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Single Cell Neurometabolomics

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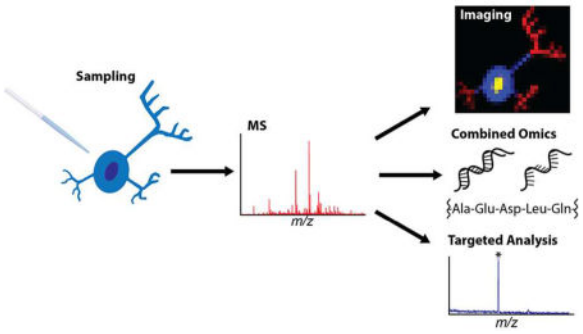
Abstract

Metabolomics, the characterization of metabolites and their changes within biological systems, has seen great technological and methodological progress over the past decade. Most metabolomic experiments involve the characterization of the small-molecule content of fluids or tissue homogenates. While these microliter and larger volume metabolomic measurements can characterize hundreds to thousands of compounds, the coverage of molecular content decreases as sample sizes are reduced to the nanoliter and even to the picoliter volume range. Recent progress has enabled the ability to characterize the major molecules found within specific individual cells. Especially within the brain, a myriad of cell types are colocalized and oftentimes only a subset of these cells undergo changes in both healthy and pathological states. Here we highlight recent progress in mass spectrometry-based approaches used for single cell metabolomics, emphasizing their application to neuroscience research. Single cell studies can be directed to measuring differences between members of populations of similar cells (i.e., oligodendrocytes), as well as characterizing differences between cell types (i.e., neurons and astrocytes), and are especially useful for measuring changes occurring during different behavior states, exposure to diets and drugs, neuronal activity, and disease. When combined with other -omics approaches such as transcriptomics, and with morphological and physiological measurements, single cell metabolomics aids fundamental neurochemical studies, has great potential in pharmaceutical development, and should improve the diagnosis and treatment of brain diseases.

Graphical abstract



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Introduction

Cellular heterogeneity is integral to most central nervous system physiological processes, including memory, neuronal network formation, function, and cellular homeostasis. Distinct cell types, such as neurons, astrocytes, and oligodendrocytes, are intertwined at the cellular scale. As a result, tissue measurements that provide information on an average set of metabolites in a region may not reflect the actual levels found in the cells of interest. Within the brain, even closely located cells can have distinct cellular and synaptic connections, and protein and metabolite profiles; these differences can be important for function. Investigation of chemical cellular heterogeneity is an expanding field of inquiry, with relevance to understanding the mechanisms of both health and disease.[1–4]

Explorations of heterogeneity at the level of the transcriptome, proteome, peptidome, lipidome, and metabolome provide key insights into brain function and dysfunction on the cellular level.[5] Single cell metabolomics is of particular interest to neuroscience due to the diverse functions of neuronal cells that are correlated to the small-molecule metabolite profiles of cell types and disease states.[6–8] The metabolites of interest range from amino acids, classical neurotransmitters, fatty acids and lipids, and small peptides, to a variety of precursor and intermediate molecules. The ability to detect and quantify this diverse set of molecules enables an enhanced understanding of neurochemical pathways and dysfunction related to disease. Each of the available measurement approaches has unique advantages and disadvantages that must be considered when adapted to the level of an individual cell. The challenges of single cell measurements involve cell isolation, sample processing, the metabolomics measurement process, and the integration of the data with other disparate datasets.

We begin with a discussion of single cell sampling techniques, followed by the mass spectrometry (MS)-based approaches that are suitable for single cell neurometabolomics, including a brief overview of some other single cell methods. Next we turn our attention to highlighting specific applications of metabolomics to single cell investigations, concluding with some thoughts about the future of the field.

Sampling for single cell analysis

Proper sampling is the prerequisite for a successful chemical analysis, especially when collecting volume-limited samples like single cells. First, careful attention needs to be paid to sampling accuracy so that only the selected individual cells or cellular components of interest are isolated. Second, although it is difficult to perform sampling without co-isolation of any extracellular content, effort should be made to minimize the effects of these interfering materials. Additionally, cell metabolism quenching or stabilization should be

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performed whenever applicable so that the samples to be measured reflect the real metabolic status of normally functioning cells.[9,10]

Sampling techniques can vary from manual manipulations to automated methods. Manual sampling can be a simple and convenient means to isolate cells and collect samples. Experience and good practice directly affect sample quality and are integral to ensuring experimental success. Manual isolation of large neurons from invertebrates, e.g., *Aplysia californica*, has been reported using surgical scissors, sharp tungsten needles, and fine-tip glass capillaries.[11–13] Manual sampling of smaller neurons and even subcellular organelles is also possible.[14–16] Micromanipulators and glass pipettes have been utilized collectively to isolate fluorescently labeled mammalian neurons from brain slices. For example, the use of a patch clamp/MS-based platform enabled the direct correlation of electrophysiological recordings to the metabolite content of single cells from rodent brain slices.[17] Recently, *in situ* microsampling from live single cells in developing *Xenopus laevis* embryos eliminated the need for dissection and cell isolation, addressing the technical gap between live single cell analysis and comprehensive untargeted metabolomics.[18] Another recent study demonstrated the use of fluid force microscopy, a modification of atomic force microscopy, to collect live-cell extracts for MS-based metabolomic analysis.[19]

Two sampling methods that require less manual handling use microscopy-guided approaches to sample cells, laser capture microdissection (LCM) and optical trapping (OT). In LCM, cell- or region-specific physical features of a target sample area are visually identified using a microscope, and then the cell(s) are removed via laser surgery. LCM has been used to isolate neurons from various brain structures, including the cortex, cerebellum, suprachiasmatic nucleus, and pituitary.[20–23] In OT, the cell is moved by a laser under the gradient force present between the high-intensity region of a focused light beam and the cell itself. Our group developed an approach that combines OT with capillary electrophoresis (CE), sampling single neurons for downstream indolamine and catecholamine measurement through fluorescence.[24] Taguchi et al.[25] demonstrated successful trapping of synaptic vesicles in a hippocampal neuron using an infrared laser, supporting the feasibility of using OT to manipulate subcellular features.

