the inhibitor, both single cells and an ensemble 10⁷ peptide loaded cells, were found to generate the same peptide fragments. However, the relative ratios of the fragments varied from cell-to-cell, whereas the ensemble consistently furnished the same ratios. Furthermore, there is a substantial difference in the cell-to-cell response to Tosedostat, consistent with the notion of significant heterogeneity at the single cell level.

Finally, Chen and colleagues employed an activity-based probe strategy to identify cathepsin proteases in the lysosome of HeLa and RAW264.7 (macrophage cell line) cells.¹⁰¹ Unlike the reporters described in the previous paragraph, ABPs don't measure catalytic activity, but rather covalently attach to the active site of the targeted protein. In this particular case, an ABP for cathepsin proteases was constructed containing an oligo-D-Arg sequence to promote cell permeability on the N-terminus and an electrophilic epoxyketone on the C-terminus (**20**). CE single cell analysis revealed two fluorescent peaks. However, the cathepsins (B, H, L1, S, F, O, and Z) contained within these peaks were identified from lysates from ABP-labeled cell populations via LC-MS.

CONCLUSION

Chemical cytometry provides the means to interrogate biochemical pathways at the single cell level, thereby enabling the identification and characterization of heterogeneity in cell populations. An array of probes has been developed to sample analytes ranging from metal ions and gases up through signaling enzymes. Although the sensitivity of chemical cytometry has reached the level of a few hundred analyte molecules, the current state of the art suffers from low throughput rates of ~50 cells/h. By contrast, mass and flow cytometry methods enjoy rates as high as 10^3 and 10^5 cells/s, respectively.^{21, 55} A number of strategies are under development to address the throughput challenge, including novel cell loading strategies, reductions in separation times, assay parallelization with arrays of separation channels or regions, and droplet-based methods for faster cell sampling.^{102,103} Improvements in these areas should move the throughput of chemical cytometry into the realm of several thousand cells/h; ultimately bolstering the quantity and quality of biochemical information that can be obtained from single cell assays.

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KEYWORDS

Analyte

A chemical species that is being identified and measured.

Capillary Electrophoresis

An electrokinetic separation method that employs capillaries that are <1 mm in diameter or micro/nanofluidic channels.

Chemical Cytometry

The use of analytical tools to measure the composition of single cells.

Dynamic Range

The range of possible analyte quantities that will result in a quantifiable signal change. Go below the dynamic range and only baseline noise will be observed, move above and the signal will reach a maximum value preventing further changes in analyte quantity from being discerned.

Electrophoretic Mobility

Describes the migration rate of an analyte under an applied electric field, which is a function of the charge, and size of an analyte in addition to the viscosity of the surrounding medium.

Limit of Detection (MLOD vs. CLOD)

The limit of detection of an analytical method refers to the lowest quantity of an analyte that can be reliably detected with 99.73% confidence from a blank measurement (i.e. requires signal to noise 3 times greater than the standard deviation of a blank measurement). MLOD describes this quantity in terms of mass units, whereas CLOD uses concentration units.

Kinases and Phosphatases

Kinases catalyze the transfer of a phosphoryl group from ATP to an acceptor functional group (typically a hydroxyl moiety) in proteins, lipids, and sugars to create phosphomonoesters. Phosphatases catalyze the hydrolysis of phosphomonoesters.

Sensor

Transforms chemical information into an analytically useful signal.

ABBREVIATIONS

Akt	protein kinase B
AML	acute myeloid leukemia
cdk4	cyclin-dependent kinase 4
CE-F	capillary electrophoresis with fluorescence detection
CE	capillary electrophoresis
CPP	cell penetrating peptide
DBZTC	1,3-dibenzothiazolinecyclohexene
DMNB	2,4,5-dimethoxy 2-nitrobenzyl
ED	electrochemical detection
EGFR	epidermal growth factor receptor
GSL	glycosphingolipids
L-Htc	7-(S)-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid
LIF	laser induced fluorescence
LOD	limit of detection
mi-RNA	micro RNA
MLODS	mass limits of detection
NADH	nicotinamide adenine dinucleotide

NO	nitric oxide
PDAC	pancreatic ductal adenocarcinoma
P13K	phosphatidylinositol 3-phosphate
PKA	Protein Kinase A
PKB	Protein Kinase B
PLC	phospholipase C
PMT	photomultiplier tube
PTEN	phosphatase tensin homolog
PTM	post-translational modification
PTP	protein tyrosine phosphatase
RA	rheumatoid arthritis
ROS	reactive oxygen species
RP	reverse phase
TNFα	tumor necrosis factor α
TPP-HE	triphenylphosphonium hydroethidine
β-APP	β -amyloid precursor protein.

