Published in final edited form as:

Anal Chem. 2017 January 03; 89(1): 314–341. doi:10.1021/acs.analchem.6b04278.

Analytical Techniques in Neuroscience: Recent Advances in Imaging, Separation, and Electrochemical Methods

Mallikarjunarao Ganesana[1], **Scott T. Lee**[1], **Ying Wang**[1], and **B. Jill Venton**[*]

Department of Chemistry, PO Box 400319, University of Virginia, Charlottesville, VA 22904

Introduction

How do we understand neuronal signaling in the brain, how neurons controls behavior, or how signaling malfunctions during disease? Neuroscientists might take two approaches: studying action potentials or studying the resultant neurotransmitters that are released. Elliott Valentine has termed these “the sparks and the soup”.[1] While cell firing can give information about how cells are connected, understanding chemical changes is necessary to truly understand neuronal communication. Measuring chemicals in the brain is challenging because of the vast number of chemicals that signal on different time and length scales. No one technique can measure over all these scales. Thus analytical chemists have developed many tools for measuring neurochemicals, and recent advances in these techniques are described in this review. The techniques are divided into three sections: (1) imaging techniques (including fluorescence, functional magnetic resonance imaging (fMRI), positron emission tomography (PET), and mass spectrometry (MS)), (2) sampling and separations techniques (including microdialysis, push-pull perfusion, high performance liquid chromatography (HPLC), capillary electrophoresis (CE) and microfluidics), and (3) electrochemical techniques (including exocytosis measurements, fast-scan cyclic voltammetry, and electrode development). Because the range of topics is broad, we concentrated on papers from the last 3 years which had technical advances. An accompanying review in this issue covers a full range of *in vivo* electrochemical sensors in depth.[2] This review shows that there has been substantial progress in the field of analytical neuroscience. Research is pushing the techniques to faster time scales, down to the millisecond, but also addressing the need to monitor chemicals chronically for days at a time. Different spatial scales are addressed: from exocytosis at single synapses, to micron-scale, regional coordination of signaling, to whole brain imaging. Analytical measurements are moving beyond traditional neurochemicals such as oxygen and dopamine, into new types of molecules, such as small molecule neuromodulators, peptides, proteins, and lipids. The end result is that no one technique can do it all; instead a better picture of the “soup” is gained by using information from many techniques in tandem.

[*]To whom correspondence should be address. jventon@virginia.edu Phone: 434-243-2132.

[1]These authors contributed equally to this work.

Imaging

Imaging has become an important tool for neuroscience and clinical research because it allows direct visualization of neurons or chemical information from the individual molecule to the whole-brain level. Fluorescence imaging is a common method to track chemical changes and new fluorescent imaging techniques expand the capability for recording neural dynamics in *in vivo*, *ex vivo*, and behaving animals. Genetically-encoded biosensors as well as small molecules and quantum dots that either bind to or mimic neurotransmitters are expanding optical imaging into the chemical domain. Whole-brain techniques such as functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) imaging continue to be popular for human studies and for tracking neurochemicals. Mass spectrometry (MS) is a newer player in the imaging world, providing a rich amount of chemical information about a sample. Different MS ionization methods have been developed that enable novel classes of molecules to be detected including lipids and neuropeptides. This section covers the recent advances in imaging for neurochemical methods, concentrating on technical advances in the past three years.

Fluorescence imaging

Fluorescence imaging is widely used to study biological processes at molecular and cellular levels in living organisms due to its good temporal and spatial resolution as well as high selectivity and sensitivity. Traditionally, fluorescent molecules or proteins have been used as labels of target cells. However, for neuroscience research, fluorescence imaging is also now being developed to track dynamic processes, such as action potentials, calcium changes, or neurotransmitter release *in vivo* and *ex vivo*. Current fluorescent imaging tools, such as fluorescent false neurotransmitters (FFNs), quantum dots, ligand-based sensors, genetically-encoded Ca^{2+} and voltage indicators, have been widely applied to study neuronal processes. Fluorescence-based imaging tools can be divided into two classes: genetically encoded fluorescent biosensors that target fluorescent proteins to specific cells and non-genetically encoded fluorescent biosensors that include synthetic chemical probes and ligand-directed chemistry. These tools demonstrate the power of fluorescence imaging to advance our understanding of functional processes in the brain.

