Categorizing Cells on the Basis of their Chemical Profiles: Progress in Single-Cell Mass Spectrometry

Troy J. Comi, Thanh D. Do, Stanislav S. Rubakhin, and Jonathan V. Sweedler*©

Department of Chemistry and the Beckman Institute for Advanced Science and Technology, University of Illinois at Urbana–Champaign, Urbana, Illinois 61801, United States

ABSTRACT: The chemical differences between individual cells within large cellular populations provide unique information on organisms' homeostasis and the development of diseased states. Even genetically identical cell lineages diverge due to local microenvironments and stochastic processes. The minute sample volumes and low abundance of some constituents in cells hinder our understanding of cellular heterogeneity. Although amplification methods facilitate single-cell genomics and transcriptomics, the characterization of metabolites and proteins remains challenging both because of the lack of effective amplification approaches and the wide diversity in cellular constituents. Mass spectrometry has become an enabling technology for the investigation of individual cellular metabolite profiles with its exquisite sensitivity, large dynamic range, and ability to characterize hundreds to thousands of compounds. While advances in instrumentation have improved figures of merit, acquiring measurements at high throughput and sampling from large populations of cells are still not routine. In this Perspective, we highlight the current trends and progress in mass-spectrometry-based analysis of single cells, with a focus on the technologies that will enable the next generation of single-cell measurements.

INTRODUCTION

Cells are the “atomic unit” of life. Inspired by Robert Hooke’s discovery of biological cells in 1665,1 scientists, evoking the philosophical musings of Marcus Aurelius,2 began to ponder: “The thing, what is it, fundamentally? What is its nature and substance, its reason for being?” These central questions set the framework for defining cell biology. Much of the early single-cell work relied on observations of cells with optical microscopy; current research has extended these investigations to the chemical and molecular regimes. Studies examining simple chemical questions about cells have detailed, extended, to the chemical and molecular regimes. Studies examining microscopy; current research has extended these investigations.

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Early successes of single-cell electrophoresis were reported from the 1950s to 1970s. In 1956, Edström3 successfully determined the relative composition of ribose nucleic acids within large, mammalian neuronal cells by microphoresis with a cellulose fiber. Separation of hemoglobin from individual erythrocytes using polyacrylamide fiber electrophoresis followed in 1965.7 Two-dimensional gel electrophoresis of proteins from single Aplysia californica neurons was reported in 1977,10 around the time single-cell mass spectrometry (MS) began to develop. In their pioneering work in the 1970s, Hillenkamp and co-workers11 used laser ablation mass analysis to generate mass spectra from tissue sections and cultured cells. They ablated several <5-μm-diameter regions on an inner-ear tissue section with a laser to obtain mass spectra containing low-molecular-weight ions at each associated laser spot.12 As another example from the 1970s, Iliffe et al.13 demonstrated single-cell gas chromatography–mass spectrometry of amino acids in an Aplysia neuron. This period also witnessed the introduction of flow cytometry and fluorescence-activated cell sorting.14 However, it was not until 1992, when James Eberwine’s group15 demonstrated that the molecular profile of a single, potentiated CA1 neuron depends on the abundance of multiple RNAs, that the field of comprehensive single-cell chemical analysis began to take shape.

After these early seminal reports, single-cell chemical characterization approaches became more robust and provided greater information, enabling astounding advances in bi-analytical techniques that have progressively revealed single-cell heterogeneity. Interdisciplinary developments include single-cell genomics and transcriptomics,16–19 electrochemistry,20 mass spectrometry of amino acids,21 single-molecule microscopy and spectroscopy,22,23 nuclear magnetic resonance,24–26 capillary electrophoresis (CE),27,28 MS,29–32 and microfluidics,33–35 to name a few. Clearly, single-cell “omics” comprises a number of rapidly growing interdisciplinary fields. We view MS as the major analytical platform for single-cell metabolomics and proteomics (SCMP) due to its versatility, multiplexed capabilities, and relatively high throughput. Modern MS instruments provide limits of detection and analyte coverages that are suitable for non-targeted SCMP. However, effective, high-throughput single-cell sampling remains a major challenge. In fact, details related to sampling often dictate the selection of the most appropriate MS instrument and experimental protocols to use for a specific investigation.

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This Perspective describes recent progress in the development of MS-based analytical techniques and the attendant cell isolation approaches used for SCMP investigations. These diverse MS-based methodologies are ideally suited for the characterization of heterogeneous cellular populations through qualitative and quantitative chemical profiling of individual cells.