References

1. Raser JM, O'Shea EK. Noise in gene expression: origins, consequences, and control. *Science*. 2005; 309:2010–2013. [PubMed: 16179466]
2. Losick R, Desplan C. Stochasticity and cell fate. *Science*. 2008; 320:65–68. [PubMed: 18388284]
3. Pegoraro G, Misteli T. High-Throughput Imaging for the Discovery of Cellular Mechanisms of Disease. *Trends Genet*. 2017; 33:604–615. [PubMed: 28732598]
4. Smallwood PM, Wang Y, Nathans J. Role of a locus control region in the mutually exclusive expression of human red and green cone pigment genes. *Proc Natl Acad Sci U S A*. 2002; 99:1008–1011. [PubMed: 11773636]
5. Heath JR, Ribas A, Mischel PS. Single-cell analysis tools for drug discovery and development. *Nat Rev Drug Discov*. 2016; 15:204–216. [PubMed: 26669673]
6. Dalerba P, Kalisky T, Sahoo D, Rajendran PS, Rothenberg ME, Leyrat AA, Sim S, Okamoto J, Johnston DM, Qian D, Zabala M, Bueno J, Neff NF, Wang J, Shelton AA, Visser B, Hisamori S, Shimono Y, van de Wetering M, Clevers H, Clarke MF, Quake SR. Single-cell dissection of transcriptional heterogeneity in human colon tumors. *Nat Biotechnol*. 2011; 29:1120–1127. [PubMed: 22081019]
7. Dash P, McClaren JL, Oguin TH 3rd, Rothwell W, Todd B, Morris MY, Becksfort J, Reynolds C, Brown SA, Doherty PC, Thomas PG. Paired analysis of TCR α and TCR β chains at the single-cell level in mice. *J Clin Invest*. 2011; 121:288–295. [PubMed: 21135507]
8. Dovichi NJ, Hu S. Chemical cytometry. *Curr Opin Chem Biol*. 2003; 7:603–608. [PubMed: 14580565]