Genetically encoded fluorescent biosensors

Genetically encoded Ca^{2+} indicators (GECIs): Genetically encoded fluorescent biosensors provide a new understanding of the dynamics of signaling events with high spatial and temporal resolution. A detailed review of genetically encoded biosensors for neuronal activity was recently published.[3] Genetically encoded Ca^{2+} indicators (GECIs), track neuronal activity and synaptic transmission based on changes in intracellular Ca^{2+} concentration. GECIs do not directly detect membrane potential or action potentials, but instead track Ca^{2+} signaling, which leads to neurotransmission. Recently, the Isacoff group designed photoactivatable GCaMP GECIs, GCaMP6s and GCaMP6f, allowing selective activation of GECIs in individual neurons from a large cell population.[4] Recent studies also developed GECIs with new emission colors. Dual-color Ca^{2+} imaging of brain activities in behaving animals was acquired using a combination of R-CaMP2, a new red fluorescent protein derived biosensor, with a green Ca^{2+} indicator.[5] The red Ca^{2+} indicator facilitates

imaging and mapping activities in deep brain areas due to the reduction of tissue scattering at longer excitation wavelengths, enabling detection and quantification of strong single action potential signals and fast kinetics *in vivo*. GECIs are also integrated with other type of indicators and optogenetic tools, such as intensity-based glutamate-sensing fluorescent reporters and optogenetic activation with channelrhodopsin-2.[6,7,8] GECIs can be used in behaving animals and are stably expressed over weeks or even months, allowing long-term imaging studies of neural dynamics in live mammals.

Genetically encoded voltage indicators (GEVIs): Genetically encoded voltage indicators (GEVIs) provide direct information on membrane potential changes, which are associated with action potentials, allowing the measurement of neural activity in cells on the millisecond scale. Recently, a new type of GEVI with fused *Acetabularia acetabulum* rhodopsin (Ace) and mNeonGreen fluorescent protein was designed, which enabled voltage-sensitive fluorescence resonance energy transfer (FRET) (Fig. 1). This new GEVI overcomes previous limitations of lack of sufficient signaling speed and dynamic range to measure action potentials *in vivo*.[9] Its faster kinetics and high brightness enabled high-fidelity imaging of individual spikes in live mice and flies. GEVIs can also be used to monitor the activity of a large number of neurons. For example, a class of GEVIs takes advantage of voltage-sensing domains (VSDs), which are surrounded by a pair of fluorescent proteins that undergo resonance energy transfer.[10] These sensors exhibit faster kinetics and can be used in living mice to report evoked cortical population responses. Additionally, an accelerated sensor of action potentials, was developed based on VSD sensors.[11] Inserting the circularly permutated green fluorescent protein (GFP), which was constructed from GCaMP3, in the extracellular loop of a VSD, yielded sufficient brightness, a large dynamic range, and fast kinetics. As such, these biosensors allow the rapid trains of action potentials to be detected and counted. Another promising GEVI called ArcLight was recently developed by the Cohen and Pieribone groups.[12] These sensors are based on fusion of the *Ciona intestinalis* voltage sensor to the pHlourin GFP, and they significantly improved signal amplitude and signal to noise ratio.[13,14] Using ArcLight and GECI to simultaneously image the odor-evoked electrical activity in the mammalian olfactory bulb *in vivo* revealed faster kinetics and a larger dynamic range for ArcLight than GECI.[14] Taken together, GEVIs provide information on both synaptic input and AP output. However, these indictors cannot be used for directly monitoring neurotransmitters or in deep tissues.

Cell-based neurotransmitter fluorescent engineered reporters (CNiFERs): The Kleinfeld group developed cell-based neurotransmitter fluorescent engineered reporters (CNiFERs) for the detection of neurotransmitter volume signaling.[15,16] CNiFERs are cultured HEK293 cells that are designed to express neurotransmitter-specific G protein-coupled receptors and the genetically encoded FRET-based Ca^{2+} indicator, TN-XXL. Activation of receptors by neurotransmitters results in the increase of Ca^{2+} in cells, and the subsequent binding of Ca^{2+} with TN-XXL leads to the color change of fluorescence through a FRET mechanism. The first CNiFER, M1-CNiFER, was engineered to express M1 muscarinic receptors for the detection of acetylcholine release into the extracellular space.[15] M1-CNiFER detected low nanomolar concentrations of acetylcholine on the second time scale. Following the success of the M1-CNiFER, two new CNiFERs, D2 CNiFER and α_{1A}

CNiFER, were created to detect dopamine and noradrenaline, respectively[16] By stereotaxically injecting CNiFERs into the targeted brain region, changes of dopamine and noradrenaline are determined *in vivo* in real time in head-restrained or behaving animals. However, these reporters cannot directly measure neurotransmitter release but only changes of Ca^{2+} concentration over time.

Non-genetically encoded fluorescent biosensors

Ligand based fluorescent probes: Several non-genetically encoded fluorescent biosensors, such as ligand based florescent probes, were developed for brain imaging. Fluorescently-labeled ligands are applied for the study of neuronal receptors, transporters and neurotransmitters. The Newman group recently developed a new fluorescent ligand for binding and visualizing serotonin transporters (SERTs).[17] This new fluorescent ligand was based on the antidepressant (S)-citalopram and had a much higher affinity binding and selectivity for SERT (Ki = 3 nM) compared with their previously reported ligand.[18] Therefore, the new ligand could be used in the future to monitor SERT in living cells. The Strømgaard group designed ligand-directed probes for visualization of ionotropic glutamate (iGlu) receptors.[19] These probes were synthesized based on fluorescent polyamine toxin analogs, using the natural product argiotoxin-636 (ArgTX-636), that blocks iGlu receptor ion channels. Pharmacological evaluation of iGlu receptors found that the analogs had affinities in the 10 nM range for NMDA and 90 nM range for AMPA receptors. Furthermore, iGlu analogs were used to visualize native NMDA receptors in hippocampal neurons.