**SETTING THE STAGE: MASS SPECTROMETRY INSTRUMENTATION IN SINGLE-CELL RESEARCH**

MS has evolved from a gas-phase, one-dimensional analytical technique into a versatile approach that provides high mass resolution, analyte coverage, and sensitivity. Several key advances in instrumentation, combined with innovative methodologies, have set performance benchmarks for an eclectic range of MS applications (for comprehensive reviews, see refs 40 and 41). Here, we focus on the aspects of MS that make it uniquely suited to single-cell analysis.

The major challenges to single-cell chemical measurements lie in the relatively small quantity of analytes, the low volume of material, and the chemical diversity of cellular constituents. SCMP measurements are made possible by improving the sensitivity and analyte coverage of analytical techniques capable of handling the small-volume (femto-scale) samples extracted from single cells (e.g., eukaryotic cells are 5–100 μm in diameter; bacterial cells range from 0.2 to 2 μm). Small molecules, such as metabolites and lipids, are often concentrated within cells, whereas peptides, proteins, and genetic material may exist at only a few copies. Ionizing intact biomolecules requires soft MS probes that minimize molecular fragmentation.

A variety of MS methods are suitable for single-cell studies. Matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) are two robust approaches for the ionization of intact peptides and proteins from single cells. Secondary ion mass spectrometry (SIMS) utilizes a focused, accelerated primary ion beam to sputter sample surfaces and has been used for sampling from cells for several decades. While traditional primary ion beams induce molecular fragmentation, newly developed cluster ion sources can desorb and ionize intact metabolites, lipids, and small peptides. Furthermore, SIMS ionization, when performed below the static limit, causes negligible damage to sample surfaces, which permits subsequent analyses of the same samples. Lastly, the speed, sensitivity, and precision of inductively coupled plasma (ICP) MS is the foundation for mass cytometry, a prominent technique for targeted single-cell analysis.

The detection limit of an MS-based platform depends on the performance of the mass analyzer. Many modern instruments offer sufficiently high ion transmission efficiency, a wide mass range, and high mass accuracy to measure cellular content, with several commercially available MS platforms that are appropriate for SCMP measurements. Among them, the time-of-flight (TOF) mass analyzer has been widely used in single-cell research because of its relatively low cost, large m/z detection window, and satisfactory performance for most MS profiling and imaging experiments, especially when fast scan rates are required. Limits of detection for TOF-MS can be below an attomole of a peptide while maintaining a mass resolution above 20 000. Spectra are acquired in tens of microseconds, though several hundred TOF spectra are frequently summed for a better signal-to-noise ratio (S/N). In “omics” work requiring high mass accuracy and mass resolution, ion cyclotron resonance (ICR) and Orbitrap mass analyzers offer superior performance. Based on the duration of the transient acquired for Fourier transformation, resolution in excess of 100 000 is routine, with an acquisition frequency of about 1 Hz. In hybrid instruments, high-resolution mass analyzers are coupled to collision cells, enabling selection of precursor ions and exact mass measurements on their fragments. Multistage fragmentation of ions (MS^n) and analysis of fragments are essential for characterization of unknowns.

Herein, we focus on the strengths, weaknesses, and future prospects of MS-based SCMP methods. From among a myriad of techniques, these were chosen to provide an overview of the field because they offer great promise for advancing single-cell research. As stated earlier, sample properties and preparation strategies oftentimes determine the appropriate MS instrument to use for a specific application. Thus, while this discussion focuses on the MS technologies, it is organized by the sampling approaches. In the first method (Figure 1A), intact tissue slices can be directly analyzed using imaging technologies that provide subcellular spatial resolution. Alternatively, targeted cells can be isolated from tissues (Figure 1B) prior to MS measurements. The success of this approach depends on prior classification of cell types and subtypes, and on the dexterity of the researcher performing the cell isolation. Finally, single-cell samples can be prepared by digesting tissues into thousands to millions of single cells (Figure 1C). Dissociation alleviates the stringent requirements of the first two methods and creates additional opportunities for cells to stabilize prior to analysis.