Microfluidic devices enable cells to be isolated and sampled using a variety of approaches, as reviewed recently.[26,27] Due to the ability to reduce fluidic volumes to the size of cells and control the laminar flow in microfluidic devices, in most cases cells can be transported one-by-one through the device. Oil droplet-based single cell isolation has been accomplished with microfluidic devices, in which individual cells are contained in a stream of droplets and segregated by the immiscible solvent from other cell-containing droplets.[28,29] Some microfluidic devices use a pneumatic membrane valve to control the passage of individual cells and isolate them from others.[30] Selected neurons have been cultured in a capillary, allowing efficient collection of cell release for follow-up MS characterization.[31]

While less commonly used for single cell metabolomic studies, fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS) also serve as efficient methods to select single cells of interest. FACS often is based on the interaction between a fluorescently labeled antibody and marker expressed on the surface of target cells. The

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fluorescently labeled antibodies are added into a cell suspension, and the cells in the suspension are sorted based on their fluorescence signal and other properties, e.g., size. Multiple research groups have used FACS to sort different types of cells in various brain regions for mRNA and protein analysis.[32,33] MACS relies on magnetic beads coated with an antibody, streptavidin, or other molecules that can specifically interact with proteins on target cells. After cell binding to coated magnetic beads, a magnetic field is applied so that only targeted or unwanted cells are retained and separated from other cells. In one example, MACS was used to sort cells and generate cultures of mammalian neuronal restricted progenitors, which later differentiated into neurons.[34] Proper sample collection is important for most measurements and becomes even more crucial as sample sizes are reduced to the single cell level. With single cell metabolomics, preserving the endogenous state of metabolites contained within the cells and minimizing their distortion becomes critical. A common strategy is to keep cells in their original microenvironment as long as possible and shorten the time spent on operations that can interrupt and change ongoing cellular activities.[2] For some techniques, such as microfluidics and electrophysiological recording, isolated cells can be placed and cultured under conditions closer to their physiological state. When cell culturing is not feasible, other approaches can be used, e.g., shock freezing and rapid cellular activity quenching using cold organic solvents.[35,36]

Characterization Methods in Small-Volume Neurometabolomics

Large-scale metabolomic measurements oftentimes use a number of preprocessing and sample conditioning steps, such as salt removal or extraction, to isolate specific analyte classes. These processes increase the robustness of the measurements; however, they often result in sample loss. Thus, to enhance metabolite detection, most single cell metabolomic methods usually uses less sample cleanup.

After the cells are isolated and prepared, the chemical measurements are performed. The two most common metabolomic approaches are CE or liquid chromatography (LC) coupled with MS and nuclear magnetic resonance (NMR). However, because NMR requires larger sample volumes and masses than single cells, NMR-based metabolomic studies are more commonly performed with tissue samples or bodily fluids, and so are not covered here.

MS is the most commonly used technique in small-volume metabolomics, due to its low detection limits and high chemical information content.[37] For single cell measurements via MS,[38] we have organized the discussion according to the ionization method used, as it often dictates other aspects of the measurement.

Electrospray Ionization (ESI) MS

Samples are typically introduced to an ESI source by direct infusion or following a separation. Much of the single cell work done via ESI-MS has involved CE–MS. CE, which separates molecules based on their electrophoretic mobility, offers the advantages of high separation efficiency and low sample volumes, and is well suited to characterizing many of the charged molecules in a single cell. For thorough perspectives on CE–MS-based metabolomics, the reader is directed to two recent reviews.[39,40]

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Neurometabolomic analyses using CE–MS have been used to characterize a broad range of metabolites, such as nucleotides and nucleotide derivatives,[41] and neurotransmitters.[42] Nemes et al.[6] profiled and quantified metabolites contained in single, freshly isolated and overnight cultured *A. californica* neurons and found a significant change in the metabolome between the two sample preparation methods for B1 neurons. They noted the presence of over 300 cell-related ion signals, and identified a fraction as relevant metabolites. Employing a whole-cell patch clamp technique following electrophysiology measurements, Aerts and colleagues[42] analyzed approximately 1–3 pL of sample extracted from individual rat thalamic cells, correlating the metabolic profiles of single cells to electrical activity. An advantage of CE–MS is that the separation can be tailored to allow characterization of a broad range of analytes over a wide dynamic range.

CE–MS- and LC–MS-based measurements offer a number of advantages, including the ability to characterize complex, large dynamic-range samples. While a disadvantage is that they tend to be lower throughput compared to direct MS measurements, usually only a few cells per hour, there have been reports of higher-throughput methods for CE–MS. One of the more recent examples is multi-segment injection, which can increase throughput by several fold.[43] The capillaries can also be embedded in devices to allow higher-speed separations. Ramsey and colleagues[44] fabricated a microchip for automated analysis of single human erythrocytes, which combined on-chip cell lysis, cellular constituent separation, and ESI-MS detection. Their method enables automated, real-time CE–MS analysis, and has the additional benefit of being relatively high-throughput at 12 cells per minute, as compared to traditional CE–MS methods.