The ligand-based probes have also been used for screening potential drugs that can selectively bind to targeted receptors. For example, the Kiyonaka and Hamachi groups recently developed a $GABA_A$ receptor ($GABA_AR$) labeling compounds that can covalently label a fluorophore near the binding sites without influencing receptor function.[20] The integration of these compounds with bimolecular fluorescence quenching and recovery enabled the construction of fluorescent $GABA_AR$ ligand biosensors on live cell surfaces. They screened small molecules that performed as negative allosteric modulators for $GABA_ARs$, finding a new modulator that almost completely inhibited GABA-induced current. This approach could be expanded to discover and develop new drugs for modulation of other neurotransmitter receptors.

Quantum dots (QDs): Quantum dots (QDs) are semiconductor nanoparticles that emit light, and the emission wavelength is tunable by changing their size, shape or materials. QDs have been widely used in bioimaging due to their high extinction coefficients and low photobleaching. The modification of QDs with antibodies or small molecule ligands allows their specific conjugation with targeted proteins or cells. For example, the Su group developed a sensitive near infrared fluorescence probe for the detection of dopamine based on the fluorescence quenching of $CuInS_2$ QDs.[21] By functionalizing $CuInS_2$ QDs with 3-aminophenyl boronic acids, the QDs formed boronate esters with vicinal diols of dopamine, resulting in fluorescent quenching. The limit of detection (LOD) was 0.2 μM in human serum samples. A novel fluorescence probe for the detection of adenosine 5'-triphosphate (ATP) was also developed.[22] In this study, $_L$-cysteine capped CdTe QDs were synthesized

that were quenched by binding with Zn^{2+}. ATP inhibited that quenching by binding the Zn^{2+} through Zn-O-P bonds due to the high affinity of phosphate groups of ATP.ATP was determined in human serum sample as well as in live cells with a LOD of 2.07 μM. These studies were proof of principle for the QD imaging concept, but were not performed in real tissue samples.

QDs were also used to target receptors of interests. The Groc and Oliet groups used QDs to monitor the diffusion of GLT-1, a glutamate transporter, at the surface of astrocytes in rat brain slices.[23] GLT-1 was labeled with QDs with red light emission of 655 nm. GLT-1 had a high mobility on astrocytes that depended on the activities of neurons and glial cells and rapidly diffused between synaptic and nonsynaptic sites, facilitating the clearance of glutamate. Recently, a new method was developed using functionalized QDs to visualize dopamine receptors *in vivo*.[24] Here, QDs with an emission maximum at 655 nm were chosen for deep brain measurements and conjugated to polyclonal antibodies. Dopamine receptors were labeled and tracked at the single nanoparticle level with minimal inflammation in acute brain slices. The high fluorescent intensity and versatile modification of QDs have resulted in the wide application of QDs in tissue imaging. However, the toxicity of QDs limits their applications *in vivo* and the non-specific binding of QDs with other molecules can interfere the imaging results.

Fluorescent false neurotransmitters (FFNs): FFNs are optical probes for monoamine neurotransmission and were introduced by the Sames and Sulzer groups. They are analogs of monoamine neurotransmitters and generally consist of small fluorescent compounds and a recognition element, such as ethylamine moiety. FFNs mimic the action of neurotransmitters such as dopamine: they are loaded into vesicles by vesicular monoamine transporters (VMAT) and released during exocytosis. FFNs studies mainly focus on synaptic control of neurotransmitter release; FFNs are loaded into vesicles and are used to visualize presynaptic terminals as well as neurotransmitter release and uptake.[25] pH-responsive FFNs have been designed, acting as dual substrates of dopamine transporter (DAT) and VMAT2, that measured the pH gradient between the cytosol and vesicular lumen.[26,27] These pH-responsive FFNs were used to identify dopamine cells and visualize DAT activity. Furthermore, FFNs acting as VMAT2 substrates revealed the accumulation of neurotransmitters in both culture and acute brain slices.[28] FFNs are useful tools for studying the probing mechanisms of vesicular release at the single cell level, facilitating monitoring of partial fusion of neurotransmitters, such as kiss-and-run. However, the actual concentration of neurotransmitters in synaptic cleft is not detected using this method.[29] In addition, these probes do not allow direct visualization of neurotransmitter release and are unable to distinguish cell populations and discriminate cell types specifically.

