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REVIEW

Small-Volume Analysis of Cell–Cell Signaling Molecules in the Brain

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Modern science is characterized by integration and synergy between research fields. Accordingly, as technological advances allow new and more ambitious quests in scientific inquiry, numerous analytical and engineering techniques have become useful tools in biological research. The focus of this review is on cutting edge technologies that aid direct measurement of bioactive compounds in the nervous system to facilitate fundamental research, diagnostics, and drug discovery. We discuss challenges associated with measurement of cell-to-cell signaling molecules in the nervous system, and advocate for a decrease of sample volumes to the nanoliter volume regimen for improved analysis outcomes. We highlight effective approaches for the collection, separation, and detection of such small-volume samples, present strategies for targeted and discovery-oriented research, and describe the required technology advances that will empower future translational science.
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INTRODUCTION

Multicellular animals are unbelievably complex and show multidimensional specialization and cooperation at the cellular level. In vertebrates, hundreds of distinct cell types are known (Alberts *et al*, 1994) and the behavior of each individual cell is carefully regulated to meet the needs of the organism as a whole under specific environmental conditions. Alteration and breakdown in the regulatory pathways affect cell function and ultimately the survival of the entire organism. Understanding the molecular mechanisms of cell-to-cell communication has thus become an active research area in both fundamental science and drug discovery.

The nervous system, and the brain in particular, have an astounding capacity to store, process, and route information. Interneuron communication is a foundation for brain function and occurs primarily by chemical mechanisms acting via fast-wired (synaptic) or slow-volume transmission through diffusion in the extracellular fluid of the extracellular space (Agnati *et al*, 2010). Many different kinds of molecules transmit information between the cells in the nervous system. Structurally, the signaling molecules used by animals range in complexity from simple gases and small organic molecules to large peptides (Cooper and Hausman, 2009). Perhaps, the most well-investigated transmitters present within the central nervous system (CNS) are amino acids (Glu, Asp, Gly, Tau, dSer, and GABA) and biogenic amines derived from aromatic amino acids such as serotonin, dopamine, and epinephrine. Closely related and metabolically associated with the well-known monoamines are trace amines (phenolamines, tyramine, and octopamine), which are found in mammalian brain and peripheral nervous tissues at substantially lower concentrations (Burchett and Hicks, 2006). Lipid messengers such as endocannabinoids are involved in short- and long-term synaptic plasticity throughout the brain (Katona and Freund, 2012; Pineiro and Falasca, 2012). The gases, nitric oxide (NO) and carbon monoxide, have unique physicochemical properties that enable them to diffuse across cell membranes, thus affecting receptors and other targets located hundreds of microns from the release site (Stern and Filosa, 2013). Neuropeptides are the largest and most functionally and structurally diverse class of cell-to-cell signaling molecules. Adding complexity, the same transmitters may act via wired and volume transmission (Agnati *et al*, 2010). The large assortment of chemical messengers and the diversity of their chemical and physical properties require a complex suite of measurement techniques and integrated approaches for dissecting the

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molecular mechanism of cell-to-cell communication in the brain.

Understanding a multifaceted biological process such as chemical signaling often begins with the measurement and identification of essential molecular players. Measuring signaling molecules in the brain continues to be an involved analytical task. One of the complexities stems from the fact that the organizational structure of the brain has an intricate hierarchical configuration, with bidirectional communication taking place between its cellular components. Two major brain cell types, neurons and glia, further classified in at least 10 subtypes by morphology and function, are interconnected into this intricate architecture. In the cerebral cortex alone, there are roughly 125 trillion synapses, and a single synapse may contain 1000 molecular-scale switches (Micheva *et al*, 2010). A sample of brain tissue as small as a few microliters includes extracellular matrix and infiltrating vasculature and whole blood, in addition to neural cells (Figure 1), with overlapping but different chemical compositions. Equally fascinating as this morphological complexity is the chemical complexity and myriad forms of intercellular communication in the nervous system (Agnati *et al*, 2010). Neurons and glia have regulated secretory pathways (Stern and Filosa, 2013) where bioactive peptides and low-molecular-weight transmitters coexist in a cell and can even be released together. Cotransmission, however, is plastic and sensitive to environmental stimuli and pathological conditions. Neurons have been shown to store and release different subsets of transmitters at different synapses (Samano *et al*, 2012). In addition, neurons and glia can release exosomes containing receptor subunits, structural proteins, mitochondrial DNA, and RNA in a process regulated by synaptic activity (Lachenal *et al*, 2011). Moreover, re-specification of transmitters may occur i*n vivo* in some neurons after synapse formation under environmental factors such as light changes (Dulcis and Spitzer, 2008).

Another challenge in the analysis of signaling is the relatively low effective concentrations of bioactive compounds present in the CNS. This is true for peptides, small transmitters, and gases that elicit their biological effects via volume transmission (Agnati *et al*, 2010) and act at nanomolar or lower concentrations far from their release sites (van den Pol, 2012). Neuropeptide binding affinity to cloned receptors is nearly 1000× higher than that of classical transmitters, often acting far from the synaptic cleft. In contrast, classical transmitters can be found at high local concentrations in the synaptic cleft (Barberis *et al*, 2011), but have short half-lives in the brain's extracellular space (Merighi *et al*, 2011). These facts suggest that the temporal dynamics and concentration profiles are different among distinct classes of bioactive compounds. The effects of peptide release develop relatively slowly and are long lasting because they are often mediated by G-protein-coupled receptors that are not spatially restricted to the synaptic structure, whereas fast amino acid transmitters operate via ionotropic receptors directly at synaptic sites on the millisecond time scale (van den Pol, 2012).