Additionally, direct infusion into an ESI-MS platform has been accomplished for metabolomic analysis. Modification of the ESI interface for improved single cell analysis has been also reported. Wei et al.[45] recently introduced one such modification to extend the analysis time of small-volume samples. Instead of a continuous electrospray, the electrospray was pulsed and synchronized to the frequency of the mass analyzer. They were able to extend the analysis time of a picoliter sample to several minutes, allowing the collection of tandem MS (MS/MS) spectra of multiple compounds from a single sample. Moreover, this method requires no or minimal sample dilution. As a result, low-abundant molecules at zeptomole levels in single HeLa cells were detected. The benefits of such a development are two-fold: the reduction of detection limits due to low sample dilution, and the ability to identify unknown molecules using MS/MS.

In another example using ESI, Gong et al.[46] inserted a tungsten probe into target single cells for metabolite enrichment, then immediately positioned it at the MS inlet with voltage applied. With the help of a solvent spray, metabolites enriched on the probe could be detected with about 30-fold increased sensitivity when compared with a traditional method in which enriched analytes are first eluted and then subjected to nanoESI-MS. The same group has more recently expanded this technology to be compatible with droplet-based microextraction.[47]

Probe-based ESI was also designed to enable *in situ* single cell metabolite analysis. The Yang group[48,49] fabricated a three-component integrated probe, composed of a dual-bore

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quartz needle for sampling, a silica capillary for solvent delivery, and a nanoESI emitter (Figure 1).[48] Once positioned into single cells of interest, cellular content was directly guided into the emitter for *in situ* real-time analysis. This introduced minimal disruption to the original cell conditions. The average analysis speed of 3 min per cell allowed a large number of cells to be analyzed within a short time period.

These probe-based ESI approaches are capable of *in situ* analysis, and extracting the contents of living cells within their native biological environment. In addition to tissue cells, progress in the analysis of live single cells in biofluids and subcellular compartments through ESI-MS has also been reported. Hiyama et al.[50] developed an ESI-MS method to measure lipids and metabolites in single tumor cells circulating in patients' blood. The individual tumor cells, sorted by flow cytometry, were introduced into a nano-tip, and then subjected to in-tip super-sonication for homogenization and MS analysis. In another example, fluorescence probes were used to reveal the location of mitochondria in live single HepG2 cells, which were aspirated into a fine tip with ionization solvents and analyzed by ESI-MS.[51]

Matrix-Assisted Laser Desorption/Ionization (MALDI) MS

Direct single cell measurement via MALDI MS analysis has a long history, especially for peptides within individual cells.[52] MALDI MS offers many advantages for single cell metabolomics, including good tolerance for salts, simple sample preparation, and attomole detection limits with little sample consumption.[53,54] One caveat is that MALDI matrix interference can limit the observation of many metabolites. A few early MALDI MS-based studies revealed neuropeptide profiles in single neurons of invertebrates such as *A. californica, Lymnaea stagnalis, Periplaneta americana*, and *Cherax destructor*.[52,55–59] Many novel neurohormones in single invertebrate neurons have been discovered by MALDI MS.[60,61]

These earlier studies isolated the cells manually. With the advent of improved automated sampling and instrumentation, the application of MALDI MS has been extended to larger numbers of smaller cells. Ong and coworkers[62] dispersed rat pituitary cells on a microscope slide, and recorded the coordinates of individual cells, with the locations used to perform automated MALDI MS measurements of thousands of targeted cells. They successfully determined major subpopulations of pituitary cells and even found rare cells containing unique features in the neuropeptide mass range. In a follow-up study, cell heterogeneity within islets of Langerhans and between different regions of the pancreas were investigated using a similar method.[63]

In addition to peptides, lipids are also well suited for single cell MALDI MS because this mass range is not affected by matrix interference. Comi et al.[64] improved the throughput of single cell MALDI MS analysis by developing an open-access software package that enables image-guided single cell MALDI MS. After acquiring whole-slide microscopy images, the software uses point-based registration to direct laser shots only to slide locations containing a single cell. Following MALDI MS screening of a large cell population, rare or representative cells with signals of interest, lipids in this case, were further analyzed by Fourier transform-ion cyclotron resonance (FT-ICR) MS for high-resolution detection

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(Figure 2A).[64] This work also points to an important trend: improvements in time-of-flight (TOF) mass analyzers and development of interfaces that couple MALDI with FT-ICR and Orbitrap MS, which have greatly improved the data obtained from tissues and cells by providing accurate mass measurements (resolution < 5ppm).[65–67]

Secondary Ionization Mass Spectrometry (SIMS)

The throughput of MALDI MS is exciting, but as noted earlier, matrix interferences tend to limit its application for metabolomics measurements. An alternative and widely used matrix-free ionization technique is SIMS, in which a focused beam of primary ions bombards the sample surface, producing secondary ions from the analyte. While fragmentation of large biological molecules is common, SIMS works well for smaller analytes, including many metabolites and lipids. SIMS also has a higher spatial resolution, oftentimes smaller than a micron, which can enable subcellular profiling.[68,69] While most SIMS instruments do not obtain MS/MS information, newer instrumentation has become available[70–72] that greatly facilitates metabolite identification. Though SIMS is often used in lipidomic[73] and targeted analyses, as described further below, it is less common for comprehensive metabolomic analyses. A 2013 study by the Vickerman group[74] evaluated TOF-SIMS specifically for metabolomics and demonstrated its ability to complement more traditional methods of metabolomic analysis. The authors found that LC–MS/MS and SIMS spectra for the same metabolite standards tended to correspond well, suggesting it is possible to use LC–MS databases to aid in metabolite identification with TOF-SIMS. In the aforementioned study by Comi and colleagues,[64] SIMS was used in combination with MALDI MS. Image-guided SIMS and MALDI MS analysis uncovered small molecules, lipids, and peptides from the same individual cell, allowing the benefits of both high-throughput and high-molecular information content from selected cells, as illustrated in Figure 2B.[64]