9. Cohen D, Dickerson JA, Whitmore CD, Turner EH, Palcic MM, Hindsgaul O, Dovichi NJ. Chemical cytometry: fluorescence-based single-cell analysis. *Annu Rev Anal Chem (Palo Alto Calif)*. 2008; 1:165–190. [PubMed: 20636078]
10. Whitmore CD, Olsson U, Larsson EA, Hindsgaul O, Palcic MM, Dovichi NJ. Yoctomole analysis of ganglioside metabolism in PC12 cellular homogenates. *Electrophoresis*. 2007; 28:3100–3104. [PubMed: 17668449]
11. Mainz ER, Gunasekara DB, Caruso G, Jensen DT, Hulvey MK, da Silva JAF, Metto EC, Culbertson AH, Culbertson CT, Lunte SM. Monitoring intracellular nitric oxide production using microchip electrophoresis and laser-induced fluorescence detection. *Anal Methods-Uk*. 2012; 4:414–420.
12. Li L, Li P, Fang J, Li Q, Xiao H, Zhou H, Tang B. Simultaneous Quantitation of Na(+) and K(+) in Single Normal and Cancer Cells Using a New Near-Infrared Fluorescent Probe. *Anal Chem*. 2015; 87:6057–6063. [PubMed: 25973531]
13. Brown RB, Audet J. Current techniques for single-cell lysis. *J R Soc Interface*. 2008; 5(Suppl 2):S131–138. [PubMed: 18426769]
14. Schrell AM, Mukhitov N, Yi L, Adablah JE, Menezes J, Roper MG. Online fluorescence anisotropy immunoassay for monitoring insulin secretion from islets of Langerhans. *Anal Methods*. 2017; 9:38–45. [PubMed: 28458724]
15. Kempe H, Schwabe A, Cremazy F, Verschure PJ, Bruggeman FJ. The volumes and transcript counts of single cells reveal concentration homeostasis and capture biological noise. *Mol Biol Cell*. 2015; 26:797–804. [PubMed: 25518937]
16. Patel TP, Man K, Firestein BL, Meaney DF. Automated quantification of neuronal networks and single-cell calcium dynamics using calcium imaging. *J Neurosci Methods*. 2015; 243:26–38. [PubMed: 25629800]
17. Proctor A, Herrera-Loeza SG, Wang Q, Lawrence DS, Yeh JJ, Allbritton NL. Measurement of protein kinase B activity in single primary human pancreatic cancer cells. *Anal Chem*. 2014; 86:4573–4580. [PubMed: 24716819]
18. Amantonico A, Urban PL, Zenobi R. Analytical techniques for single-cell metabolomics: state of the art and trends. *Anal Bioanal Chem*. 2010; 398:2493–2504. [PubMed: 20544183]
19. Rubakhin SS, Romanova EV, Nemes P, Sweedler JV. Profiling metabolites and peptides in single cells. *Nat Meth*. 2011; 8:S20–29.
20. Cecala C, Rubakhin SS, Mitchell JW, Gillette MU, Sweedler JV. A hyphenated optical trap capillary electrophoresis laser induced native fluorescence system for single-cell chemical analysis. *Analyst*. 2012; 137:2965–2972. [PubMed: 22543409]
21. Trouillon R, Passarelli MK, Wang J, Kurczy ME, Ewing AG. Chemical analysis of single cells. *Anal Chem*. 2013; 85:522–542. [PubMed: 23151043]
22. Ouimet CM, D'Amico CI, Kennedy RT. Advances in capillary electrophoresis and the implications for drug discovery. *Expert Opin Drug Discov*. 2017; 12:213–224. [PubMed: 27911223]
23. Sun X, Niu Y, Bi S, Zhang S. Determination of ascorbic acid in individual rat hepatocyte cells based on capillary electrophoresis with electrochemiluminescence detection. *Electrophoresis*. 2008; 29:2918–2924. [PubMed: 18546168]
24. Dickinson AJ, Armistead PM, Allbritton NL. Automated capillary electrophoresis system for fast single-cell analysis. *Anal Chem*. 2013; 85:4797–4804. [PubMed: 23527995]
25. Wang XL, Li LJ, Li ZY, Wang J, Fu HY, Chen ZZ. Determination of ascorbic acid in individual liver cancer cells by capillary electrophoresis with a platinum nanoparticles modified electrode. *J Electroanal Chem*. 2014; 712:139–145.
26. Guo XF, Arceo J, Huge BJ, Ludwig KR, Dovichi NJ. Chemical cytometry of thiols using capillary zone electrophoresis-laser induced fluorescence and TMPAB-o-M, an improved fluorogenic reagent. *Analyst*. 2016; 141:1325–1330. [PubMed: 26814594]
27. Li Q, Chen P, Fan Y, Wang X, Xu K, Li L, Tang B. Multicolor Fluorescence Detection-Based Microfluidic Device for Single-Cell Metabolomics: Simultaneous Quantitation of Multiple Small Molecules in Primary Liver Cells. *Anal Chem*. 2016; 88:8610–8616. [PubMed: 27503398]
28. Shehadul Islam M, Aryasomayajula A, Selvaganapathy P. A Review on Macroscale and Microscale Cell Lysis Methods. *Micromachines*. 2017; 8:83.