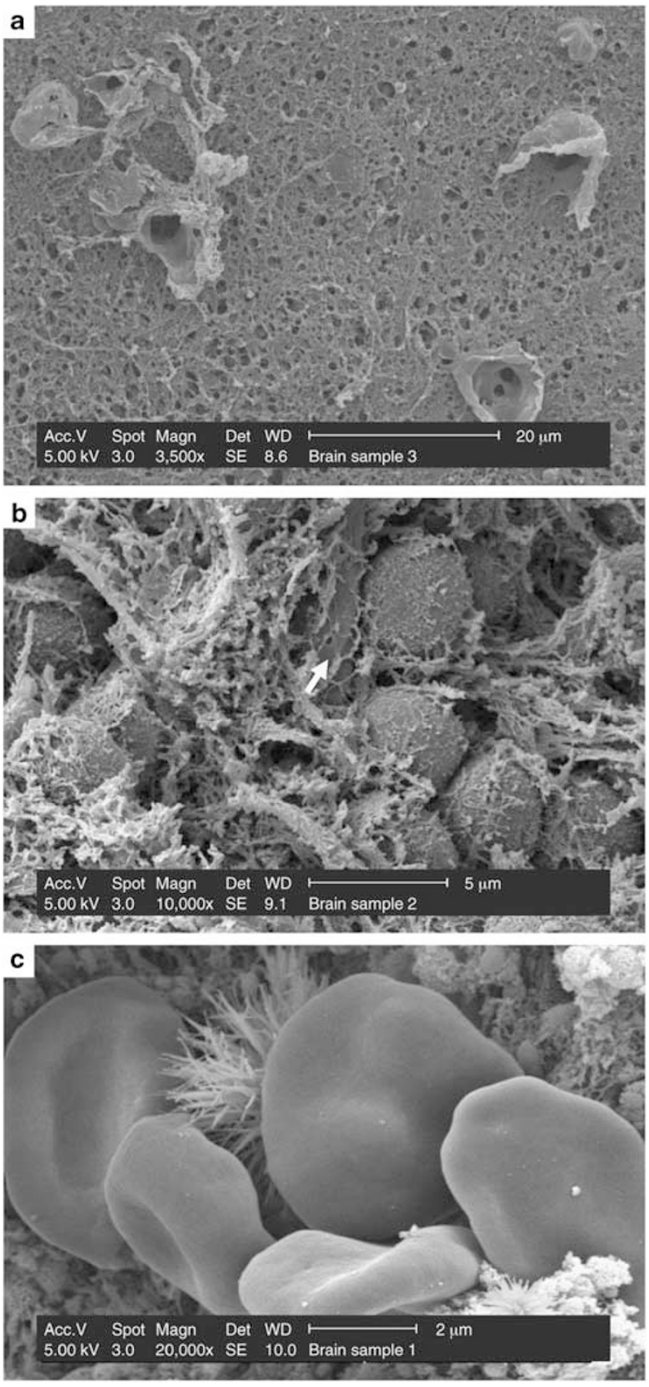

Figure 1. Scanning electron micrograph of rat brain tissue. (a) Torn blood vessels are seen on the surface of a tissue slice and (b) individual cortical neurons are seen among the extracellular matrix. Elongated structure near neurons (arrow) is apparently a vasculature element; (c) erythrocytes seen among salt crystals on the dry fractured surface of a brain slice.

Given this structural and chemical complexity, measuring signaling compounds in the brain with relevance to specific experimental paradigms continues to challenge analytical science. Although most chemically information-rich measurement approaches require large samples representing hundreds of thousands of cells, nanoliter and picoliter

volume analyses offer many advantages for bioanalytical measurements in the nervous system. Decreasing the tissue sample size to the near cellular scale reduces the structural and chemical heterogeneity of the sample, improves the selectivity of measurement, and offers greater potential to correlate sample chemistry with biological function and pathology.

The tools and techniques that are able to isolate and characterize cellular and subcellular samples from heterogeneous cell populations continue to advance in their capabilities. The tremendous growth in the availability of commercial instruments with ever-increasing sensitivity and performance opens up many new avenues of study. However, the sampling aspects of nanoliter volume analyses still require considerable skill and practice, making them less user-friendly and more difficult to move into a core research facility. At this time, individual cell and other small-volume assays appear better suited to multidisciplinary collaborations between technologists and neuroscience laboratories, with these studies aided by moderate cross-training by each group.

In the following sections, several approaches that are well suited for characterizing nanoliter-volume samples for their chemical content are described, with particular attention to measurement, quantification, and structural characterization of neurotransmitters, neuromodulators, and metabolites in individual neurons, small populations of neurons, cerebral–spinal fluid, cellular releasates, and brain tissue.

DOWNSIZING WITH SUCCESS

Sampling Strategies for Microanalysis

Unlike DNA/RNA-based molecular techniques, bioanalytical measurements of small-volume samples lack a significant signal amplification step. Clever sampling methods, careful sample handling, precise measurement, and sensitive detection are paramount to a successful analysis. Multiple sampling techniques exist for nanoliter volume analysis of peptides and small molecules, as reviewed recently (Cecala and Sweedler, 2012). Not surprisingly, obtaining a pure sample of a tissue region, single-cell, subcellular area, or organelle—with minimal sample dilution and degradation during extraction, cleanup, and analysis—can be difficult. Sampling strategies can be categorized as manual and automated, whereas samples can be classified as solid and liquid.

Manual sample isolation, considered by some as time-consuming and tedious, is one of the more common approaches and is often unavoidable. For example, sampling of live individual neurons and subcellular neural regions from ganglia, nerves, or other anatomical structures is carried out primarily by hand. In our group, hundreds of samples of individual, live invertebrate, and mammalian nerve cells, and even individual dense core vesicles, have been manually isolated under stereomicroscopic observation from intact ganglia with sharpened tungsten carbide dissection needles (Fuller *et al*, 1998; Rubakhin *et al*, 2003, 2006; Romanova *et al*, 2004, 2007, 2012; Ye *et al*, 2008). In another approach, individual cells or miniature defined morphological regions have been removed from frozen or fixed tissue sections for neuropeptide analysis (Neupert *et al*, 2012).

The tools of choice in cell-scale manual sampling are sharpened tungsten needles, super-fine-tip forceps, or small scissors with a micron-scale diameter tip. Glass capillaries can be pulled using pipette pullers to make fine, sharp tips that can be used to cut through soft tissue that might adhere to a cell of interest (Neupert *et al*, 2012). Alternatively, a pulled glass capillary can be altered by breaking the tip to create a tip diameter slightly larger than the cell of interest, burned to round the edges, and then used to aspirate gently a selected cell for subsequent cleanup and analysis steps while ensuring minimal carryover from surrounding tissue and media. Connecting a glass micropipette to a vacuum line via a length of tubing helps in manipulating the pressure within the capillary for optimal cell capture and transfer. Manual cell isolation can be quite precise (Figure 2), but its success depends on the researcher's fine motor skills. Manual sampling for neurotransmitter measurements in miniature *Drosophila* brains have been successfully performed from whole freeze-dried flies (Berglund *et al*, 2013). Freeze-drying dehydrates tissue under reduced pressure by sublimating the water from the tissue directly into the gas phase (Lowry, 1953), thereby preserving tissue structural integrity and morphology.

The manual isolation skills and associated sample manipulations involved in nanoliter volume analyses require significant training to master; once mastered, these tools allow cell-to-cell neurochemical variability to be probed.

Several non-contact isolation methods present more complex but less labor-intensive alternatives. The combination of tapered glass capillary tips with an optical trap have been used to introduce individual intact pinealocytes into a fused silica capillary for the analysis of indolamines and catecholamines by capillary electrophoresis (CE) with laser-induced native fluorescence (Cecala *et al*, 2012). Laser capture microdissection (LCM) has emerged as a powerful, high-resolution and high-accuracy tool for isolation of specific cell types from tissue sections (Emmert-Buck *et al*, 1996). More recently, combined with expressed fluorescent tags, LCM has been used to isolate small neurons from *Drosophila* brain sections (Iyer and Cox, 2010). Popular uses of LCM include DNA, RNA, and proteomics applications (Espina *et al*, 2006). Isolation of individual live cells from suspensions shows promise for applications of high-throughput single-cell and even organelle microanalysis. A variety of methods, including flow cytometry and microfluidic and array-based platforms, allow cell targeting based on fluorescent tags or even metabolic activity (Nilsson *et al*, 2009; Suzuki *et al*, 2009).