Fluorescent molecular logic gates: A new category of non-genetically encoded biosensors was recently developed to label neurotransmitters directly. These small molecules have reactive functional groups, such as aldehydes or hydroxides, that react with target analytes or the environment to trigger the fluorescence of the biosensors. Recently, a series of fluorescent molecular logic gates based on the coumarin-aldehyde system were developed for probing primary amine neurotransmitter release in different logic formats, such as YES

and AND. A fluorescent turn-on sensor (NeuroSensor 521, NS521) was generated for selectively labeling and imaging high concentrations of norepinephrine and dopamine in the acidic environment of secretory vesicles.[30] This single-input YES logic gate becomes fluorescent after reacting with monoamine, and labeled monoamine cannot cross the vesicular membrane due to its positive charge. Thus, a derivative, ExoSensor (ES517), was designed for imaging only active neurotransmitter released in the synaptic cleft upon exocytosis.[31] By integrating a pH-sensitive functionality into the scaffold, this biosensor bound to primary amine neurotransmitters, but was only fluorescent in the neutral (pH 7.4) extracellular environment after it was deprotonated and the fluorescence was switched on. Furthermore, a three-input AND gate was designed that was triggered on in the presence of glutamate, Zn^{2+} and a pH around 7.4.[32] A NeuroSensor 539 (NS539) was developed with enhanced photophysical property, which is modified from NS521 but has a thiophene group instead of a phenylmethoxy group. The fluorescence is 48 fold brighter for norepinephrine and 57 fold brighter for glutamate, while providing an overall low background.[33] Recently, NS715 was developed, based on the structure of NS539, that had an electron-rich 1,2,3,4-tetrahydroquinoxaline framework.[34] This sensor demonstrated a turn-on near-infrared red fluorescence response to serotonin at 715 nm. These fluorescent molecular logic gates have great potential for helping to elucidate mechanisms of exocytosis, since the actual neurotransmitters are monitored. They are suitable for labeling neurotransmitters in vesicles, but not in the extracellular space, due to the low extracellular concentrations of neurotransmitters.

Fluorescence imaging conclusions—Fluorescent imaging tools provide a method to visualize neural activity and neuronal communication. Genetically-encoded biosensors are useful because they allow monitoring of membrane potentials or calcium changes at targeted cells on the millisecond time scale. Other fluorescent biosensors enable a better picture of the dynamics of neurotransmitters, either by mimicking neurotransmitters or binding the receptors of interest with small molecules. The newly developed NeuroSensor molecules that actually bind neurotransmitters are the first to sense neurotransmitters directly and will be useful for studies of vesicle dynamics and content. These NeuroSensor probes are relatively new and new chemistries will need to be developed to expand the number of molecules detected. Fluorescent probes are especially useful for brain slice or cell experiments; *in vivo* experiments are always challenging due to fluorescence intensity, possible toxicity of probes, and imaging in deep tissue. New advances in red and near-IR probes are facilitating imaging in deeper tissues. In addition, the kinetics of the probes binding to the targeted proteins or molecules must also be optimized. Employing such powerful fluorescence imaging techniques in neuroscience provides the opportunity to do high temporal-spatial resolution measurements. Future studies can implement multiplexed fluorescent detection to enhance a deeper understanding of the brain function. For example, combining FFNs with GECIs and GEVIs makes it possible to investigate synaptic function and plasticity at circuit level.[29]

Functional magnetic resonance imaging (fMRI)

Functional magnetic resonance imagining (fMRI) is a non-invasive neuroimaging technique that evaluates brain structure and function. A typical fMRI measures brain activity by

detecting changes in oxygen levels associated with blood flow. Blood oxygenation level dependent (BOLD) contrast monitors increases in brain oxygen that accompany brain activity. Metabolism increases with brain activity; metabolites and small molecules signal for vasodilation, which brings more nutrients as well as an oversupply of oxygen to the active area. Therefore, neuronal activity is indirectly measured based on blood oxygenation level using BOLD fMRI. The efficacy of fMRI has been improved by developing different contrast agents to target analytes, allowing a molecular fMRI signal specific to a molecule of interest. fMRI can be used for longitudinal studies in the same animal over time to study the interaction between neuronal activity and behavior as well as the effects of acute and chronic drug treatments.

BOLD fMRI—BOLD fMRI is currently one of the most popular techniques to study neurological diseases and investigate alterations in brain function. For example, the Nedelman group examined the effects of Huntington's disease (HD) on neural activity. Using fMRI, differences in brain activity affected by odor were identified between wild type, heterozygous, and homozygous HD gene knock-in mice. There was a reduced change in BOLD signal intensity by almond odor in the glomerulus of the olfactory bulb in homozygous HD mice compared to the other phenotypes, suggesting a deficit in olfactory sensitivity.[35] In addition, fMRI was used to identify a possible functional imaging biomarker in the human brain that indicates premanifest HD (preHD).[36] The fMRI signal in the right-frontal oculomotor cortex of a preHD patient was activated during a behavioral task. Moreover, the average BOLD signal changes in the frontal oculomotor cortex had a linear relationship with the probability to develop the disease in the next 5 years, a correlation that could help develop early therapeutic approaches to prevent or delay the onset of HD. Furthermore, fMRI was used to study the extent to which schizophrenia symptoms and antipsychotic treatment depends on estrogen in female rats.[37] Treatment with estradiol, an estrogen, enhanced BOLD activation in the mesocorticolimbic, habernular and olfactory pathways which are implicated in schizophrenia symptoms. In addition, treatment with chronic haloperidol, an antipsychotic, and estradiol also increased the BOLD signal in rat brain.[38] Taken together, BOLD fMRI is a useful approach for studies of brain function and related diseases.