29. Keithley RB, Metzinger MP, Rosado AM, Dovichi NJ. Manipulating ionic strength to improve single cell electrophoretic separations. *Talanta*. 2013; 111:206–214. [PubMed: 23622546]
30. Dickinson AJ, Meyer M, Pawlak EA, Gomez S, Jaspers I, Allbritton NL. Analysis of sphingosine kinase activity in single natural killer cells from peripheral blood. *Integr Biol (Camb)*. 2015; 7:392–401. [PubMed: 25786072]
31. Proctor A, Zigoneanu IG, Wang Q, Sims CE, Lawrence DS, Allbritton NL. Development of a protease-resistant reporter to quantify BCR-ABL activity in intact cells. *Analyst*. 2016; 141:6008–6017. [PubMed: 27704073]
32. Kang S, Nieuwenhuis AF, Mathwig K, Mampallil D, Lemay SG. Electrochemical single-molecule detection in aqueous solution using self-aligned nanogap transducers. *ACS Nano*. 2013; 7:10931–10937. [PubMed: 24279688]
33. Nemes P, Knolhoff AM, Rubakhin SS, Sweedler JV. Single-cell metabolomics: changes in the metabolome of freshly isolated and cultured neurons. *ACS Chem Neurosci*. 2012; 3:782–792. [PubMed: 23077722]
34. Liu JX, Aerts JT, Rubakhin SS, Zhang XX, Sweedler JV. Analysis of endogenous nucleotides by single cell capillary electrophoresis-mass spectrometry. *Analyst*. 2014; 139:5835–5842. [PubMed: 25212237]
35. Onjiko RM, Moody SA, Nemes P. Single-cell mass spectrometry reveals small molecules that affect cell fates in the 16-cell embryo. *Proc Natl Acad Sci U S A*. 2015; 112:6545–6550. [PubMed: 25941375]
36. Lombard-Banek C, Moody SA, Nemes P. High-Sensitivity Mass Spectrometry for Probing Gene Translation in Single Embryonic Cells in the Early Frog (*Xenopus*) Embryo. *Front Cell Dev Biol*. 2016; 4:100. [PubMed: 27761436]
37. Chen Y, Arriaga EA. Individual acidic organelle pH measurements by capillary electrophoresis. *Anal Chem*. 2006; 78:820–826. [PubMed: 16448056]
38. Yang Q, Zhang X, Bao X, Lu H, Zhang W, Wu W, Miao H, Jiao B. Single cell determination of nitric oxide release using capillary electrophoresis with laser-induced fluorescence detection. *Journal of Chromatography A*. 2008; 1201:120–127. [PubMed: 18561938]
39. Xu X, Arriaga EA. Chemical cytometry quantitates superoxide levels in the mitochondrial matrix of single myoblasts. *Anal Chem*. 2010; 82:6745–6750. [PubMed: 20704362]
40. Xu X, Thompson LV, Navratil M, Arriaga EA. Analysis of superoxide production in single skeletal muscle fibers. *Anal Chem*. 2010; 82:4570–4576. [PubMed: 20446672]
41. Deng B, Wang Z, Song J, Xiao Y, Chen D, Huang J. Analysis of doxorubicin uptake in single human leukemia K562 cells using capillary electrophoresis coupled with laser-induced fluorescence detection. *Anal Bioanal Chem*. 2011; 401:2143–2152. [PubMed: 21858496]
42. Deng B, Wang ZM, Zhou ZH, Liu YM, Yang XL, Song J, Xiao YX. Quantitative study of cellular heterogeneity in doxorubicin uptake and its pharmacological effect on cancer cells. *Biomed Chromatogr*. 2014; 28:1393–1401. [PubMed: 24687250]
43. Dickinson AJ, Hunsucker SA, Armistead PM, Allbritton NL. Single-cell sphingosine kinase activity measurements in primary leukemia. *Anal Bioanal Chem*. 2014; 406:7027–7036. [PubMed: 24980601]
44. Xu F, Zhao H, Feng X, Chen L, Chen D, Zhang Y, Nan F, Liu J, Liu BF. Single-cell chemical proteomics with an activity-based probe: identification of low-copy membrane proteins on primary neurons. *Angew Chem Int Ed*. 2014; 53:6730–6733.
45. Zhang ZX, Guo XF, Wang H, Zhang HS. Capillary electrophoresis strategy to monitor the released and remaining nitric oxide from the same single cell using a specially designed water-soluble fluorescent probe. *Anal Chem*. 2015; 87:3989–3995. [PubMed: 25707954]
46. Mainz ER, Serafin DS, Nguyen TT, Tarrant TK, Sims CE, Allbritton NL. Single Cell Chemical Cytometry of Akt Activity in Rheumatoid Arthritis and Normal Fibroblast-like Synoviocytes in Response to Tumor Necrosis Factor alpha. *Anal Chem*. 2016; 88:7786–7792. [PubMed: 27391352]
47. Proctor A, Sims CE, Allbritton NL. Chemical fixation to arrest phospholipid signaling for chemical cytometry. *Journal of Chromatography A*. 2017; 1523:97–106. [PubMed: 28528682]