Once isolated, a solid biological sample may either be analyzed using techniques such as direct cell/tissue

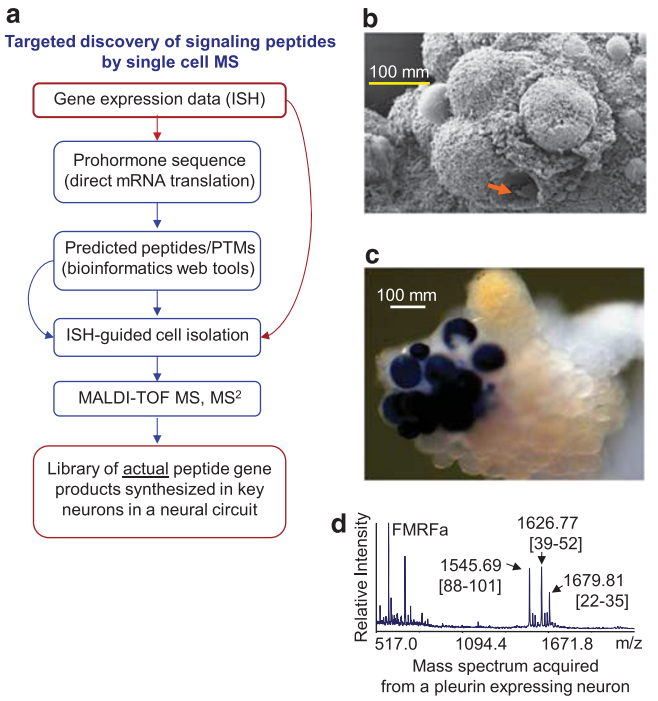

Figure 2. Targeted approach for characterization of neuropeptides. (a) Tried and true workflow for discovery of novel neuropeptides by single-cell mass spectrometry (MS). (b) Scanning electron micrograph of the *Aplysia* buccal ganglion fixed and imaged after manual isolation of a giant motoneuron for MS analysis. The arrow points to where an isolated neuron was located in a live ganglion before isolation. The image illustrates the precision of manual cell isolation but is not related to the data in (c) and (d). (c) *In situ* staining showing the localization of pleurin prohormone to a cluster of neurons in the pleural ganglion of *Aplysia*. The image is courtesy of L Moroz and has been modified from Moroz *et al* (2006). (d) Actual single-cell mass spectrum obtained on one of the pleurin-expressing neurons shown in (c). Peptides predicted from the pleurin prohormone sequence are labeled by their amino acid positions on the prohormone. FMRF, peptide is also labeled; MALDI-TOF, matrix-assisted laser desorption-time-of-flight; ISH, *in situ* hybridization staining; PTMs, posttranslational modifications.

matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) or analytes can be extracted and measured using a variety of approaches. Individual cells may yield a sample volume of nanoliters, as in the case with large neurons such as human dorsal root ganglion neurons ($\sim$100 µm) and even larger invertebrate neurons, but typical cells may be $<$20 µm in diameter and have a volume in the picoliter range. Optimization of analyte extraction from cells or tissue must often be carried out in a compound-specific manner, taking into account analyte stability and solubility in a given extraction solvent. Cold extraction solutions are typically used for small-molecule analysis, as enzymatic activity is generally reduced at lower temperatures, whereas neuropeptides are most often extracted in highly acidic conditions. Temperature and extraction solution additives have been optimized by several groups for mammalian cell metabolomic analyses (Sellick *et al*, 2009).

Sampling and measuring signaling molecules *in vivo* at their release sites in an intact brain is an important goal. Microdialysis is one of the most common *in vivo* sampling techniques for continuous monitoring of drugs and/or endogenous compounds in the extracellular fluid and has been used to study many areas of the brain in different model systems (Sharp and Zetterström, 2006; Behrens and Li, 2010). One advantage of this sampling technique is the reduction of protein in the dialysate, which eliminates the concern of neurotransmitter degradation due to enzymatic activity and streamlines the sample cleanup before analysis. Constant replacement of the sampling fluid with fresh perfusate allows coupling of this sampling method to microfluidic devices for further separation and analysis. As changes in the concentration of classical neurotransmitters in the extracellular space occur in milliseconds, several groups have worked to improve temporal and spatial resolution, enabling sampling on release time scales and in much smaller regions than traditional microdialysis. The Kennedy group (Wang *et al*, 2009) reported an inventive way to reduce dispersion along a sampling channel by introducing a segmented flow system into an online microdialysis setup. By creating discrete aqueous analyte plugs that are surrounded by an oil phase, diffusion broadening is limited to the droplet, and temporal resolution is improved overall. This technique has been used to study both amino acids (Wang *et al*, 2009) and acetylcholine (Song *et al*, 2012) from the rat brain. Furthermore, by coupling this segmented flow system to a push–pull perfusion system, Glu was detected with 7 s temporal resolution and a spatial resolution of 0.016 mm^2, an 80-fold improvement over traditional microdialysis (Slaney *et al*, 2011). The Ismagilov group (Chen *et al*, 2008) reported a lab-on-a-chip platform, the chemistrode, that consists of a droplet-based delivery system that stimulated single cells and re-formed the released solution into a segmented plug with high spatial resolution ($<$15 µm) and high temporal resolution (50 ms).

Following sampling, the fractionation/separation of analytes before their characterization often increases the confidence of analyte identification and allows greater numbers of analytes to be characterized from a sample. A particular problem inherent to separations of volume-limited samples is the ability to manipulate small volumes to introduce them into a compatible separation platform without sample losses or excessive dilution. An overview of specialized interfaces for sample introduction into micro-separation platforms is presented elsewhere (Cecala and Sweedler, 2012). Microseparations with a range of detection schemes, including fluorescence, electrochemical detection, and MS, allow sensitive and selective analyses of single-cell and other small-volume brain samples.

Microseparation Methods

Here we outline several separation approaches and highlight the characteristics that define their utility for small-volume neuroscience work. Although conventional liquid chromatography (LC) using a narrow-bore column is well suited for neurochemical analysis and is available in many core

facilities, its use for nanoliter-volume samples is less routine. The integration of liquid chromatographic separations into microfluidic platforms enhances sensitivity and rapid analysis of volume-limited samples (Rainville, 2011; Lin *et al*, 2012).

CE separations are easily scaled to sample volumes in the femtoliter to nanoliter volume range, show unsurpassed femtomole to zeptomole mass detection limits with appropriate detection modalities (Ramsay *et al*, 2009), consume miniscule volumes of buffer, are low cost compared with LC systems, and are more often used as a result (Guihen and O'Connor, 2010). Another benefit of CE, especially for neurochemical applications, is that separation is based on the differential migration of charged molecules; many of the neurotransmitters and neuromodulators in the nervous system are charged over a range of pH values, and thus can be separated from the cellular milieu under typical CE conditions. CE has been successfully used for discovering new serotonin metabolites (Squires *et al*, 2006), and characterizing the content of single cells (Nemes *et al*, 2012) and single vesicles (Omiatek *et al*, 2009). Chiral analysis of excitatory acids from the brain by CE has been reported (Wang *et al*, 2011; Wagner *et al*, 2012). In addition, CE has a potential for high-throughput analysis, and separation times as fast as 20 s have been reported for catecholamines, as well as a fourfold increase in sensitivity by using short separation capillaries with small inner diameters (Grundmann and Matysik, 2011). A sampling approach that further enhances the concentration sensitivity of CE separations is single-drop microextraction; analyte enrichment ranging from ~7000-fold for chiral amino acids and up to ~30 000-fold for fluorescent dyes has been reported (Alothman *et al*, 2012). CE is equally suitable for separation of neuropeptides that are zwitterions and whose overall charge is a function of the pH of its surroundings. Capillary isoelectric focusing can separate peptides based on their isoelectric points, and has been used for the characterization of crustacean neuropeptides (Zhang *et al*, 2012) and human cerebrospinal fluid (Ye *et al*, 2011).