SIMS has been successfully employed to investigate lipid and fatty acid distribution and abundance changes in tissue section and single cells.[75,76] As one example, distribution of vitamin E on the cell membrane of individual *A. californica* neurons was uncovered using TOF-SIMS.[77] Metabolite visualization through SIMS has also been extended into the 3D spatial world. Lipids and lipid fatty acid side chain distributions were revealed as a function of depth in isolated *X. laevis* oocytes, perhaps the first demonstration of 3D biomolecular imaging in a real biological system.[78] Our group has also implemented both MALDI and SIMS together with optical microscopy to perform high-throughput profiling of neurons.[62,79]

Other matrix-free ionization approaches

Several other ionization methods have been applied to individual cell measurements, including direct desorption electrospray ionization (DESI), laser ablation electrospray ionization (LAESI), and nanophotonic ionization, all with different measurement capabilities and advantages. At present, DESI is rarely applied to individual cell measurements,[80,81] partly because of spatial resolution and detection sensitivity issues, but we expect its application to increase.

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LAESI is a variant of MS that is useful for metabolomics; a mid-IR laser ablates a sample *in situ*, the plume from which is then intercepted by an electrospray for MS analysis.[82] LAESI allows *in situ* metabolite sampling directly from tissue and single cells. In addition, LAESI has a clear chemical background that is analogous to ESI. The technique has been shown to distinguish subcellular features in lipid profiles between the vegetal and animal poles of *X. laevis* eggs, in addition to being used to detect 52 small metabolites and 92 various lipids.[83] Despite being a relatively new ionization method, LAESI has the potential to make great contributions to small-scale metabolomics as the technology improves. The *in situ* capabilities in particular make this an ideal technique for experiments on cultured brain tissue, though without single cell resolution.

Nanophotonic ionization using nanoscale silicon materials to perform surface-assisted laser desorption/ionization MS enables matrix-free analysis of small molecules with low detection limits. The architecture of the silicon structures has been shown to have a significant effect on ionization efficiency[84] and sensitivity.[85] The technique's applicability to single cell analysis was demonstrated by the Vertes lab;[86] using nanophotonic ionization to analyze individual yeast cells deposited on a nanopost array chip, they achieved detection of analytes in the zeptomole range. The same group demonstrated the technique's metabolomic capabilities by screening more than 600 metabolite standards.[87]

Mass spectrometry imaging (MSI)

The approaches discussed thus far are used to assay a user-selected sample, such as a single cell, for its metabolomic content. Methods that sample a location on a surface can be adapted to an imaging mode where the sampling location is rastered across the surface, building an image of a sample one location at a time (there are other modes of acquiring an image, but this is the most common). This approach has been used since the 1960s with SIMS, and was adapted to MALDI and other ionization methods to form the collection of measurement techniques known as MSI. MSI has been used to study tissues down to the individual cell level, although when the cells are not separated, the information obtained will be from a blend of cells; in the brain, for example, astrocytic processes, oligodendrocyte extensions, and nerve terminals can be within the same micron field of view of a focused laser. Thus, even when achieving a several micron resolution, below the size of many cells, multiple cells may be sampled. However, MSI has the advantage of measuring many metabolites while simultaneously preserving spatial information.

Differences between MSI and conventional MS measurements of a cell involve data collection and processing. For example, during MALDI MSI, the spectrum at each location across the sample is recorded along with the (X, Y) coordinates, defining the location being analyzed. Signals from different mass-to-charge ratios (*m/z)* at each analyzed position are extracted and processed to construct the distribution map of each *m/z*, with intensity shown in a false color scale. An area of rapid growth is the development of approaches to improve the spatial resolution. Common spatial resolutions achieved with MALDI MSI are between 10 and 200 μm, which hinders accurate measurement of single cells from tissues. However, higher spatial resolutions at 2 to 10 μm have been reported,[65,88,89] with recent work from the Spengler group[90] achieving 1.4 μm resolution while detecting lipids, peptides, and other

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metabolites within individual cells. An alternative way to improve the spatial resolution of MSI is to use SIMS instead of MALDI. SIMS has a reported spatial resolution of 50 nm to 1 μm,[38] with different molecular information obtained across this range. In all cases, there is a tradeoff between detection limits and resolution; as the sample probe diameter is decreased, the number of molecules within the probe decreases. For example, decreasing a probe beam from 50 μm to 5 μm should reduce the number of molecules 100-fold; at some point, the measurement becomes limited by instrumental detection limits and not the probe beam geometry, a situation common in SIMS with its smaller probe diameters.

Single Cell Metabolomic Analyses

Enhancing metabolite coverage in single cells

Metabolites within an individual cell account for a vast array of functionalities, but the analytes are present over many orders of magnitude, at levels ranging from millimolar to less than nanomolar. One issue is that nanomolar levels of a molecule in a picoliter volume sample are present at zeptomole amounts, which are not usually characterizable. Because of the analyte amounts, an individual cell's metabolomic measurement uncovers fewer metabolites than larger-volume measurements. As highlighted in the previous sections, the specific fraction of the metabolome characterized depends on sample properties as well as the figures of merit for the measurement approach used. Our group has demonstrated metabolic heterogeneity in neuronal cell types from both *A. californica* and rat, and more than one hundred chemical features have been putatively identified with CE–MS,[7,42] and we have been able to detect metabolic differentiation between subcellular regions of single cells.[91] Onjiko et al.[92] used CE–MS to distinguish more than 80 molecular features in *X. laevis* embryonic cells and were able to metabolically distinguish cell fates of the differentiated cells.