48. Li L, Fan Y, Li Q, Sheng R, Si H, Fang J, Tong L, Tang B. Simultaneous Single-Cell Analysis of Na(+), K(+), Ca(2+), and Mg(2+) in Neuron-Like PC-12 Cells in a Microfluidic System. *Anal Chem*. 2017; 89:4559–4565. [PubMed: 28335593]
49. Omiatek DM, Dong Y, Heien ML, Ewing AG. Only a Fraction of Quantal Content is Released During Exocytosis as Revealed by Electrochemical Cytometry of Secretory Vesicles. *ACS Chemical Neuroscience*. 2010; 1:234–245. [PubMed: 20368748]
50. Wang X, Ma Y, Zhao M, Zhou M, Xiao Y, Sun Z, Tong L. Determination of glucose in human stomach cancer cell extracts and single cells by capillary electrophoresis with a micro-biosensor. *Journal of Chromatography A*. 2016; 1469:128–134. [PubMed: 27688173]
51. Li X, Hu H, Zhao S, Liu YM. Microfluidic Platform with In-Chip Electrophoresis Coupled to Mass Spectrometry for Monitoring Neurochemical Release from Nerve Cells. *Anal Chem*. 2016; 88:5338–5344. [PubMed: 27111409]
52. Smits AH, Lindeboom RG, Perino M, van Heeringen SJ, Veenstra GJ, Vermeulen M. Global absolute quantification reveals tight regulation of protein expression in single *Xenopus* eggs. *Nucleic Acids Res*. 2014; 42:9880–9891. [PubMed: 25056316]
53. Patabadige DE, Mickleburgh T, Ferris L, Brummer G, Culbertson AH, Culbertson CT. High-throughput microfluidic device for single cell analysis using multiple integrated soft lithographic pumps. *Electrophoresis*. 2016; 37:1337–1344. [PubMed: 26887846]
54. Cecala C, Rubakhin SS, Mitchell JW, Gillette MU, Sweedler JV. A hyphenated optical trap capillary electrophoresis laser induced native fluorescence system for single-cell chemical analysis. *Analyst*. 2012; 137:2965–2972. [PubMed: 22543409]
55. Kovarik ML, Shah PK, Armistead PM, Allbritton NL. Microfluidic chemical cytometry of peptide degradation in single drug-treated acute myeloid leukemia cells. *Anal Chem*. 2013; 85:4991–4997. [PubMed: 23590517]
56. Fresta CG, Hogard ML, Caruso G, Melo Costa EE, Lazzarino G, Lunte SM. Monitoring carnosine uptake by RAW 264.7 macrophage cells using microchip electrophoresis with fluorescence detection. *Anal Methods*. 2017; 9:402–408. [PubMed: 29104617]
57. Sadeghi J, Patabadige DE, Culbertson AH, Latifi H, Culbertson CT. Out-of-plane integration of a multimode optical fiber for single particle/cell detection at multiple points on a microfluidic device with applications to particle/cell counting, velocimetry, size discrimination and the analysis of single cell lysate injections. *Lab Chip*. 2016; 17:145–155. [PubMed: 27909706]
58. Wang XL, Ma YF, Yao XM, Wang J, Yin M. Determination of dopamine in rat less differentiated pheochromocytoma cells by capillary electrophoresis with a palladium nanoparticles microdisk electrode. *Rsc Adv*. 2013; 3:24605–24611.
59. Omiatek DM, Santillo MF, Heien ML, Ewing AG. Hybrid capillary-microfluidic device for the separation, lysis, and electrochemical detection of vesicles. *Anal Chem*. 2009; 81:2294–2302. [PubMed: 19228035]
60. Chen Q, Wu J, Zhang Y, Lin JM. Qualitative and quantitative analysis of tumor cell metabolism via stable isotope labeling assisted microfluidic chip electrospray ionization mass spectrometry. *Anal Chem*. 2012; 84:1695–1701. [PubMed: 22242916]
61. Yang M, Nelson R, Ros A. Toward Analysis of Proteins in Single Cells: A Quantitative Approach Employing Isobaric Tags with MALDI Mass Spectrometry Realized with a Microfluidic Platform. *Anal Chem*. 2016; 88:6672–6679. [PubMed: 27257853]
62. Sinkala E, Sollier-Christen E, Renier C, Rosas-Canyelles E, Che J, Heirich K, Duncombe TA, Vlassakis J, Yamauchi KA, Huang H, Jeffrey SS, Herr AE. Profiling protein expression in circulating tumour cells using microfluidic western blotting. *Nat Commun*. 2017; 8:14622. [PubMed: 28332571]
63. Kennedy RT, Stclair RL, White JG, Jorgenson JW. Chemical-Analysis of Single Neurons by Open Tubular Liquid-Chromatography. *Mikrochim Acta*. 1987; 2:37–45.
64. Suzuki T, Miyazono A, Baba K, Sugawara R, Kamiyama T. LC-MS/MS analysis of okadaic acid analogues and other lipophilic toxins in single-cell isolates of several Dinophysis species collected in Hokkaido, Japan. *Harmful Algae*. 2009; 8:233–238.
65. Kajiyama S, Harada K, Fukusaki E, Kobayashi A. Single cell-based analysis of torenia petal pigments by a combination of ArF excimer laser micro sampling and nano-high performance