**DIRECT TISSUE ANALYSIS: PLACING SINGLE CELLS INTO CONTEXT**

Mass spectrometry imaging (MSI), an information-rich approach for direct tissue analysis, provides unprecedented details on the chemical composition of tissue and cell specimens. Typically, an MS image is acquired by sampling a regularly spaced grid on a thin tissue section or dispersed cell population, collecting a mass spectrum at each spot. MSI is an attractive option when determining the spatial context of individual cells within tissues is important, or when single-cell isolation is not feasible. Different MS ionization methods facilitate the successful analysis of numerous biochemical classes, including proteins, small peptides, lipids, and metabolites (Figure 2). MALDI-MSI (Figure 2A) is the most...
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which are utilized during cell mating. Subsequent to this report, in aminoethylphosphonolipid at highly curved fusion pores, a decrease in abundance of phosphatidylcholine and an increase in the plasma membrane of Tetrahymena. The images revealed a decrease in abundance of phosphatidylcholine and an increase in aminoethyolphospholipid at highly curved fusion pores, which are utilized during cell mating. Subsequent to this report, a variety of cell types have been analyzed by SIMS imaging, providing subcellular distributions of lipids, metabolites, and small molecules.

Elemental secondary ions can be characterized by a magnetic sector analyzer equipped with up to seven detectors set to particular m/z values—a technique referred to as nanoSIMS. State-of-the-art nanoSIMS is quantitative, can achieve spatial resolution <50 nm, and allows 3D chemical mapping. NanoSIMS has been applied for subcellular-resolution imaging of metabolic pathways, interacting microorganisms, and microbial communities.43,55–59 The main drawbacks are relatively low sample throughput and the high cost of isotope-labeled substrates. Nevertheless, the clever use of isotopes allows nanoSIMS to interrogate the 3D composition of representative cell subtypes.

Recent developments with polyatomic and cluster ion sources have expanded the biochemical coverage of SIMS by allowing direct measurement of intact molecules below m/z 2000. The cluster ion sources achieve primary ion beam diameters approaching 1 μm, equivalent to high-resolution MALDI sources.60 Complementary MS imaging, non-MS analyses, and matrix-enhanced reagents have been incorporated to improve molecular coverage and quantitation of SIMS imaging. Aspects of the sample preparation pipeline contribute significantly to the spatial integrity of measured molecular distributions. SIMS is especially sensitive to minute amounts of environmental contamination, as analysis is restricted to the topmost layer of the surface. While primary ion beams may be focused to tens of nanometers, obtaining such high spatial resolution is still extremely challenging.

Most MSI experiments are non-targeted and label free, but at the pixel widths required for subcellular imaging, only abundant compounds will be detectible. Imaging mass cytometry (Figure 2C), can improve the limits of detection for specific compounds by using affinity-based probes to selectively localize target antigens. As a direct analog to immuno-gold staining used with electron microscopy, imaging mass cytometry couples metal-conjugated antibodies developed for mass cytometry with a laser or ion beam, allowing antigen localization in tissue sections and individual cells. Giesen et al.63 used imaging mass cytometry with a high spatial resolution laser ablation system to localize 32 proteins and post-translational modifications (PTMs) at 1 μm resolution to delineate cell heterogeneity in human breast cancer tissue sections. Angelo et al.64 adapted the mass cytometry pipeline to SIMS imaging, effectively improving the spatial resolution of the method to 50 nm. The chelated metal isotope adducts generated secondary ions, which were analyzed via a magnetic sector mass spectrometer equipped with multiple detectors. The technique, referred to as multiplexed ion beam imaging (MBI), was successfully applied to human breast cancer samples to reveal tumor immunophenotypes. The current acquisition rate for MBI is 2 h for a 0.250 mm² field-of-view for 10 distinct targets.65

Rastering the desorption probe over large areas, as in MSI, effectively analyzes each cell, but does so at the expense of throughput and considerable cost in instrument time and assay sensitivity. At the Nyquist frequency to resolve individual cells, each cell should be sampled at least four times; this divides the cellular analytes among each pixel and may cause some compounds to fall below the limit of detection. Still, the drive to acquire higher resolution MS images has spurred the development of improved ion beam optics, sensitive mass

Figure 2. Several MSI methods obtain single-cell resolution. (A) Application of a matrix is required for MALDI-MSI and must be optimized to maintain native spatial distributions. While spatial resolution is poorer than with SIMS, MALDI ionization is much softer, such that intact lipids and peptides are detectable. (B) SIMS provides the highest spatial resolution with focused primary ion beams but is limited in analyte coverage, typically detecting fragment ions and small compounds. (C) Imaging mass cytometry is capable of targeted localization of protein antigens with resolutions similar to SIMS.

common technique used in tissue imaging. A recent review by Römpf and Spengler highlights several successful studies in which MALDI-MSI provided detailed histological information on phospholipids, drug molecules, neuropeptides, and tryptic peptides at (or close to) the single-cell level. While 10–35-μm pixel widths are common, MALDI-MSI at 3 μm spatial resolution was performed on the lateral ventricle region of a coronal mouse brain section to image phospholipids.67