The analysis of nonpolar compounds is possible through the use of a CE variant, micellar electrokinetic chromatography (MEKC). MEKC is a variation of electrophoresis that is optimized for the separation of electrically neutral analytes by the addition of an ionic micelle to the running buffer, with the effect similar to that of a stationary phase in an LC separation. MEKC has been commonly used for fast, high-efficiency separation of amino acid transmitters in microdialysis samples, as reviewed in detail elsewhere (Viglio *et al*, 2012).

Nanoliter and picoliter volumes permit separation of biological samples in microfluidic devices that are characterized by faster and oftentimes massively parallel performance. Implemented on miniaturized platforms that function according to the scaling rules (Livak-Dahl *et al*, 2011), microfluidic separations benefit from highly controlled transport of particles suspended in the fluids. The turbulence-free nature of microscale flow allows for the segregation of sample components and the formation of sharp gradients, which is highly useful for investigating concentration-dependent biological phenomena such as cell signaling (Mellors *et al*, 2013). As one example, microchip electrophoresis has recently been used to monitor neurotransmitter concentrations and blood–brain barrier permeability (Nandi *et al*, 2010).

Detection Platforms Compatible with Microanalysis

There are multiple sensitive detection methods that are suitable for nanoliter volume analysis. These include electrochemical, fluorescence, and mass spectrometric detection, which have been reviewed by Trouillon and co-workers, 2013). Electrochemical detection allows for the real-time, sensitive and quantitative analysis of biomolecules. Suitable for miniaturization and microfabrication, this method is very adaptable for measurements in small volumes, but is limited to electroactive compounds. Electrochemical methods for neurotransmitter detection include amperometry and voltammetry, which vary in sensitivity, and in chemical and temporal resolution. Electrochemical detection has been successfully implemented in analyses of nanomolar concentrations of biogenic amines from volume-limited samples such as individual fruit fly heads (Powell *et al*, 2005; Berglund *et al*, 2013), fly brain regions (Kuklinski *et al*, 2010), and individual fly larva brains (Fang *et al*, 2011).

CE has traditionally been used with fluorescence detection. In one example, a combination of CE with two-color fluorescence allowed the investigation of glycolipid catabolism in primary rat cerebella neurons with 500 ymol to 1 zmol sensitivity (Essaka *et al*, 2012). Laser-induced fluorescence (LIF) is the most sensitive detection technique used with CE (Pentoney and Sweedler, 1997); it can not only detect but quantify neurotransmitters in minute volumes of sample based on their retention time and area under the peak curve, and can be used to confirm analyte identity by spectral characteristics relative to known standards (Wise and Shear, 2006; Hatcher *et al*, 2008; Fossat *et al*, 2012). A dynamic range of nine orders of magnitude and 120 ymol sensitivity have been reported for glycosphingolipid metabolites (Dada *et al*, 2011). Applications of this approach to the analysis of single cells and small-volume biological samples have been reviewed elsewhere (Szoko and Tabi, 2010; Lin *et al*, 2011), with several examples highlighted in the following sections of this article.

MS permits the label-free identification of almost any analyte, can provide structural information for unknown analytes, and thus presents a detection method with the highest possible information content. MALDI MS and nanoelectrospray ionization (nanoESI) MS perhaps are the most suitable for microanalysis. In MALDI (Hillenkamp and Peter-Katalinic, 2007), analyte molecules are typically incorporated into organic matrix crystals that absorb light. The sample is then irradiated with a pulsed laser, causing

the vaporization and ionization process. The ionized molecules of interest are then measured using an appropriate mass analyzer, oftentimes, a time-of-flight system. With its salt tolerance, attomole sensitivity, minimal sample consumption, and ease of sample preparation, MALDI MS, has been successfully applied to a wide range of analytes, including lipids, oligosaccharides, nucleic acids, peptides, proteins, and other polymers. It has become an excellent approach for qualitative profiling of larger molecular weight signaling molecules such as neuropeptides (Li and Sweedler, 2008; Chen and Li, 2010). MALDI MS can be used for direct analysis of volume and mass-limited biological samples, in solid or liquid states and without purification, but occasionally the approach is paired with a separation platform off-line. High-throughput analyses of single cells deposited from suspension and picoliter-volume aliquots have been demonstrated using high-density microarrays for MS. This approach can anchor samples on 100 μm spots, which is equal to 250 sample recipients per cm^2, or 250-fold higher sample density than what is currently available from commercial sample plates for MALDI MS (Urban *et al*, 2010). Applications of MALDI MS to small-volume analyses of signaling molecules are discussed in the next section.

ESI is an effective detection method for CE separations because charged molecules eluting from the CE capillary can be ionized and introduced into the mass analyzer via ESI conditions. CE-MS (see Figure 3a) is widely used to measure and identify bioactive peptides (Ye *et al*, 2011), metabolites (Nemes *et al*, 2011, 2012; Nautiyal *et al*, 2012; Gholipour *et al*, 2013), classical neurotransmitters (Lapainis *et al*, 2009), and amino acids (Moini, 2013). Alternatively, microdialysis sampling can be directly coupled to ESI-MS via nanodroplet segmented flow for *in vivo* chemical monitoring of neurotransmitters, metabolites, and drugs in the live brain (Song *et al*, 2012). As another hyphenation solution, CE separations have been integrated into microfluidic devices that also serve as electrospray emitters, although sample loading for small-volume analysis remains a barrier to high-throughput analysis (Mellors *et al*, 2008; Sun *et al*, 2010; Lin *et al*, 2012). A combination of ESI-MS and LIF detection with CE is utilized for many small-molecule applications, including chiral amino acid analysis (Simó *et al*, 2010). MS confirmation of analytes detected using LIF is important; however, transferring a particular separation method from a CE-LIF instrument to one with MS detection is challenging because of LIF buffer incompatibilities with MS detection. Recent CE-MS work has shown that separations using smaller inner diameter capillaries (eg, 5 μm) were shown to be less affected by buffer composition (Grundmann and Matysik, 2011).