- liquid chromatography (HPLC)-mass spectrometry. *Journal of Bioscience and Bioengineering*. 2006; 102:575–578. [PubMed: 17270726]
66. Chen Q, Yan G, Gao M, Zhang X. Ultrasensitive Proteome Profiling for 100 Living Cells by Direct Cell Injection, Online Digestion and Nano-LC-MS/MS Analysis. *Anal Chem*. 2015; 87:6674–6680. [PubMed: 26061007]
67. Yang NJ, Hinner MJ. Getting across the cell membrane: an overview for small molecules, peptides, and proteins. *Methods Mol Biol*. 2015; 1266:29–53. [PubMed: 25560066]
68. Falanga A, Galdiero M, Galdiero S. Membranotropic Cell Penetrating Peptides: The Outstanding Journey. *Int J Mol Sci*. 2015; 16:25323–25337. [PubMed: 26512649]
69. Koren E, Torchilin VP. Cell-penetrating peptides: breaking through to the other side. *Trends Mol Med*. 2012; 18:385–393. [PubMed: 22682515]
70. McLaughlin S, Aderem A. The myristoyl-electrostatic switch: a modulator of reversible protein-membrane interactions. *Trends Biochem Sci*. 1995; 20:272–276. [PubMed: 7667880]
71. Nelson AR, Borland L, Allbritton NL, Sims CE. Myristoyl-based transport of peptides into living cells. *Biochemistry*. 2007; 46:14771–14781. [PubMed: 18044965]
72. Mainz ER, Wang Q, Lawrence DS, Allbritton NL. An Integrated Chemical Cytometry Method: Shining a Light on Akt Activity in Single Cells. *Angew Chem Int Ed*. 2016; 55:13095–13098.
73. Proctor A, Wang Q, Lawrence DS, Allbritton NL. Development of a peptidase-resistant substrate for single-cell measurement of protein kinase B activation. *Anal Chem*. 2012; 84:7195–7202. [PubMed: 22881604]
74. Yang S, Proctor A, Cline LL, Houston KM, Waters ML, Allbritton NL. beta-Turn sequences promote stability of peptide substrates for kinases within the cytosolic environment. *Analyst*. 2013; 138:4305–4311. [PubMed: 23785707]
75. Turner AH, Lebhar MS, Proctor A, Wang Q, Lawrence DS, Allbritton NL. Rational Design of a Dephosphorylation-Resistant Reporter Enables Single-Cell Measurement of Tyrosine Kinase Activity. *ACS Chem Biol*. 2016; 11:355–362. [PubMed: 26587880]
76. Li L, Li Q, Chen P, Li Z, Chen Z, Tang B. Consecutive Gated Injection-Based Microchip Electrophoresis for Simultaneous Quantitation of Superoxide Anion and Nitric Oxide in Single PC-12 Cells. *Anal Chem*. 2016; 88:930–936. [PubMed: 26639182]
77. Caruso G, Fresta CG, Siegel JM, Wijesinghe MB, Lunte SM. Microchip electrophoresis with laser-induced fluorescence detection for the determination of the ratio of nitric oxide to superoxide production in macrophages during inflammation. *Analytical and Bioanalytical Chemistry*. 2017; 409:4529–4538. [PubMed: 28555342]
78. Ye X, Rubakhin SS, Sweedler JV. Detection of nitric oxide in single cells. *Analyst*. 2008; 133:423–433. [PubMed: 18365109]
79. Zhang ZX, Guo XF, Wang H, Zhang HS. Capillary Electrophoresis Strategy to Monitor the Released and Remaining Nitric Oxide from the Same Single Cell Using a Specially Designed Water-Soluble Fluorescent Probe. *Anal Chem*. 2015; 87:3989–3995. [PubMed: 25707954]
80. Holmstrom KM, Finkel T. Cellular mechanisms and physiological consequences of redox-dependent signalling. *Nat Rev Mol Cell Biol*. 2014; 15:411–421. [PubMed: 24854789]
81. Caruso G, Fresta CG, Siegel JM, Wijesinghe MB, Lunte SM. Microchip electrophoresis with laser-induced fluorescence detection for the determination of the ratio of nitric oxide to superoxide production in macrophages during inflammation. *Anal Bioanal Chem*. 2017; 409:4529–4538. [PubMed: 28555342]
82. Ye F, Huang Y, Xu Q, Shi M, Zhao S. Quantification of taurine and amino acids in mice single fibrosarcoma cell by microchip electrophoresis coupled with chemiluminescence detection. *Electrophoresis*. 2010; 31:1630–1636. [PubMed: 20401902]
83. Dishinger JF, Reid KR, Kennedy RT. Quantitative monitoring of insulin secretion from single islets of Langerhans in parallel on a microfluidic chip. *Anal Chem*. 2009; 81:3119–3127. [PubMed: 19364142]
84. Fahy E, Cotter D, Sud M, Subramaniam S. Lipid classification, structures and tools. *Biochim Biophys Acta*. 2011; 1811:637–647. [PubMed: 21704189]