MSI at nanometer resolution can be achieved by SIMS imaging, which employs a tightly focused, accelerated primary ion beam for desorption and ionization (Figure 2B). SIMS is suitable for mapping elements, metabolites, small molecules, lipids, and peptide fragments at subcellular resolution (for a review on the fundamentals of SIMS, see Boxer et al.68). Several primary ion beams are suitable for biological analyses. High-energy and reactive sources may provide sufficient ion current to afford submicron spatial resolution but tend to fragment the chemical bonds of larger molecules.68 Ostrowski et al.69 utilized an indium liquid metal ion beam focused to 200 nm to examine the plasma membrane of Tetrahymena. The images revealed a decrease in abundance of phosphatidylcholine and an increase in aminoethyolphospholipid at highly curved fusion pores, which are utilized during cell mating. Subsequent to this report, a variety of cell types have been analyzed by SIMS imaging, providing subcellular distributions of lipids, metabolites, and small molecules.

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analyzers, and optimized sample preparation protocols. We expect instrument capabilities will continue to progress and cellular resolution will become standard in commercial MALDI-MS instrumentation over the next few decades. A limitation to the continued development of smaller pixel sizes is the absolute abundance of compounds within a given region. A 1-μm pixel contains just over 1% of the area of a 10-μm-diameter cell, requiring analyte concentrations 2 orders of magnitude higher to be observable in a single pixel as opposed to the entire cell. Compounding this effect for MALDI-MS is the compromise between analyte extraction and delocalization during matrix application.

Imaging mass cytometry circumvents these issues with the application of rare-earth-labeled antibodies. Each antibody holds several hundred isotope atoms, which amplifies the signal from a single binding event. The shortcomings of mass cytometry imaging are inherited from affinity labels: the a priori selection of antigens, cost of generating antibodies, and limited plexity (though not as severe as fluorescence probes). We envision mass cytometry imaging experiments will be performed on a tissue section following non-targeted MSI acquisition, similar to work performed with immunohistochemistry. Such an experiment could place the non-targeted data into the context of more traditional cell subtyping to improve biomarker identification. As subcellular MSI resolution becomes more widespread, the distinction between imaging and single-cell analysis will be less pronounced. The capability to examine each cell within its native environment would revolutionize medical, pharmaceutical, and fundamental research.

SPECIFIC CELL-TYPE TARGETING: MEETING THE NEEDS FOR SEPARATION AND QUANTITATION

When molecular characterization is the paramount experimental objective, measurements that do not provide spatial information can be undertaken. Additional analytical dimensions, such as separation and quantitation, can be coupled with MS to enable information-rich single-cell measurements. CE is a qualitative and quantitative technique used in analyses of single cells and subcellular compartments. It features rapid analyte separations based on the electrophoretic mobility of molecules, including those with the same molecular weights (e.g., diastereomers), with high resolving power and low sample consumption (a microliter or less).³¹,³² Many aspects of CE have greatly progressed in recent decades, and include the development of advanced separation modes and nanoscale sampling, and the interface of CE with different detection methods.³⁰,⁶⁷ While CE is powerful on its own, it is even more productive when coupled with optical, electrochemical, or MS-based detection. For example, CE-MS provides a label-free and unique characterization method for investigation of endogenous biomolecules in complex cellular mixtures (Figure 3). Hyphenating CE with other detection modalities, such as laser-induced fluorescence, allows targeted cell analysis based on chemical signatures, but those approaches are limited to molecules with native fluorescence and those that can be tagged with a fluorophore via derivatization chemistry.⁷ Single-cell metabolomics studies using CE-ESI-MS have demonstrated detection limits for molecules in the low nanomolar range, high-efficiency separations, and increased analyte coverage. The injection of only 0.1% of the total content from a single Aplysia californica metacerebral cell (150 μm in diameter) yielded unambiguous detection of more than 100 compounds.⁶⁸

Preconcentration methods further improve analyte coverage, especially when initial concentrations of extracted analytes are below the detection limits of MS systems.³⁰,⁴² Improvements in sheathless CE-MS interfaces have allowed investigation of complex bioanalytical problems, as in the characterization of protein isoforms and combinatorial PTMs reported by Yates and co-workers.⁶⁹,⁷⁰ Recent examples from Dovichi⁷¹ and Neme⁷²,⁷³ of the developing Xenopus laevis embryo demonstrate the great promise for CE-MS-based single-cell proteomics.