A new modality of fMRI was developed, real-time fMRI-based neurofeedback (rtfMRI-NF). The goal of using this technique is to diagnose abnormal brain connectivity or neural activation, and train a subject to change it by providing them feedback. Real time fMRI (rtfMRI) was firstly published by the Hyde group, who used fMRI data to measure localized brain activity and correlate it with behavior and cognition.[39] The Weiskopf group tested whether rtfMRI-NF was able to manipulate region-specific brain activity.[40] Researchers trained participants to simultaneously control brain activity in the supplementary motor area and parahippocampal cortex, brain regions associated with motor and memory functions. Trained participants learned to voluntarily control both brain areas to provide a specific behavioral effect; for example, the motor reaction times were reduced and the ability of memory encoding was improved. rtfMRI-NF was also used to study emotional regulation in healthy individuals who were trained for neurofeedback to downregulate the amygdala when viewing aversive pictures.[41] These results demonstrated that fMRI neurofeedback enhanced

the functional connectivity of the amygdala and rtfMRI-NF could be used to influence the network of emotion processing and regulation, which may be beneficial for the treatment of patients with severe emotional dysregulation. However, a drawback of fMRI is the requirement of fully immobilized experimental subjects and lack of high temporal resolution.

The integration of electroencephalography (EEG) and rtfMRI is a multimodal neuroimaging technique that offers a unique approach to characterize brain activity under normal function and during disorders. Simultaneous EEG and rtfMRI neurofeedback in healthy subjects were used to track metabolic and electrical activities to identify self-regulation of human brain activity.[42] Participants could fulfill both fMRI and EEG feedback under the positive emotion induction task. One important application of EEG-rtfMRI is to diagnosis patients with epilepsy. The Carmichael group focused on the effect of the intrinsic connectivity network in pediatric focal epilepsy.[43] A decrease in activity was found in the intrinsic connectivity network using EEG-fMRI signals and the functional connectivity within visual and attention network also decreased, which had a transient influence on the functional organization of the brain.

Molecular fMRI—Traditional BOLD fMRI, as described above, measures oxygen changes as a correlate of brain activity, but does not measure any brain neurotransmitters directly. Molecular fMRI, developed by the Jasanoff group, uses a specialized molecular MRI contrast agent to measure chemicals as a function of neural activity. These MRI contrast agents are paramagnetic heme proteins with altered T1-weighted MRI signals derived from the bacterial cytochrome P450-BM3 (BM3h).[44] BOLD fMRI is based on oxygenation of hemoglobin in blood as hemoglobin acts as a contrast agent and changes its magnetic property when it binds to oxygen. In contrast, the BM3h sensor can bind individual molecules to change its magnetic properties and affect the MRI signal. Dopamine release dynamics were mapped in the ventral striatum using BM3h-9D7, a variant of BM3h with high specificity for dopamine.[45] This technique is a major advancement because it permits the quantitative assessment of different neurochemicals, other than oxygen, with MRI. However, it is in an early development phase and requires invasively delivered contrast agent to site-specific brain areas. Also, the development of fMRI contrast agents is still a major challenge due to blood-brain-barrier permeability.

Molecular fMRI can also be used to study extracellular Ca^{2+} fluctuations. In a recent study, the Angelovski group generated ultrasmall nanoparticles, that specifically interacted with Ca^{2+} with dissociation constant (K_d) of 1.9 mM.[46] These Ca^{2+} contrast agents were derived from gadolinium chelates, DOTAGA, and consisted of ultrasmall and rigid platforms covalently coupled with Ca^{2+} contrast agents. This is the first report that demonstrated the dynamics of Ca^{2+} using molecular fMRI *in vivo*. However, this method has not yet been used in brain.

fMRI conclusions—fMRI is a promising methodology for understanding brain function and BOLD fMRI is now routinely used in both rodent models and human patients to track neural activity. Multimodal combinations, such as EEG/fMRI, allow simultaneous recording of hemodynamic and electrophysiological activities that provides a better understanding of

brain function. However, the spatial and temporal resolutions still need to be improved. Molecular fMRI, as a new experimental tool, allows molecular-level specificity for analytes, such as neurotransmitter release and calcium signaling. Thus, fMRI is now being expanded past the traditional monitoring of oxygen. Molecular fMRI imaging of calcium in organs or tissues is still a developing technique compared with GECIs and lacks the specificity to image calcium activity in specific receptors or transporters. A remaining challenge is to successfully design contrast agents and introduce them into humans. Thus, the improvement of probe delivery and probe chemistries are crucial in the future.