Efforts to improve metabolite coverage require enhanced sampling approaches; for example, the mismatch between collected sample volumes and the injected volumes used in CE needs to be addressed. One approach is to inject more analyte onto the capillary via analyte stacking. This involves concentrating metabolites into a narrow zone at the entrance to the separation capillary. Liu et al.[41] used a large-volume sample stacking method to improve the detection of endogenous nucleotides in an individual *A. californica* neuron. They detected 51 fg of material, which is 200 times less than in previous studies. CE–MS stacking has also been used for detection of drugs and metabolites in urine samples, with limits of detection as low as 2 ng/mL.[93]

Additional improvements to metabolome coverage can also made by developing metabolite-specific CE–MS conditions. Although in some cases, methods that successfully increase metabolome coverage are not initially demonstrated on individual cells, these approaches could be adapted to small-volume sample measurements. For instance, in one study, a fused silica capillary was coated with poly-(N,N,N',N')-tetraethyldiethy-lenetriamine, N-(2-hydroxypropyl) methacrylamide to shorten separation time and enhance signal intensity for metabolites suffering from poor negative-mode MS response.[94] The authors reported detection of 87 metabolites in orange juice and 142 metabolites in red wine, demonstrating the method's applicability to expand the detectable metabolite pool.

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Sheathless CE–MS interfaces have also been reported to enhance metabolite coverage. Ramautar et al.[95] used a porous tip sprayer as a CE–MS interface to eliminate the need for a sheath liquid. Due to the improved ionization efficiency, they were able to detect three times more molecular features, improve sensitivity by two orders of magnitude, and expand metabolome coverage for polar ionogenic metabolites in human urine samples. Other recent works have also demonstrated the use of sheathless CE–MS in enhancing anionic metabolite profiling, although still in bulk samples as opposed to single cells.[96,97] While metabolome coverage is inherently limited in single cell analysis due to the low concentration of many small molecules in single cells, such advancements will prove useful when paired with improvements in CE–MS sensitivity.

Mapping the single cell metabolome

As highlighted above, one goal of MSI is to characterize the metabolites that are present, and their localization and abundance. In a rat cerebral ischemia reperfusion model, MALDI MSI was used to obtain diverse metabolite profiles in both single mammalian cells and brain sections. The density, intensity, and distribution of over 30 metabolites covering nucleotides, cofactors, sugars, amino acids, lipids and carboxylic acids were revealed.[98] Another study used quantitative MALDI MSI together with CE–MS and visualized a spatiotemporal behavior of adenylates and NADH regulated by ischemia reperfusion.[99] Application of SIMS for single cell measurements from tissue slices has been reported. Karlsson et al.[100] used high-resolution SIMS to probe the biochemical changes *in situ* altered by neurotoxin-induced brain pathology. In addition to endogenous metabolites, MSI can be used to study drugs, nutrients, and their related metabolites in cells, toward a fundamental understanding of *in vivo* distribution and metabolism of drugs.[101,102] Fernandez-Lima and colleagues[102] employed a 3D TOF-SIMS system to investigate the delivery and distribution of a chemotherapeutic drug in single cells with 250 nm spatial resolution. Chemical maps of molecular markers indicated the presence of the drug on the cell surface instead of the nucleus. In addition to profiling metabolites at cellular or subcellular resolution across tissue slices, MALDI and SIMS imaging have also been used directly on dispersed cells. Lanni et al.[72] created a MALDI/SIMS dual source instrument with 5–250 μm resolution. Using the hybrid instrument, they imaged cultured *A. californica* neurons and neuronal networks (Figure 3).[72] Recently, an atmospheric pressure MALDI MSI instrument was reported with 1.4 μm lateral resolution, over 100,000 mass resolution, and an average below 2 ppm accuracy.[90] The authors were able to localize 220 peptides, lipids, and metabolites in cilia and oral grooves in a single *Paramecium caudatum*. The highest spatial resolution MSI involves nanoSIMS; Lovrić et al.[103] employed nanoSIMS and correlated the MS data with transmission electron microscopy. By combining these two techniques, they measured the distribution and dynamics of newly synthesized dopamine across single nanometer neuroendocrine vesicles in neuron-like cells.

Targeted metabolite analysis

The goal of many -omics measurements is untargeted analysis; however, there are a number of important targeted metabolic single cell assays. While -omics scale measurements lead to insight into function and disease states, complementary targeted analysis provides critical information for many studies. There are also some metabolites that are difficult to

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characterize using traditional MS-based metabolomic methods. For instance, D-amino acids have been established as being present at biologically relevant concentrations and, in the case of D-serine, shown to have functional significance in the human brain.[104] Many metabolites, such as both L- and D-amino acids, can be detected after derivatization, followed by CE with laser-induced fluorescence (LIF) detection. Thus, CE-LIF is effective for chiral measurements from single neurons and other cells.[105–107]

Metabolite-specific probes have also been employed. Fluorescent probes designed for cysteine, glutathione, and hydrogen peroxide were used in a microfluidic electrophoresis platform for single cell analysis; 12 fmol, 840 amol, and 0.49 amol of cysteine, glutathione, and hydrogen peroxide, respectively, were quantified through a multichannel fluorescence detector.[108] Heath et al.[109] successfully utilized immunoreactivity-based probes, implemented them in a barcode chip, and achieved simultaneous quantitation of metabolites such as cyclic adenosine monophosphate, cyclic guanosine monophosphate, glutathione, and proteins in single cells.