Though capable of sensitive, quantitative analysis, a limitation of CE is its low throughput. Even a state-of-the-art CE platform operates at a rate of less than one cell per minute.⁷⁴,⁷⁵ Typical separations, performed in longer capillaries, can last between 5 and 60 min to achieve optimal resolution; however, chip-based CE devices do increase throughput. Moreover, the duration of a set of experiments may be constrained by the endurance of intact cells within a physiological solution prior to analysis (a few hours), which ultimately limits the number of cells that can be assayed from one population.⁷⁶ Further constraining throughput, each sample and target analyte requires an optimal set of CE
conditions, including background electrolyte, chiral selectors, pH, separation voltage, and temperature, among others.

To increase throughput, researchers have focused on the development of automated cell-handling modules that are compatible with a wide range of background electrolytes and analyte classes. CE columns can be embedded in, or coupled with, microfluidic devices that permit fluorescence-activated cell sorting (FACS) and automatic cell trapping, culturing, sorting, and lysis prior to CE separation. Higher peak capacities are achieved by combining multiple capillary columns in series to provide complementary separation dimensions. Examples include the velocity gap mode, which manipulates the electrical fields on connected capillaries with conductivity detection at the joint, and 2D-CE, which employs orthogonal separation conditions in connected capillaries.77

Recent advances in CE have overcome technical hurdles for the detection and separation of chiral molecules, such as D/L-amino acids and peptide diastereomers,78 at a resolution and sensitivity that is currently inaccessible by other label-free, MS-coupled mobility spectrometry or spectroscopy approaches.79 Furthermore, these molecules are separated non-destructively with minimal loss, which is another advantage of CE over MS-based separation methods. In addition, performing the separation postionization can introduce additional complexity due to the formation of protomers (molecular isomers that differ only in the site of protonation).80 However, many conditions used in chiral separations have yet to be made compatible with ESI-MS, awaiting future optimization.

Owing to its superb sensitivity and prospects for high throughput, CE-MS has become a method of choice for separation-based, quantitative analyses of single cells. Compared to other single-cell techniques, CE-MS applications that directly introduce cells into the capillary for lysis and separation reduce the time between cell rupture and analyte characterization. Such rapid analyses limit unwanted side reactions and degradation that lead to non-specific profile variations. The future of high-throughput CE-MS offers a unique approach to classify cell types and identify new subtypes, which will provide complementary profiles to other methods.

- **DISSOCIATED AND CULTURED CELL SAMPLES: SEARCHING FOR CELL SUBTYPES AND RARE CELLS**

In the final approach discussed here, cells are either separated from tissue sections by dissociation or cultured. Once in solution, cells may be labeled for mass cytometry, or deposited onto a surface for single-cell profiling. The native connections between cells in the tissue are dismantled and extraction is more limited than with specific cell isolation, but dissociated cell measurement approaches can have a higher per-cell throughput than the cell-based MS methods described above.

Mass cytometry is one of the most versatile MS-based techniques for multiplexing single-cell measurements on an “omics” scale. As briefly mentioned when discussing MSI, mass cytometry operates much like flow cytometry, in which fluorescently labeled markers, including antibodies, are used to characterize the presence of a panel of antigens in large populations of individual cells. However, instead of fluorescence labels, mass cytometry uses rare earth metal isotope tags with high plexity (Figure 4A). The binding of the conjugates to cellular events from debris and distinguish single cells from doublets or aggregates of cells. Metal calibration beads are also...
spiked into each sample to serve as internal standards.\textsuperscript{85} Cell-based multiplexing methods, such as mass-tag cell barcoding,\textsuperscript{65,87} can be utilized to reduce antibody consumption, acquisition time, and eliminate cell-to-antibody ratio-dependent effects.\textsuperscript{81,83} For example, a binary barcoding can utilize \( n \) rare metal isotopes to uniquely label \( 2^n \) individual cell samples before they are mixed, stained, and analyzed in one batch.\textsuperscript{88} Mass cytometry has assisted the discovery of complex aspects of single-cell chemistry, including different stages of the cell cycle, phenotypes and signaling responses, cytokine expression, and cell viability.\textsuperscript{82,88–92}

Cell surface markers, the degree of expression, and PTM events can be used to identify cellular phenotypes and distinguish cell populations. For example, a single-cell mass cytometry study using 31 distinct transition and rare earth metal isotopes to label two antibody staining panels revealed 24 distinct immune cell populations in bone marrow during hematopoiesis.\textsuperscript{89} Currently, mass cytometry surpasses other MS-based single-cell techniques in the total number of analyzed cells per experiment. Newell et al.\textsuperscript{93} combined mass cytometry with combinatorial peptide-major histocompatibility complex staining to analyze samples of 84 million T-cells for distinct phenotypes and their ability to recognize viral epitopes.