Positron emission tomography (PET)

Positron emission tomography (PET) is a high sensitivity *in vivo* imaging technique for investigation of neural activity. This technique uses radioactively labeled molecules (tracers) that are injected into the bloodstream and metabolized by cells. These tracers decay and emit positrons, which can be detected and spatially mapped. PET is used to map cellular processes associated with brain activity. For example, the combination of PET with voxel-wise analysis allowed the mapping of the serotonin and opioid systems in human brain. The PET tracers were a serotonin transporter tracer, [^{11}C]MADAM, and a μ-opioid tracer, [(11)C]carfentanil.[47] This study revealed a high degree of overlap between the expression of serotonin and opioids in different brain regions, such as the anteromedial thalamus and dorsolateral prefrontal cortex, which are relevant to the regulation of pain. In another example, the Hooker group developed a new method to monitor transient glucose changes in human brain, overcoming the traditional low temporal resolution of PET.[48] In this method, [^{18}F]fluorodeoxyglucose (FDG) was constantly infused intravenously to provide a baseline PET activity, and fast dynamic changes of glucose metabolism were detected with 5 min temporal resolution.

DREADD-assisted metabolic mapping (DREAMM)—DREADD-assisted metabolic mapping (DREAMM) is a new biobehavioral imaging methodology that was recently introduced by the Michaelides and Hurd groups. DREAMM combines the techniques of designer receptors exclusively activated by designer drug (DREADD) and FDG as well as PET.[49] In DREAMM, PET images of whole brain activity are collected with high spatial resolution as DREADD is used to remotely activate or inhibit specific neurons that cause changes in brain activity. Thus, the integration of these two techniques provides dynamic, cell type-specific functional circuit imaging in the whole brain. DREAMM was used to manipulate brain activity in freely-moving rats after infecting their medium spiny neurons with a viral vector that expressed an inhibitory G-protein coupled receptor.[50] The inhibition of medium spiny neurons resulted in a discrete response in different brain regions, which was determined by changing of FDG uptake. These findings demonstrated the potential applications of DREAMM to image brain activity and cell-type-specific function in behaving animals. In another example, DREAMM was applied to study the inhibition of prodynorphin neurons in the rat brain to study the high comorbidity of opiate abuse with major depressive disorder.[51] In this study, inhibited prodynorphin neuronal activity was mapped using PET; both opiate addiction and depression showed the common changes in expression of prodynorphin, suggesting a shared neuropathological feature in these diseases.

PET conclusions—In summary, PET offers the important advantage of visualizing brain activities involved in blood flow and molecule metabolism with high sensitivity. However, the drawback of this technique is the requirement of delivering small radioactive tracers into the bloodstream. Also, it has low spatial resolution, on the millimeter scale, and temporal resolution on the minute time scale. DREAMM is a new approach that provides direct imaging of cellular activities with high cell-type specificity in live animals, expanding the application of PET to brain function studies. Compared to fMRI, DREAMM is advantageous because it is a direct technique that can be done in freely moving, not immobilized, animals. DREAMM is not particularly rapid, as the modulation of DREADD occurs on the order of minutes.[52] Therefore, improving temporal resolution remains an important challenge. Development of new radioactive tracers for PET will broaden the area of neurobiological studies in the future.

Mass Spectrometry (MS)

Imaging mass spectrometry (MS) is another important analytical technique in neuroscience research. In MS, analytes are ionized and then separated based on their mass-to-charge ratios. The development of soft ionization techniques, such as matrix-assisted laser desorption ionization (MALDI), secondary ion mass spectrometry (SIMS), and desorption electrospray ionization (DESI) allows direct identification and quantification of analytes, such as proteins, peptides, neurotransmitters and metabolites in neural tissues. A review article by Spengler covers a variety of MS techniques and biological applications.[53] Here, we focus on MALDI-MS, SIMS, DESI-MS, and nano-DESI MS.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS)—MALDI-MS imaging has been extensively adopted for studying biological tissues. The Gile group demonstrated the first successful MALDI imaging in tissue samples of the rat pituitary gland and pancreas in 1997.[54] With MALDI, the tissue must be coated with a matrix; laser irradiation of the sample causes the analytes to ionize and vaporize. The matrix is generally acidic for the analysis of proteins or lipids, which are protonated and ionized into positively charged ions. The time of flight (TOF) mass analyzer is often used to determine the mass to charge ratio (m/z). By moving the laser around the sample, the m/z of compounds at different locations is measured.