Quantification Approaches for Small-Volume Samples

Concentrations of signaling molecules in cells and the extracellular space are dynamic and defined by the balance between synthesis, release, reuptake, and degradation. Measurement methods that generate concentration- or mass-dependent responses may yield quantitative information based on comparisons of signal intensity between sample cohorts. When known compounds are involved, absolute quantitation is possible by using standards and appropriate calibration curves. Here, we outline several approaches that have worked well for assessing concentration changes in the smallest samples of brain tissues. The combination of CE-LIF, radioisotope labeling and radionuclide detection has been successfully applied to demonstrate synthesis, accumulation, and release of D-aspartate in neurons (Scanlan *et al*, 2010). The quantitation of biogenic amines has been performed by MEKC with electrochemical detection (Berglund *et al*, 2013). When quantitation of multiple or unknown bioactive compounds is desired, MS-based quantitation methods are effective because they do not require prior knowledge of sample composition. MALDI MS, in particular, is a flexible platform for measuring small samples, including those that are solid (eg, tissue and cells) or liquid (eg, extracts). In combination with stable isotopic labeling, this method has been used for both relative and absolute quantitation of peptides in single neurons and central nerves (Rubakhin and Sweedler, 2008).

Database Resources for Identification of Unknowns

The advantage of using microseparation methods combined with MS is the ability to identify the unknown and oftentimes unexpected components of the samples, either via accurate mass or a combination of mass and molecular fragmentation patterns. Most mass spectrometer manufacturers provide software to handle data analysis and compound library searching of both in-house libraries as well as external mass spectrometric public libraries. Several issues complicating the use of the freely accessible chemical databases include error propagation due to redeposition of spectral libraries between databases and erroneous stereochemical and covalent bond descriptors found when chemical identifiers such as InChI and SMILES are interconverted between structures and names (Williams *et al*, 2012).

Mass spectral databases such as the Human Metabolome Database (HMDB) (http://www.hmdb.ca/) (Wishart *et al*, 2009) and METLIN offer straightforward search options with ~8000 and ~25 000 compounds, respectively. Mass spectra for known compounds can also obtained through a search of common name, CAS, SMILES, or InChI. More advanced search parameters, such as structure-based searches capable of searching uploaded or MOL, SDF, CDX structure files or image files, can be used at the publicly accessible database websites such as ChemSpider (http://www.chemspider.com/) and Chemical Entities of Biological Interest (http://www.ebi.ac.uk/chebi/) (Hastings *et al*, 2013). In addition, METLIN offers a data analysis tool for LC-MS data sets called XCMS online. Users can upload data and after a few simple mouse clicks, be presented with

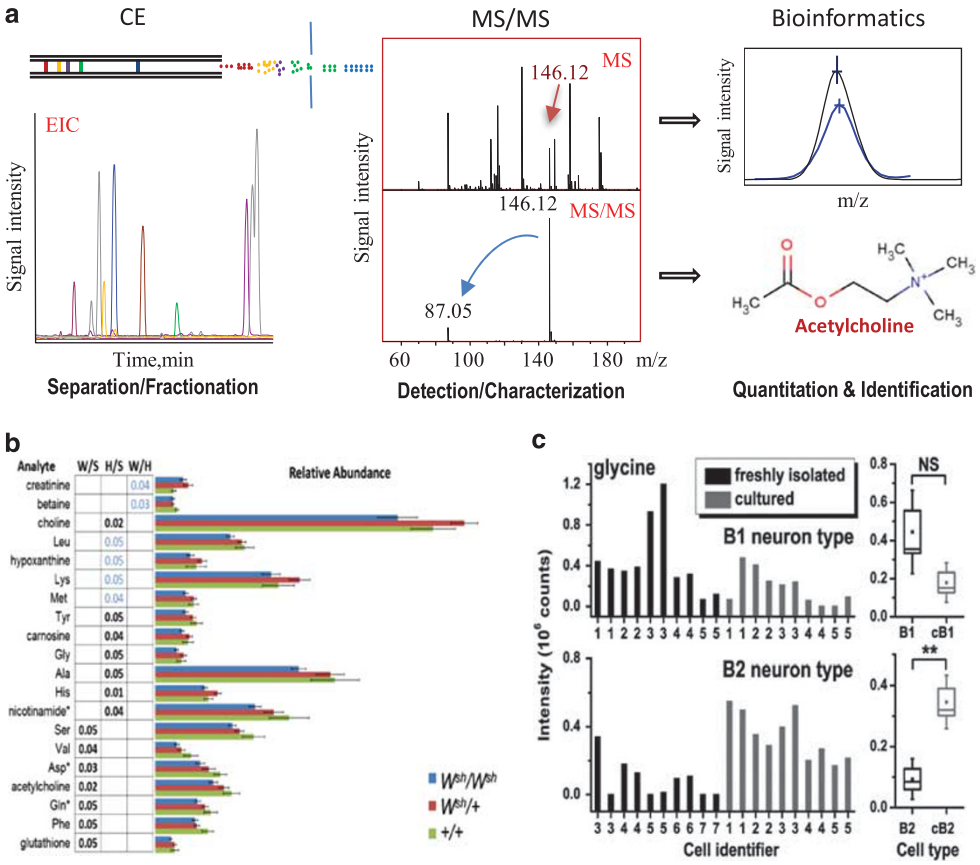

Figure 3. Small-volume metabolomics provides unmatched molecular information on the chemical content of nanoliter-volume samples. Here, a capillary electrophoresis-mass spectrometry (CE-MS) platform is used for qualitative and quantitative characterization of cell-to-cell signaling molecules. (a) Overview of a CE-MS analysis. CE fractionates complex biological samples, which are then introduced to the mass spectrometer via electrospray ionization. Following the measurement of the mass-to-charge ratio of each detectable compound, selected ions are fragmented, and the mass-to-charge of the fragments determined, oftentimes permitting the determination of primary ion structure *de novo*. Querying the MS data against appropriate chemical and mass spectral databases with the help of bioinformatics tools leads to the identification of analytes and chemical characterization of the sample. EIC, extracted ion electropherogram; *m/z*, mass-to-charge ratio; MS, mass spectrometry; MS/MS, tandem mass spectrometry. (b) Statistical results of the CE-MS metabolomics data from individual hippocampi from wild type +/+ (W) and mast cell-deficient W^{sh}/W^{sh} (S), $W^{sh}/+$ *(H)* mice. Even though there are only about 500 mast cells per mouse brain on average, their lack is correlated to profound neurochemical changes; a total of 20 distinct analytes consisting of amino acids, classical neurotransmitters, and nucleosides exhibit statistically significant differences in relative abundance among tested genotypes. Bars show the mean of normalized abundances measured in the biological replicates for each genotype; error bars represent standard error; *p*-values from the corresponding Student's *t*-tests are tabulated to the left. Statistically significant differences in the levels of these analytes ($p \leq 0.05$) are also supported by gene expression data. Metabolites associated with differentially expressed metabolic pathways identified by gene expression analysis are marked with black bold in the table. An asterisk indicates that the intensity is reflective of the second peak in the isotopic series due to its high concentration. WT (W), $W^{sh}/+$ (H), W^{sh}/W^{sh} (S). (Adapted with permission from Knolhoff *et al* (2013); Copyright 2013 American Chemical Society.) (c) Statistical analysis of the CE-MS metabolomics data from identified individual *Aplysia* neurons. The data show cell identity-dependent variations in the neuron chemistry under culture conditions. As one example, an inhibitory neurotransmitter glycine is accumulated in the B2 neurons only. Bars correspond to individual cells measured in technical duplicates. Square, box, and whisker represent statistical median, standard error, and confidence interval, respectively. NS labels statistically insignificant variations, and asterisk (*) and two asterisks (**) mark *p*-values below 0.05 and 0.005, respectively. (Adapted with permission from Nemes *et al* (2012); Copyright 2012 American Chemical Society.)