A technical inefficiency of mass cytometry lies in the nebulization of single cells, which stochastically loses approximately 70\% of the cells in the process of forming droplets.\textsuperscript{81} Although this loss does not inherently introduce a significant sampling bias, improvements in cell introduction efficiency would reduce cell consumption. The sensitivity of mass cytometry is greatly affected by the loading of metal atoms on each antibody. The metal chelating chemistry facilitates a maximum of \( \sim 100 \) metal reporter ions per antibody molecule.\textsuperscript{81} Mass cytometry can seamlessly measure 58 or more different parameters simultaneously, though this requires \textit{a priori} knowledge about the cells and well-defined molecular targets with specific antibodies. The limited number of commercially available rare metal isotopes also limits the number of antigens that can be measured simultaneously. Currently, 37 stable lanthanide isotopes that are compatible with metal chelating chemistry are available at sufficient purity.\textsuperscript{81} While antibodies can recognize a wide range of antigens, mass cytometry is less effective for smaller molecules, such as metabolites and peptides, which may not be accessible to antibodies or cross-linked by fixation. These molecules can be specific biomarkers for disease-transformed cells.\textsuperscript{94} Therefore, the complexity of multidimensional single-cell analysis is another area worth improving,\textsuperscript{95} including new affinity agents that can bind small-molecule metabolites.

Mass cytometry is poised to extend the capabilities of many immunofluorescence methods beyond the limitation of fluorescence spectral overlap. In a clinical setting, the rapid and accurate quantification of numerous biomarkers can facilitate deeper subtyping of tissue sections or biopsy samples. Though mass cytometry requires preselection of antigens, it should continue to find application in targeted cell population profiling. While mass cytometry can profile cellular states at given points in time with high throughput and plenitude, an important caveat is that cells are destroyed by the ICP torch, preventing follow-up characterization of selected cellular subtypes.

A distinct non-targeted approach involves dispersing cells onto sample surfaces where they are analyzed with an MS microprobe. In contrast to MSI, the contents of one cell are completely sampled during a single analysis. Manual placement of cells is a low-throughput implementation of this type of handling.\textsuperscript{43} A higher throughput method is to disperse cells sufficiently such that no neighbors are within the microprobe region. With the correct choice of seeding density, separated cells greatly relax instrumental sampling requirements and allow more stringent extraction procedures, further increasing analyte sensitivity. As described below, two methods of dispersed cell sampling have been developed recently for MALDI-MS analysis of single cells, one based on constrained cell positions and the other on randomly seeding the cells.

The first cell-dispersed approach involves constraining the cell positions. A variety of microfluidic constructs are available for trapping single cells for subsequent high-throughput analysis. Microdroplet arrays can systematically trap single cells in microwells, allowing subsequent profiling by ESI-MS.\textsuperscript{96} The sensitivity of the trapping depends on the ratio of the diameters of the cell and the microwell, limiting the sizes of analyzed cells. The current implementation also requires manual sampling of each well. For high throughput sampling, Zenobi and co-workers\textsuperscript{97} developed an omniphobic, patterned surface specifically for constraining microdroplets of MALDI matrix solution, called microarrays for MS (MAMS) (Figure 4B). By depositing cells into these microwells, their contents remain isolated due to the omniphobic microarray walls. This isolation allows the application of more rigorous extraction methods, such as shock freezing\textsuperscript{98} as analytes neither severely dilute nor become contaminated by nearby cells. Cell deposition in MAMS is achieved by a variety of methods, including piezoelectric printing of cellular solutions\textsuperscript{99} or submerging the surface in a cell solution.\textsuperscript{97,100} Each well contains a variable number of cells described by a Poisson distribution.\textsuperscript{98} As such, with a cell concentration generating the maximum probability of wells containing one cell (average, \( \lambda = 1 \)), approximately 37\% of wells are occupied by one cell. Another 37\% of the wells are empty, with the remaining 26\% containing two or more cells. Orthogonal methods, such as optical microscopy, can enumerate the cell counts in each well. Once cell number and positions are determined, cellular analytes are extracted and samples are coated with MALDI matrix. The contents are analyzed by simply collecting spectra at each predetermined point in a regular array. Unlike subcellular MSI, the required positional accuracy and laser spot size are easily achieved by most commercial instruments.