MALDI is a powerful technique for monitoring lipid changes, facilitating determination of the relative abundance and spatial distribution of specific lipids. For example, local alterations of membrane phospholipids were investigated in the brain of a transgenic mouse model of Alzheimer's disease with MALDI imaging.[55] MS imaging technology alongside immunostaining methods showed that the distribution of lysophosphatidylcholine and sulfatide were changed in transgenic Alzheimer's mice. Also, a correlation between amyloid plaque and phospholipid distribution in brain tissue was discovered for the first time, suggesting a potential application of MALDI imaging in disease prediction.

MALDI is also used to image the distribution of neuropeptides in various animal models. The Li group examined the relationship between salinity stress and neuropeptide content in *Carcinus maenas* with isotopic dimethyl labeling.[56] The abundance of neuropeptides was

associated with the salinity stress but the different salinity levels did not significantly affect the localization of the neuropeptides. In another study, they adopted multidimensional mass spectrometric platforms to characterize the expression and distribution of neuropeptides in the spiny lobster *Panulirus interruptus* brain.[57] Here, 51 endogenous neuropeptides were successfully identified for the first time and 31 novel neuropeptides were sequenced. The use of multifaceted approaches made it possible to perform discovery neuropeptidomics on crustacean species with limited sequence information. Recently, the Sweedler group developed a method to characterize the release of neuropeptides from select neurons by the combination of a microfluidic device and MALDI-TOF.[58] In this study, bag cell neurons of *Aplysia californica* were loaded and incubated in polyimide coated capillary. After stimulation, the released neuropeptides were collected by a capillary-based, particle-embedded monolithic capillary and then eluted and transferred to the MALDI matrix for MS characterization. Neuropeptides are important neurotransmitters that are present at low concentrations and MS provides the sensitivity to detect them at the very low levels at which they exist in the brain. Imaging MALDI is now providing a spatial map of this peptide location and facilitating discovery of new peptides in a variety of species.

MALDI was also used to visualize the spatial distribution of drugs. The Yost group introduced a method to measure absolute concentrations of cocaine in brain tissue samples.[59] Deuterated cocaine was applied as an internal standard, which significantly improved the signal reproducibility and calibration curve linearity in combination with wide-isolation MS/MS analysis. The quantitative results obtained from this method were comparable to those obtained from liquid chromatography (LC)-MS/MS. Imaging of drugs will allow an understanding of spatial heterogeneity of drug localization.

Recently, a new MALDI approach was developed to image and quantify common neurotransmitters, such as tyrosine, glutamate, and dopamine in mouse brain regions.[60] Most small molecule neurotransmitters are not directly detectable using MALDI because of their poor laser desorption and ionization efficiencies. Using pyrylium salts to react with primary amines, high ionization efficiency pyridinium ions are formed which can be detected by MALDI. Neurotransmitter distributions were detected in brain tissue sections with spatial resolution of 15 μm. In addition, the absolute concentration of neurotransmitter was determined by using deuterated neurotransmitters as standards. This deuterated standard method overcomes a traditional challenge of in-tissue detection but it requires long synthetic procedures with deuterated matrix and complicated sample preparation. In general, quantitation of MALDI has been one of the main challenges, as spatial heterogeneity of the spot the laser is hitting and heterogeneous matrix/analyte mixtures lead to different ionization efficiencies. Thus, this pyrylium labeling technique, along with deuterated standards, allows MALDI to simultaneously measure the distribution and concentration of neurotransmitters with high spatial resolution.

Secondary ion mass spectrometry (SIMS)—Secondary ion mass spectrometry (SIMS) is another powerful MS technique used to identify and image chemical compounds with high spatial resolution. SIMS uses an ion beam to hit the sample surface to produce secondary positive and negative ionized compounds. One advantage of SIMS imaging is that the sample can be analyzed without any specific pretreatment, such as matrix modification,

thereby shortening the process for sample preparation. SIMS coupled to TOF acquires mass spectra with high sensitivity and mass resolution of compounds with m/z below 1500 in biological samples.[61]

SIMS was used to study the distribution of lipids and metabolites in tissues. For example, TOF-SIMS was successfully employed to image the distribution of native lipids and metabolites in the human spinal cord by the Ewing group.[62] Here, multivariate statistical analysis, such as principal component analysis and maximum autocorrelation factor, were performed on SIMS imaging data to discriminate gray matter in different neuroanatomical regions. Also, high concentrations of intracellular choline were identified in α-motor neurons at submicrometer spatial resolution. In another study, TOF-SIMS was used to visualize changes in lipid and metabolite distributions correlated with amyotrophic lateral sclerosis.[63] Lysophosphatidylcholine and its fragments were increased in the ventral horn of amyotrophic lateral sclerosis tissue due to the death of neurons by inflammation (Fig 2). However, cholesterol, triglycerides and vitamin E were significantly deceased compared to healthy tissue and these compounds are associated with demyelination and neurodegeneration. Another TOF-SIMS study examined cholesterol distribution in the cerebral cortex of control and Alzheimer's samples.[61] Cholesterol increased in the lower half of the cortex in Alzheimer's samples, suggesting that the accumulation of cholesterol can be a therapeutic target. The new information from MS imaging techniques such as SIMS demonstrates that lipid changes may be important to both tracking and treating many diseases.