85. Jiang D, Sims CE, Allbritton NL. Single-cell analysis of phosphoinositide 3-kinase and phosphatase and tensin homolog activation. *Faraday Discuss.* 2011; 149:187–200. discussion 227–145. [PubMed: 21221426]
86. Keithley RB, Weaver EM, Rosado AM, Metzinger MP, Hummon AB, Dovichi NJ. Single cell metabolic profiling of tumor mimics. *Anal Chem.* 2013; 85:8910–8918. [PubMed: 24011091]
87. Essaka DC, Prendergast J, Keithley RB, Palcic MM, Hindsgaul O, Schnaar RL, Dovichi NJ. Metabolic cytometry: capillary electrophoresis with two-color fluorescence detection for the simultaneous study of two glycosphingolipid metabolic pathways in single primary neurons. *Anal Chem.* 2012; 84:2799–2804. [PubMed: 22400492]
88. Essaka DC, White J, Rathod P, Whitmore CD, Hindsgaul O, Palcic MM, Dovichi NJ. Monitoring the uptake of glycosphingolipids in *Plasmodium falciparum*-infected erythrocytes using both fluorescence microscopy and capillary electrophoresis with laser-induced fluorescence detection. *Anal Chem.* 2010; 82:9955–9958. [PubMed: 21043509]
89. Lee KJ, Mwongela SM, Kottegoda S, Borland L, Nelson AR, Sims CE, Allbritton NL. Determination of sphingosine kinase activity for cellular signaling studies. *Anal Chem.* 2008; 80:1620–1627. [PubMed: 18197698]
90. Whitmore CD, Hindsgaul O, Palcic MM, Schnaar RL, Dovichi NJ. Metabolic cytometry. Glycosphingolipid metabolism in single cells. *Anal Chem.* 2007; 79:5139–5142. [PubMed: 17567107]
91. Huang W, Proctor A, Sims CE, Allbritton NL, Zhang Q. Fluorous enzymatic synthesis of phosphatidylinositides. *Chem Commun.* 2014; 50:2928–2931.
92. Keithley RB, Rosenthal AS, Essaka DC, Tanaka H, Yoshimura Y, Palcic MM, Hindsgaul O, Dovichi NJ. Capillary electrophoresis with three-color fluorescence detection for the analysis of glycosphingolipid metabolism. *Analyst.* 2013; 138:164–170. [PubMed: 23154386]
93. Boardman A, Chang T, Folch A, Dovichi NJ. Indium-tin oxide coated microfabricated device for the injection of a single cell into a fused silica capillary for chemical cytometry. *Anal Chem.* 2010; 82:9959–9961. [PubMed: 21033750]
94. Anandappa G, Turner NC. Targeting receptor tyrosine kinases in HER2-negative breast cancer. *Current opinion in oncology.* 2013; 25:594–601. [PubMed: 24100346]
95. Dornan D, Settleman J. Dissecting cancer heterogeneity. *Nat Biotechnol.* 2011; 29:1095–1096. [PubMed: 22158362]
96. Phillips RM, Bair E, Lawrence DS, Sims CE, Allbritton NL. Measurement of protein tyrosine phosphatase activity in single cells by capillary electrophoresis. *Anal Chem.* 2013; 85:6136–6142. [PubMed: 23682679]
97. Ong ILH, Yang KL. Recent developments in protease activity assays and sensors. *Analyst.* 2017; 142:1867–1881. [PubMed: 28487913]