results showing statistics, chromatograms, and putative METLIN identities. A recent tool, MetaboSearch (http://omics.georgetown.edu/MetaboSearch.html) (Zhou *et al*, 2012), aids in MS-based metabolite identification through the searching of multiple MS databases (HMDB, Madison Metabolomics Consortium Database (http://mmcd.nmrfam.wisc.edu/) (Cui *et al*, 2008), METLIN, and LIPID MAPS (http://www.lipidmaps.org/) (Fahy *et al*, 2007)) simultaneously. Mass spectral databases typically link out to each other, as well as to KEGG (http://www.genome.jp/kegg/) (Kanehisa *et al*, 2012), PubChem (http://www.ncbi.nlm.nih.gov/pccompound) (Bolton *et al*, 2008), and others, with additional resources regarding a particular compound of interest. One exciting new website, Chemicalize.org (http://www.chemicalize.org/), offers an easy-to-use interface that provides most physical properties of a molecule and helps in deciding which approach to take for optimizing a separation.

The aforementioned methods for sampling, separation, measurement, detection, identification, and quantification

of biologically active compounds in femtoliter to nanoliter sample volumes are readily applicable for studying the CNS in animal models of brain disorders, and potentially also in human subjects. The following section describes specific applications that benefit the most from these small-volume workflows, and highlights less explored but promising approaches.

TECHNOLOGY ROADMAP TO CELL–CELL SIGNALING

Scaling sample volumes to the level of the cell opens a number of new opportunities for probing brain chemistry. The chemical complement of individual members of defined cell populations can be inventoried, compared, and qualitatively assessed to reveal biomarkers of physiological or developmental states. Disparate elements of neuronal circuits underlying complex behaviors or physiological processes can be characterized to find those elements that show the greatest change, even for surprisingly subtle behavioral perturbations. Dynamic biochemical alterations relevant to a disease state can be monitored. The literature reviewed below demonstrates that these areas are being explored in a range of animal models by small-volume analysis approaches.

Power of One: Single-Cell Analysis for Circuit and Pathway Characterization

The heterogeneity of a cellular population can result from stochastic gene expression, the deterministic nature of biochemical events occurring in each cell making up the population, and for cells such as neurons with a complex topology, their physicochemical environment, and cell connectivity. Because of the increased ability to study individual cells, a clearer understanding of the effects of the cellular surroundings has emerged, with the awareness that the cellular microenvironment contributes more to cell-to-cell variability than stochastic mechanisms can account for (Swain *et al*, 2002; Stockholm *et al*, 2007; Raj and van Oudenaarden, 2008; Snijder and Pelkmans, 2011). Of course, neurons within a network that have thousands of connections to other neurons are unique, especially as cells with distinct connections will then respond differently to activity.

Pioneered as a method for investigating electrical connectivity in the neurons, single-cell analysis acquired new dimensions with the advent of bioanalytical MS, as it offers unparalleled chemical information content. One of the more successful MS platforms for chemical profiling of single nerve cells in the circuit or pathway context is MALDI MS. Targeted analysis of neuropeptides in single cells or subcellular regions (Figure 2) greatly benefits from prior genomic information because limited sample amounts often are not sufficient for *de novo* structural characterization (Romanova *et al*, 2009). By knowing which genes are expressed in a cell of interest, a library of peptides encoded by the prohormones can be compiled and used as a reference for interpretation of spectra by peptide mass fingerprinting. Groundbreaking research, as reviewed by Li *et al* (2000), demonstrated that neuronal circuits can be studied by MALDI MS at the single-cell level, and their chemical content correlated to animal behavior and to specific physiological states. Combined with molecular biology and electrophysiology, single-cell MS is effective for examining neuronal architecture, and for delineating neuronal circuits that regulate physiology and control of instinctive and learned behaviors (Jarecki *et al*, 2010; Jing *et al*, 2010; Vilim *et al*, 2010). Investigations of signaling peptides and their involvement in neural plasticity, learning, memory, complex behavior, and various physiological functions at the level of the individual cell have been successful in invertebrate models (Jimenez *et al*, 1994; Christie *et al*, 2010; Li and Smit, 2010; Neupert and Predel, 2010; Neupert *et al*, 2012). Given the simplicity of their nervous systems and the accessibility of identifiable neurons, invertebrates provide unsurpassed single-neuron resolution and coupling to molecular mechanisms. Currently, single-cell MS is widely being used to characterize novel prohormones (Romanova *et al*, 2012) and differential prohormone expression (Romanova *et al*, 2007), map the cellular localization of peptide transmitters (Jimenez *et al*, 2006; Neupert *et al*, 2007, 2012; Yew *et al*, 2009), and analyze neuropeptide release at identified release areas (Fan *et al*, 2011).

Low-molecular-weight transmitters are also actively measured in defined neuronal networks mediating complex behavior, often with fluorescence detection. Using CE-LIF, the Gillette group (Hatcher *et al*, 2008) quantitatively measured how 5-HT and related indole metabolites fluctuate with hunger state in well-characterized serotonergic neurons of the feeding motor network of the predatory sea-slug *Pleurobranchaea californica*. Silver and collaborators (Nautiyal *et al*, 2012) found that a significant contribution of serotonin to the hippocampal milieu is associated with mast cell activation, which contributes to behavioral and physiological functions of the hippocampus, and later compared the transcriptome and the CE-MS defined metabolome of hippocampi from normal and mast cell-deficient mice (see Figure 3b) (Knolhoff *et al*, 2013).

Although neuropeptide transmitters and neuromodulators can be characterized in well-defined neuronal circuits, gaseous transmitters such as NO are typically measured using indirect approaches. Various fluorescent NO indicators (Ye *et al*, 2010), electrochemical sensors (Arafah *et al*, 2013), or reporters of NO synthase activity (Ye *et al*, 2008; Potgieter *et al*, 2010) have been used to track NO in defined neuronal populations and regulatory pathways. The analytical techniques enabling NO detection at single-cell levels include fluorescence microscopy, CE with LIF detection, and electrochemistry (Ye *et al*, 2008). Using fluorescence confocal microscopy, CE, MS, and pharmacological tools, we verified the NO production in specific identified *A. californica* neurons (Ye *et al*, 2010). NO-selective amperometry has been used for dynamic measurements of NO in

injured leeches, demonstrating that NO production is regulated through the endocannabinoid system (Arafah *et al*, 2013).

Freedom of Exploration: Untargeted Analysis of Physiologically Important Compounds on a Small Scale

While targeted analysis of bioactive compounds is initiated by a biologically rationalized hypothesis, untargeted analysis is often driven by technological development. High-throughput platforms that comprehensively interrogate the genome, transcriptome, proteome, peptidome, and even secretome in entire organisms or select tissues are certainly desirable in discovery-oriented studies. An important advantage of such approaches is that *a priori* information on the identity of the compound(s) to measure is not required, and one can measure changes in entire classes of compounds such as RNA, DNA, proteins, and metabolites. Untargeted analyses have the potential to uncover never-before-documented chemical relationships that may lead to novel pharmacological targets.