An important targeted approach for studying selected metabolites within single cells is electrochemical detection. Easily oxidizable neurotransmitters, such as dopamine and norepinephrine, are well suited for electrochemical-based approaches as they offer nano- to micromolar detection limits, and can be used to assay selected cells, cellular release, and individual exocytosis events, as reported in several recent studies. Mao and colleagues[110] fabricated a carbon fiber microelectrode and achieved the first measurement of exocytosis of endogenous ascorbate from cultured adrenal chromaffin cells via single-cell amperometry. A gold-ring pipette electrode was used to successfully detect catecholamines released from individual ingle chromaffin cells.[111] In addition, to measure release from living cells, electrochemistry has been used to uncover single cell spatial behavior. Ewing and coworkers[112] packed over 60 thin film microelectrodes into an array, and measured chemicals released from different locations over a single cell surface during exocytosis. Further details on many of the recent advancements in the use of single cell electrochemistry for neuroscience research can be found in a variety of recent reviews.[113–116]

Integrating metabolomics with other -omics-scale measurements

Investigations in the different -omics disciplines have greatly enhanced scientists' understanding of specific biological systems, such as DNA, RNA, proteins, peptides, metabolites, and lipids. A trend but also a challenge is to integrate the -omics fields of study, gain access to complimentary information from different classes of molecules, and obtain a more comprehensive insight on processes occurring in biological systems as a whole. While these interdisciplinary efforts have rarely been extended to individual cells, examples of small-volume combinations have been reported. Knolhoff et al.[117] used CE–MS to measure metabolite changes in small caudal hippocampal samples in mice with a mast cell mutation. They also employed gene array technology to collect transcriptome data from the same region. Combined analysis of the transcriptomic and metabolomic data led to the notation of pathways altered due to the loss of mast cells, which were evident from both analyses. In another example, Zebrafish embryos were subjected to metabolomic analysis using NMR and LC–MS, and transcriptomic analysis using DNA microarray.[118] Changes in metabolite

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and mRNA levels were continuously tracked throughout five embryo developmental stages at 12 time points. Correlating the transcriptome to the metabolome at concurring and adjacent time points revealed metabolism dynamics and underlying gene expression under temporal change, in a manner that improved upon traditional methods, which typically yield dichotomous results (such as up- and down-regulation).

Future Perspectives

As one of the important -omics used in systems biology, metabolomics applies analytical tools coupled with bioinformatic approaches to determine the identity, abundance, flux, pathways, and even distribution of small molecular metabolites in biological systems. Metabolites are changed by a pathogenic status; accordingly, their comprehensive study helps to uncover mechanisms of action and facilitate the diagnosis of devastating diseases such as cancer. In brain cancer, for example, several investigations of serum and tumor tissue metabolomics using NMR or MS have been performed for tumor diagnosis, grade determination, type differentiation, etc.[119–121] With further improvements to single cell measurement approaches, which promise to lead to new insights in the diagnosis and treatment of brain tumors, it becomes feasible to understand the cell-by-cell transition from a normal to cancerous state.

The potential of single cell metabolomics has been recognized at the national level, as both metabolomics and single cell analysis were early foci of the National Institute of Health's Common Fund Initiatives.[122] While a number of approaches have been outlined here that provide details on the chemical content of individual cells, this is a rapidly evolving measurement field, and further enhancements to performance are needed. Future technologies will boost the ability to probe the single cell brain metabolome. Obvious advances that are still needed include more complete metabolome coverage, the addition of details on metabolite localization through MSI,[123] improvements in small-scale sampling,[91] hyphenated detection modalities such as MS and vibrational spectroscopy. Perhaps the most important goal involves integration of the cellular "omics," including transcriptomics, peptidomics, and proteomics. As our understanding of how gene transcription, translation, protein modifications, and metabolites interact to determine cell phenotype and fate, the integration of the data obtained will allow the most complete and accurate understanding of individual cells and their role in health and disease. The resulting single cell information will require improved approaches for processing the unique and large individual cell datasets. Collaborative efforts have resulted in shared data and common data formats for MSI, but these standards have yet to be applied to single cell data. Metabolomics as a field is rapidly evolving and being applied to precision medicine.[124]