Using this methodology, the metabolic profiles of several single-celled microorganisms were investigated, showing quantities of nucleoside di- and triphosphates, as well as lipids unique for each species, with concentrations proportional to the number of cells within a given well; Raman spectra were also obtained and correlated with a given microwell.\textsuperscript{97} Further experiments correlated fluorescence and Raman microspectroscopy acquired from the freshwater algae \textit{Haematococcus pluvialis} and combined the images with MS measurements to discriminate between encystment stages.\textsuperscript{100} In addition, using \textit{Saccharomyces cerevisiae} as a model organism, Zenobi and co-workers\textsuperscript{98} investigated the metabolic consequences of environmental and genetic perturbations on several metabolites, recapitulating population-level changes and discriminating genotypic differences.

Advantages of MAMS include the capabilities to thoroughly extract analytes from deposited cells and ensure each sample is isolated from nearby cells, limiting cross contamination. However, the efficiency for random seeding is low (only 37\%
of wells contain single cells) and the spatial constraints of the microwells limit investigations of long-range cellular outgrowth and changes related to cell-to-cell signaling. Theoretically, MAMS could facilitate studies of interactions between small cell populations. With conventional random seeding or printing, the likelihood of two cells from each of two populations occupying the same well is $0.37^2 = 14\%$; however, the cases when a well is occupied by more than one cell of each type are also interesting. This would allow investigations into the competition between malignant and immune cells for small populations of each, generating a large, random assortment of populations on a single device. FACS could also be used as an enabling, selective cell deposition technology coupled to MAMS. Precise seeding of specific, preselected phenotypes could construct complex cell distributions to allow full utilization of each MAMS device.

An alternative method for high throughput analysis of isolated, individual cells involves randomly dispersing them on a surface, and using optical microscopy to precisely locate the dispersed cells on a transparent indium tin oxide-coated glass slide. Suspensions of cells are deposited onto conductive surfaces and the cells allowed to attach to the substrate. High-contrast, fluorescence images of a nuclear stain deliver a simple data set to locate individual cells. Registration of the microscopy image with the mass spectrometer coordinate system provides the location of each selected cell. Once MALDI matrix is applied, the laser is positioned over each cell in turn and a spectrum acquired (Figure 4C). In this initial report, microscopy-guided single-cell MALDI-MS was coupled to principal component analysis-based outlier detection to perform an unsupervised analysis in a population of dispersed pituitary cells. Several peptides were detected at high S/N from individual pituitary cells, including arginine vasopressin, oxytocin, and α-melanocyte-stimulating hormone. Additional MS profiling of cells from pancreatic islets of Langerhans demonstrated single-cell sensitivity to canonical peptide hormones, including intact insulin, glucagon, pancreatic polypeptide, and somatostatin. In a follow-up study on single islet cells, the levels of peptide hormones were used to classify cells into traditional histological classes, showing good agreement with previous reports. Furthermore, cell-type-specific peptide heterogeneity was compared between the dorsal- and ventral-derived islets, with results indicating an increased abundance of processed pancreatic polypeptide within ventral-derived γ-cells. The peptides were not previously observed endogenously, and the anatomical heterogeneity in peptide processing would be difficult to detect with bulk measurements.

Successful analyte profiling using microscopy-guided MALDI-MS largely depends on accurate cell positioning under the laser probe, requiring the ability to locate a 10-μm cell over a ~20 cm$^2$ microscope slide. Assuming a random seeding, the probability of individual cells being sufficiently far apart is determined by a spatial Poisson point process, which has the same form as a Poisson distribution. Again, at ideal conditions, only 37% of the seeded cells will be sufficiently spaced for analysis, but there is a relatively large area available for seeding. As such, the total number of cells analyzed in a given footprint will be larger than with reported examples of MAMS. Furthermore, long-range interactions should be easier to observe, as there is no physical barrier between cells. Coupling with FACS may be more difficult, as the cells in droplets impacting the surface could migrate without being confined in omniphobic wells.

While both high throughput studies described above used MALDI-MS, these methodologies could be adapted to work with other microprobe-based MS analyses such as DESI and SIMS, and liquid microjunction probes.