High-throughput, 'omics protocols are not easily adapted for small samples, in part, because the limited amount of material available is not sufficient to achieve systematic analysis of unknowns. Nevertheless, information-rich detection techniques, often powered by MS, push the boundaries of analytical possibilities and initiate new, biologically relevant inquiries. In a study combining CE separation, MS identification, and chemometric quantification, we investigated cellular heterogeneity among physiologically well-characterized identified neurons of *A. californica*. Out of 300 detected species, 144 were structurally identified, and 50 quantified, revealing surprising differences, not only between neuronal types but also among individual neurons of the same type (Nemes *et al*, 2011). These observations contribute new evidence about the physiological differences in neuron phenotypes. In a follow-up study, we explored how culturing conditions affect the metabolic profile of individual neurons and found statistically significant changes in the levels of amino acids and small transmitters linked to cell culture conditions (see Figure 3c) (Nemes *et al*, 2012). With proper experimental design, small-volume exploratory measurements of biological samples may lead to the discovery of biomarkers for disease states. Using CE-MS, Ibanez *et al* (2012) undertook a non-targeted examination of the metabolic differences in cerebrospinal fluid samples from subjects having different cognitive status related to Alzheimer's disease progression and revealed possible metabolic disease progression biomarkers.

In Vitro Manipulation of Neural Cells: Lab-on-Chip Platforms

In recent years, on-chip systems have been used for neuroscience applications, bringing with them a unique set of capabilities for studying cell-to-cell communication on a small scale (Pearce and Williams, 2007; Wang *et al*, 2009; Taylor and Jeon, 2010; Croushore and Sweedler, 2013). Lab-on-chip devices readily address the issue of cell-to-cell heterogeneity, as they can recreate a network or individual cells of interest for study in a defined environment *in vitro*. Small culture chamber volumes (μl to nl) reduce the dilution encountered in dish-based cultures, improving release collection and leading to better detection of cell-to-cell signaling molecules. In addition, the advent of microvalves (Unger *et al*, 2000) and complex fluid handling systems has allowed greater control and manipulation of the extracellular space such as the application and removal of chemical stimuli and nutrition factors. Finally, these systems can be coupled to a range of information-rich detection platforms.

What neuroscience questions can be best answered using the unique and growing capabilities of microfluidic lab-on-chip devices? These devices have been used to culture a wide range of cells, from mammalian cortical (Kunze *et al*, 2011) and hippocampal (Millet *et al*, 2007) neurons to molluscan *A. californica* neurons (Croushore *et al*, 2012). Of course, when culturing using an *in vitro* microenvironment, conditions such as temperature, waste removal, and media replenishment must be taken into account. Several groups have reported designs that improve cell survival *in vitro* (Tourovskaia *et al*, 2005; Millet *et al*, 2007; Millet and Gillette, 2012). Various compartmentalized device designs have enabled studies on axonal transport, injury, and regeneration (Hosmane *et al*, 2010; Taylor *et al*, 2010; Majumdar *et al*, 2011; Taylor and Jeon, 2011). Compartmentalized devices have been used for single molecule tracking of retrograde axonal transport by observing quantum dot-labeled nerve growth factor in endosomes within cultured dorsal root ganglion neurons (Zhang *et al*, 2010). Innovative microfluidic models of the *in vitro* blood–brain barrier have drawn attention due to their potential to provide insights into its role in the CNS and drug delivery. These systems, which oftentimes consist of brain endothelial cells cocultured with neurons and/or astrocytes, have been shown to mimic effectively the *in vivo* environment and regulate the transport efficiencies of rat endothelial cells (Lippmann *et al*, 2011; Abbott *et al*, 2012; Prabhakarpandian *et al*, 2013). Microfluidic models promise to advance our basic understanding of the biological signaling in the blood–brain barrier physiology, knowledge that can have implications in drug discovery research.

Another successful application of lab-on-a-chip devices is the measurement and manipulation of cellular release to understand the conditions that govern it. Control and fluid handling capabilities are achieved by the incorporation of microvalves, which permit selective stimulation of cellular networks or single cells and regulation of the stimulation duration. Several groups have instituted precise chemical stimulations of cells into devices (Sabounchi *et al*, 2006; Jo *et al*, 2007). More recently, our group designed a system that used microvalves to control the stimulation of

low-density cultured neurons within a device (Croushore *et al*, 2012). A difference in the onset of neuropeptide release was observed for two different chemical stimulations by increasing the duration of chemical stimulation to the cellular network maintained in the device.

A number of lab-on-a-chip applications have focused efforts toward improving signaling molecule detection, including sample preparation on chip (Wei *et al*, 2010), surface modifications (Jo *et al*, 2007; Zhong *et al*, 2012), and on-chip detection (Dishinger *et al*, 2009). The Kennedy group (Dishinger *et al*, 2009) reported an automated approach that quantified insulin release from 15 single islet cells using an on-chip electrophoresis channel and an immune assay for detection. We reported a microfluidic system with C18 functionalized channels to capture and quantify neuropeptide release on-chip with MS detection (Zhong *et al*, 2012). Rather than quantifying the peptide level via MS peak height, the length of the channel producing appreciable peptide signal from adsorption was used as a measure of peptide amount in release. A linear relationship between peptide amount and band length has been suggested by prior modeling and validated using known neuropeptides.

An advantage of lab-on-chip systems is their fully controllable integration and ability to function as high-throughput chemical screening tools (Livak-Dahl *et al*, 2011). The use of these devices is gaining popularity for investigations of chemical stimuli on live cells and even entire small organisms such as the nematode *Caenorhabditis elegans*. A complex, droplet-based microfluidic device integrated with a floatage-based trap array and a tapered immobilization channel array coupled to fluorescence imaging was used in a pharmacological evaluation of neurotoxins on whole-animal mobility, neuron degeneration, and oxidative stress in individual *C. elegans* (Shi *et al*, 2010). Another microfluidic device combined with *in vivo* optical neurophysiology has been used to study proprioceptive properties in the worm motor circuit (Wen *et al*, 2012). A simpler combination of a microfluidic system with optical microscopy allowed monitoring of the male response to hermaphrodite-conditioned medium containing mating signals (Chung *et al*, 2011).

Chemical Maps with Mass Spectrometry Imaging

Non-optical imaging has become an important tool in brain research. Widely used noninvasive technologies for visualization of brain structures (computer-assisted tomography, laser Doppler ultrasound, magnetic resonance imaging) and function (positron emission tomography, diffusion tensor magnetic resonance imaging, electroencephalography and functional magnetic resonance imaging) provide diagnostic information on a range of neurological conditions and therapy effects. MS-based chemical imaging, in contrast, is a cutting edge tool for laboratory neuroscience research that interrogates and catalogs various classes of physiologically important molecules in brain samples, and provides their location, amounts, and temporal dynamics. However, it is both invasive and destructive to the sample. Mass spectrometry imaging (MSI) offers unmatched information content and high throughput and is suitable for chemical imaging of metabolites, transmitters, lipid, peptides, and even proteins in entire brain sections or specific brain region sections, cultured cells of any origin, and *in vitro* reconstructed neuronal networks.