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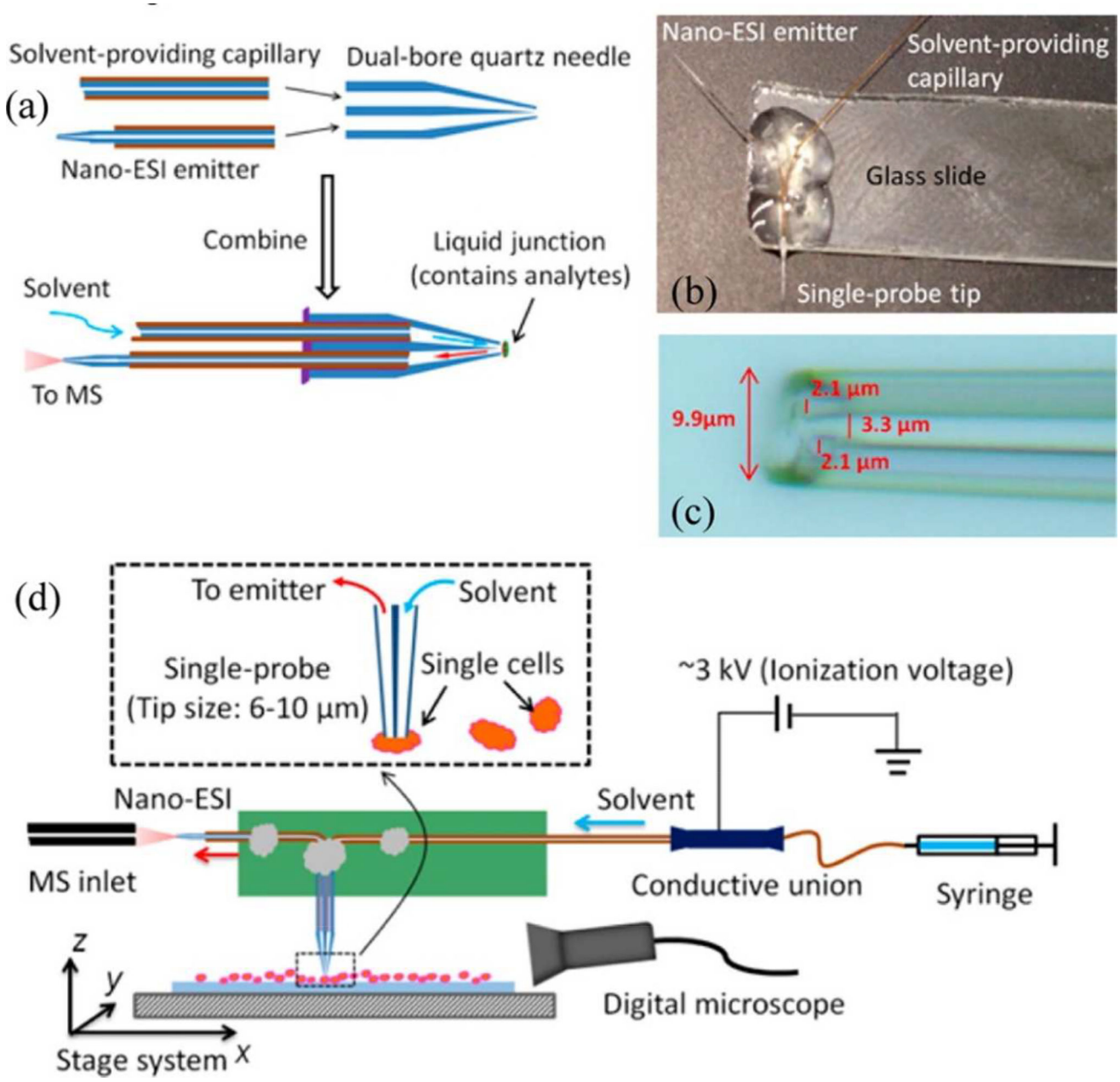

Figure 1.

(a) Fabrication steps and structure of a Single-probe for ESI-MS; (b) photograph of a working Single-probe; (c) 40× magnification of the Single-probe tip with measurements obtained from the calibrated digital microscope; (d) setup schematic of an *in situ* real-time single cell MS analysis. Reproduced with permission from Pan, N., Rao, W., Kothapalli, N. R., Liu, R., Burgett, A. W. G., and Yang, Z. (2014) The single-probe: A miniaturized multifunctional device for single cell mass spectrometry analysis, Anal. Chem. 86, 9376–9380 (ref.[48]). Copyright 2014, American Chemical Society.

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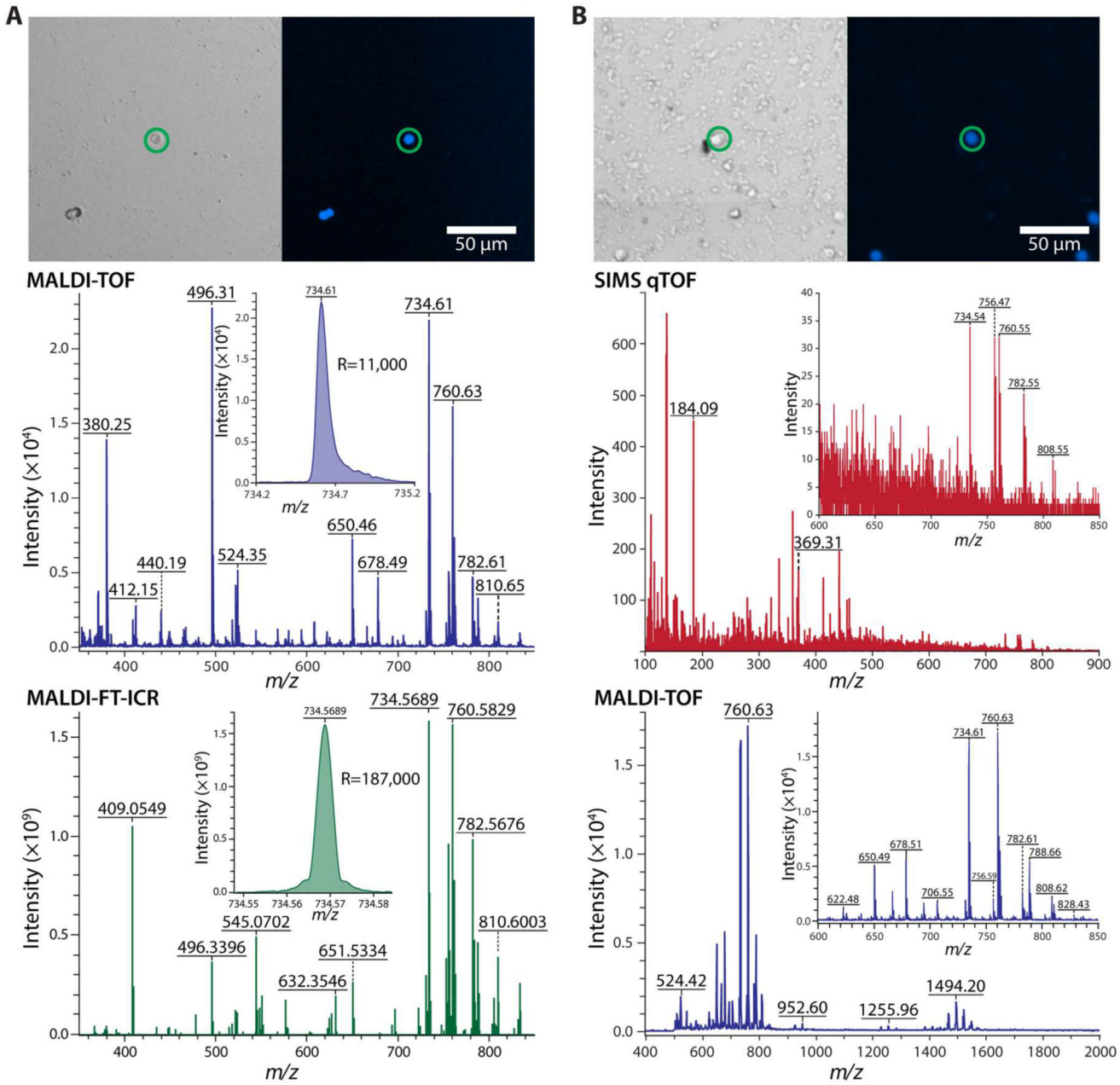