An exciting aspect of dispersed-cell methods is the ease with which they can be coupled with complementary analytical methods, e.g., combining with optical microscopy to count the number of cells in each MAMS well or locate cell bodies. A clear extension of the methodology is the use of exogenous or endogenous probes or reporters to provide pre-MS subtyping of cells. For example, transfection of cells with fluorescent probes could simplify rare cell detection within a population. Any spatially localized analytical technique capable of sampling from a surface is readily adapted to provide additional information on analyzed cells. Vibrational microscopy, a non-destructive profiling method, could be used to generate further information on cellular contents. Additional MS experiments are also possible, if performed in the correct order. Unlike MSI, the data sets are easily combined based on the unique cell location, greatly simplifying data fusion. For sample preparation, we expect to see FACS utilized in more powerful and efficient seeding setups. Precise deflection of cell-containing droplets would allow placement of suitable numbers of cells at evenly spaced intervals. Combined with appropriate molecular biology and pharmacology tools, interactions between different cell types could be assayed, as described earlier.

Finally, an intriguing aspect of MALDI-MS is that only a small fraction of the cell is consumed for analysis. Material remaining on the substrate is available for subsequent, follow up analysis by tandem MS or other methods on the same cell. The prospects are especially exciting for the integration of MALDI-MS-based profiling with orthogonal analytical and biochemical approaches. High-throughput MALDI-MS could provide a non-targeted, label-free profile of thousands of cells within a population. Utilizing multidimensional analysis on such a data set would facilitate the selection of individual cells that are representative of a given subclass. Focusing subsequent assays on the characteristic cells would reduce the number of analyses required to practically characterize an entire population. For instance, preselecting cells with MALDI-MS would greatly enhance the effective throughput of CE or single-cell transcriptomics by targeting cells that provide the most information on the population composition.

### OUTLOOK AND CONCLUDING REMARKS

Mass spectrometry is an information-rich analytical technology, positioned at the forefront of single-cell metabolomics, peptidomics, and proteomics. Progress thus far has been impressive. Current-generation instruments display exquisite sensitivity for the multiplexed, label-free measurement of hundreds of biomolecules from cellular samples. With careful sample preparation, analyte separation, and/or labeling, relative and absolute quantitative MS analysis of single cells becomes feasible. Issues with single-cell investigations arise from sampling, during the transition from organism to the instrument. Manual sample manipulation is suitable for detailed analysis of a small subset of cells; however, this sampling approach is less applicable for the characterization of large-scale cellular heterogeneity in complex structures. Automatic profiling of an entire tissue section by MSI can collect spectra from thousands of cells, but has not solved issues related to
matrix effects and subdividing cell contents. In contrast, representative populations of dispersed cells may be seeded on surfaces for microprobe-based MS analysis. By physically separating cells, MALDI matrix application can be optimized to improve analyte extraction and limit matrix effects from nearby cells, allowing the identification of rare individuals within a population. Sample throughput is enhanced over MSI, albeit at the cost of locational context within the native tissue. Each method offers a unique set of performance characteristics that are suitable to approach a given biological question.

Beyond more advanced instrumentation, a key shortcoming to the methods discussed herein is their limited utilization outside of MS research groups. Mass cytometry is gaining momentum as an alternative to flow cytometry by providing rapid, quantitative assessments of hundreds of antigens at a rate of thousands of cells per hour. These targeted methods, together with label-free MS analyses, greatly enhance the capabilities of SCMP-MS for discovery and hypothesis-driven investigations. Wider acceptance of single-cell MS technologies as practical analytical methods will broaden the breadth of questions addressed by SCMP-MS and facilitate its further integration with more routine genomics and transcriptomics approaches. Streamlining the workflows and simplifying data interpretation will encourage further acceptance by a wider multidisciplinary user base.

Willard Quine once said, “Physics investigates the essential nature of the world, and biology describes a local bump.” The advent of single-cell MS created an opportunity to explore changes in “local bumps” at a finer resolution than ever before. Through interdisciplinary investigations, we are beginning to discover the low-abundance cellular minorities in homogeneously bulk populations of cells that may cause drastic phenotypic changes. Sampling techniques that provide high throughput, high spatial and/or temporal resolution, and broad molecular coverage enable the determination of individual cellular properties while discriminating between unusual cell profiles and statistical noise. The body of work produced in SCMP, aligned with results gathered with transcriptomics and genomics, allows detailed understanding of changes occurring in individual cells during normal and pathological states, with promising applications in medicine.

■ AUTHOR INFORMATION

Corresponding Author
*jseweddle@illinois.edu

ORCID

Jonathan V. Sweedler: 0000-0003-3107-9922

Notes

The authors declare no competing financial interest.

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