98. Dickinson AJ, Hunsucker SA, Armistead PM, Allbritton NL. Single-cell sphingosine kinase activity measurements in primary leukemia. *Anal Bioanal Chem.* 2014; 406:7027–7036. [PubMed: 24980601]
99. Nguyen LT, Oien NP, Allbritton NL, Lawrence DS. Lipid pools as photolabile “protecting groups”: design of light-activatable bioagents. *Angew Chem Int Ed.* 2013; 52:9936–9939.
100. Brown RB, Hewel JA, Emili A, Audet J. Single amino acid resolution of proteolytic fragments generated in individual cells. *Cytometry A.* 2010; 77:347–355. [PubMed: 20187109]
101. Chen D, Fan F, Zhao X, Xu F, Chen P, Wang J, Ban L, Liu Z, Feng X, Zhang Y, Liu BF. Single Cell Chemical Proteomics with Membrane-Permeable Activity-Based Probe for Identification of Functional Proteins in Lysosome of Tumors. *Anal Chem.* 2016; 88:2466–2471. [PubMed: 26810843]
102. Kim JJ, Sinkala E, Herr AE. High-selectivity cytology via lab-on-a-disc western blotting of individual cells. *Lab Chip.* 2017; 17:855–863. [PubMed: 28165521]
103. Mellors JS, Jorabchi K, Smith LM, Ramsey JM. Integrated microfluidic device for automated single cell analysis using electrophoretic separation and electrospray ionization mass spectrometry. *Anal Chem.* 2010; 82:967–973. [PubMed: 20058879]

Figure 1. Separation-based Chemical Cytometry

(A) A reporter is introduced into the cell. (B) The reporter is modified in the cell by or binds to its target molecule. (C) A cell is lysed and the cell contents along with the reporter are introduced into the separation column. (D) The cell contents are separated in the column. (E) The identify and amounts of unmodified and modified reporter are quantified from the separation trace.

Figure 2. Separation and Detection Methods for Chemical Cytometry

The most commonly employed separation methods in chemical cytometry are liquid chromatography and capillary electrophoresis. Both separation techniques can be coupled to the three major analyte detection strategies, electrochemistry (usually amperometry), mass spectrometry (commonly employing an electrospray ion source), and fluorescence detection (laser or diode-based excitation). (A) A cell is (B) introduced into the separation channel (C) followed by detection and (D) data collection.

Figure 3. Measured Analytes in Single Cells

Diagram of a cell highlighting the analyte classes that have been measured by chemical cytometry.

Figure 4. Range of Separation Based Chemical Cytometry Measurements

Chemical cytometry has been used to measure analytes with a wide range of molecular weights with excellent mass limits of detection (MLOD) by mass spectrometry (dark blue), electrochemistry (red), or fluorescence (light blue).