Figure 2.

Sequential analysis of the same individual cell with two separate MS systems. Once a cell has been located in the optical image (top), its location remains fixed through multiple analyses, allowing two instruments to probe the same set of selected cells. (A) MALDI-TOF MS (middle) of a rat cerebellum-derived cell followed by MALDI-FT-ICR MS (bottom). MALDI-TOF MS provides high throughput screening of thousands of cells to highlight rare or representative individuals. Here, FT-ICR MS provides high mass resolution and high mass accuracy for unequivocal elemental composition of selected cellular contents. (B) SIMS profiling (middle) followed by MALDI-TOF MS (bottom) with a DHB-coated, SCN-derived cell. SIMS provides information on small molecule compounds while MALDI-TOF

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MS effectively detects larger species, such as lipid dimers and peptides. The insets demonstrate overlap of intact lipid coverage from each modality. Reproduced with permission from Comi, T. J., Neumann, E. K., Do, T. D., Sweedler, J. V.: microMS: A python platform for image-guided mass spectrometry profiling. J. Am. Soc. Mass Spectrom. 28, 1919–1928 (2017) (ref.[64]). Copyright American Society for Mass Spectrometry 2017, with permission from Springer.

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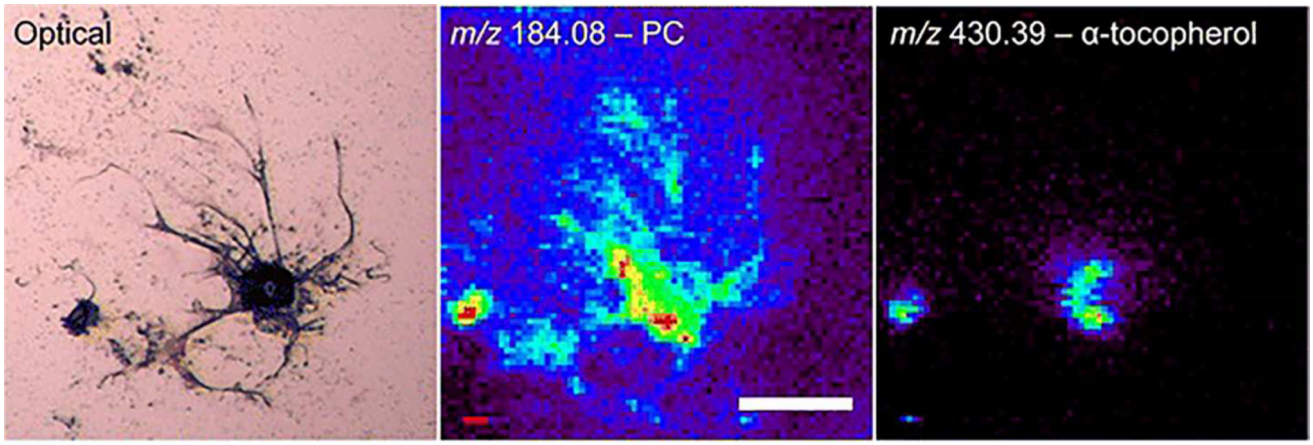

Figure 3.

Distribution of metabolites in cultured *A. californica* buccal neurons was performed by C_{60}-SIMS imaging. Cells were cultured on silicon tiles and stabilized with glycerol. C_{60}-SIMS ion images revealed that phosphocholine (PC) and α-tocopherol showed different locations in neurons. Cell bodies and processes are apparent in the PC image (*m/z* 184.08, 0–200 counts), while α-tocopherol accumulated almost exclusively within the cell bodies (*m/z* 430.39, 0–50 counts). Adapted with permission from Lanni, E. J., Dunham, S. J., Nemes, P., Rubakhin, S. S., and Sweedler, J. V. (2014) Biomolecular imaging with a C60-SIMS/MALDI dual ion source hybrid mass spectrometer: Instrumentation, matrix enhancement, and single cell analysis, J. Am. Soc. Mass Spectrom. 25, 1897–1907 (ref.[72]). Copyright 2014, American Society for Mass Spectrometry, with permission from Springer.