Although most methods in microanalysis experience a bottleneck in throughput because of sampling, MSI conveniently eliminates the need for microdissection/extraction/purification steps; the approach can be used to assay defined tissue regions, individual cells, and subcellular domains from conventional tissue sections. Just like MS profiling, MSI generates direct molecular/structural information and spatial localization within the sample. The quality of the chemical map depends on the resolution parameters for mass and space. Mass resolution determines the chemical specificity and accuracy of the measurement, whereas spatial resolution, defined as lateral and depth, specifies the level of morphological detail attainable by a particular MSI technique. Depending on the ionization technique used, the spatial resolution obtained from MSI can be as small as 50 nm (Klitzing *et al*, 2013), allowing for single-cell and subcellular chemical mapping, although oftentimes the resolution is much less. Refining spatial resolution capabilities to obtain a more accurate cellular localization of compounds of interest drives the development of improved systems. A tradeoff of better spatial resolution is that there are fewer molecules within the sample spot, leading to fewer analytes being detected.

Although a number of approaches using MSI have been developed (Chen and Li, 2010; Verhaert *et al*, 2010; Janfelt and Norgaard, 2012; Vismeh *et al*, 2012), MALDI and secondary ion mass spectrometry (SIMS) are the most widely used ionization methods, each well suited to specific classes of analytes. Most recently, MALDI MSI has been used to create localization maps for neuropeptides with resolutions ranging from 20 to 100 μm in the nervous system from several invertebrate models (Zimmerman *et al*, 2009; Chen *et al*, 2010; Jia *et al*, 2012; Mark *et al*, 2012; Ye *et al*, 2013), as well as from mammals (Monroe *et al*, 2008) and from neuronal cell cultures (Zimmerman *et al*, 2011). Owing to the larger size of invertebrate neurons, *de novo* sequencing of novel peptides has been possible in MALDI MSI studies (Chen *et al*, 2010; Jia *et al*, 2012). Chemical maps of bioactive compounds facilitate functional insights, even in complex organisms. For example, region-specific distribution profiles of neuropeptides in the brain of an animal model of Parkinson's disease by MALDI MSI revealed a correlation between dyskinesia severity and levels of des-tyrosine α-neoendorphin in the striatum (Ljungdahl *et al*, 2011). In another study, the neurochemical response to cocaine was investigated (Uys *et al*, 2010). Even though MALDI MSI is less common for the analysis of low-molecular-weight compounds due to chemical interferences from matrix ions, Sugiura *et al* (2012) studied acetylcholine

distributions in the mouse hippocampus and spinal cord on the 50 μm scale. With atmospheric pressure MALDI MSI, the Spengler group (Guenther *et al*, 2011) demonstrated neuropeptide imaging of the mouse pituitary gland at the cellular scale (5 μm spatial resolution), along with structural verification by tandem MS for some of the detected peptides.

In contrast to MALDI, SIMS is a matrix-free approach in which a specimen, such as a tissue section, is sputtered with a focused primary ion beam that ejects secondary ions from the surface of the sample. The secondary ions are then guided into a mass analyzer for measurements. SIMS ionization is more energetic and so fragments larger molecules, limiting its application to small molecules (typically less than 800 Da), but achieves higher spatial resolution than MALDI. SIMS MSI is often used to investigate the subcellular localization of lipids and lipophilic species in brain sections and cultured neurons (Passarelli and Winograd, 2011; Tucker *et al*, 2012; Passarelli *et al*, 2013). Other applications of SIMS MSI for the analysis of biological samples are thoroughly reviewed elsewhere (Lanni *et al*, 2012). For further reading on the strengths, weaknesses, and complementarity of existing MSI methods, we recommend two recent comprehensive reviews (Vickerman, 2011; Trim *et al*, 2012).

FUTURE RESEARCH DIRECTIONS

Small-volume analysis has experienced tremendous technical progress in the past decade. We envision it becoming an essential element in modern neurotherapeutic discovery, with growth anticipated in fundamental neuroscience, diagnostics, and drug development. In terms of fundamental research, identifying the molecular pathways involved in neurological disorders and psychiatric disease presents a challenge in neuroscience, one that will be best addressed by methods that supply high chemical information content, spatially resolved data, and temporal information on chemical dynamics.

MS-based platforms for label-free profiling and imaging, and single-cell applications in particular, can contribute to a better understanding of the complexity of the neuronal circuit and pathway. Future work will focus on the development of improved sampling, increasing mass and spatial resolution to enable MSI, and identification of bioactive molecule localization at the subcellular scale. In fact, MS and MSI, in particular, have yet to realize their potential to complement existing gene expression and immunohistochemical maps (such as those found in the Allen Brain Atlas, http://www.brain-map.org/) by generating maps of final gene products. This will be especially useful for neuropeptides whose sequences are often inferred from their mRNA transcripts. Peptides are created from gene products that have alternate splicing, post-transcriptional proteolytic processing, and other modifications that cannot yet be reliably predicted from genetic information, and thus have to be investigated on the peptide level using direct detection methods such as MSI.

The ability to correlate behavior with dynamic changes in native bioactive compounds and drugs from precise brain locations should accelerate the development of new treatments for neurological diseases. Methods will become available that enable cerebral microdialysis to provide continuous sampling of endogenous and exogenous molecules of interest from the extracellular fluid of the brain in freely moving animals, and perhaps even from patients with several types of brain injury. Innovative research using the successful hyphenation of microdialysis to sensitive detection modalities has demonstrated the great potential of this approach for achieving real-time quantitative monitoring of metabolites and neurotransmitters, and for its applicability in neuropharmacokinetic drug studies. In conjunction with microdialysis, CE and MS can be used for identification of potential biomarkers in cerebrospinal fluid for clinical diagnosis of relevant disorders, and for monitoring therapeutic interventions. To enable these approaches for use in medical settings, future efforts should concentrate on miniaturization of sampling probes, making cerebral microdialysis less traumatic—just as lumbar puncture has become—and thus more applicable to human volunteers for facilitation of drug testing and discovery. A limitation in the small-volume analysis of cerebral fluid samples has been the dispersion of the analyte band during transfer from the dialysis probe to the analytical system, which limits temporal resolution. Although exciting progress has been reported with the advent of segmented flow microdialysis and low-flow push-pull perfusion, further improvements in temporal resolution of *in vivo* monitoring methods are required to follow accurately neurochemical dynamics.

Finally, microfluidic devices and lab-on-chip platforms have become more widely used for *in vivo* and *in vitro* analyses of signaling compounds, as they incorporate multiple functions, including cell culture, stimulation, separation, and detection, at better sensitivity than bulk analysis methods. The portability and small footprint of lab-on-chip devices have the potential to revolutionize high-content screening, allowing clinical use of cell-based assays for determining drug efficacy and toxicity. The translation of exciting original ideas into medical applications requires standardization of protocols, validation of day-to-day reproducibility, and robustness of microfluidic systems, which remains a significant and ongoing endeavor.

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