Recently, the capabilities of three MS methods were compared, nanoparticle-assisted laser desorption ionization mass spectrometry (NP-LDI), MALDI and gas cluster ion beam SIMS (GCIB SIMS). [64] By using GCIB SIMS, intact lipids were detected with m/z between 700 to 900 in both white and gray matter areas of the brain slices. However, NP-LDI only selectively detected lipids in the white matter areas. In addition, MALDI had high sensitivity for phosphatidylcholine lipids with m/z less than 800 while it required data normalization to achieve a better ion image. Therefore, GCIB SIMS provides more information about lipid imaging with high spatial resolution and is more effective in bioimaging and lipidomics studies. However, this instrument is expensive and multivariate analysis is sometimes difficulty to interpret.

Desorption electrospray ionization mass spectrometry (DESI-MS)—Desorption electrospray ionization mass spectrometry (DESI) is an ambient technique invented by the Cook group in 2004.[65] Unlike MALDI, which requires the co-crystallization of sample with matrix at atmospheric pressure, DESI does not require a matrix chemical or further sample preparation and does not damage samples. In DESI, ions are generated by spraying the sample surface with charged solvent drops from electrospray, causing sample to ionize. The sample ions are then transferred through an atmospheric pressure ion-transfer inlet to the mass spectrometer.

DESI was used to simultaneously study neurotransmitters and neuroactive drugs in brain tissue sections.[66] Neurotransmitters are difficult to ionize, so a chemical derivatization method was developed by adding a charge to the primary amine group on the target

molecules. As a result, neurotransmitters and their metabolites (including dopamine, 3-methoxytyramine, serotonin, glutamate, glutamine, aspartate, GABA and adenosine) (Fig 3) and neuroactive drugs (including amphetamine, sibutramine, and fluvoxamine) were simultaneously identified using DESI. Thus, DESI can provide information about complex mixtures in tissue.

DESI was further applied to cancer diagnosis. For example, DESI was used to define the brain tumor margins based on the lipid pattern of tumor cells.[67] Lipid species in the brain tissue slice were ionized and their distinct distributions and abundances were visualized by DESI, providing tumor information such as cell grade and concentration. Therefore, the boundaries between healthy and tumor cells can be distinguished. Discrimination of human brain tumor types has also been achieved by assessing lipid and metabolite signals from tissue smears using DESI-MS.[68] Tumors, such as glioma, meningioma, and pituitary tumors, have their own phospholipid derived signals in MS and can be readily recognized by multivariate statistics with high sensitivity and selectivity. Thus, these methods lay the foundation for an intraoperative application of DESI-MS. DESI has gained popularity because it can detect a wide variety of compounds including, lipids, neurotransmitters, metabolites and drugs with high sensitivity and selectivity. DESI is not as useful for neuroproteomics. It is matrix free but has lower spatial resolution compared with MALDI and SIMS.

Nanospray desorption electrospray ionization mass spectrometry (nano-DESI MS)—Nanospray desorption electrospray ionization (nano-DESI) is a complementary form of DESI developed by the Laskin group in 2010.[69] One disadvantage of DESI is that the desorbed analyte ions can be splashed onto the surroundings of the substrate, resulting in the transport of analytes on the surface. Nano-DESI overcomes this drawback by using two capillaries to transport analytes into the mass spectrometer. Analytes are desorbed into a solvent bridge, which is generated between two capillaries and the sample surface. A high voltage is created between the primary capillary and the MS inlet to pump the solvent from the primary capillary to the solvent bridge. The solvent dissolves the analytes and then flows from the second capillary to the MS inlet. This technique enables independent control of desorption, ionization and transportation of analytes, and was recently used for tissue imaging.[70]

The composition of mouse spinal cords was characterized by integrating nano-DESI with inverted light microscopy, where biomolecules, including lipids, sugars, and peptides, were detected in the mass range from 300 Da to 16 kDa.[71] Specially, the transition of hemoglobin expression was identified from fetal to adult stages. Also, two β-thymosins were visualized in developing embryonic spinal cord. In another example, the Zare group incorporated nano-DESI with an LTQ Orbitrap mass spectrometer to study proteins in the mouse brain and MYC-induced lymphoma tissue sections.[72] Proteins, such as ubiquitin, β-thymosin, myelin basic protein, and hemoglobin, were mapped and identified. Importantly, ubiquitin and thymosin that lost amino acid residues in the C-terminus were discovered in lymphoma, and this alteration of protein structure was not always detectable using common immune-based approaches.

The matrix effects in nano-DESI MS were evaluated in middle cerebral artery occlusion mice brain tissues.[73] The ionization efficiency of each analyte is affected by the presence of cations, resulting in an inability to accurately map molecular distribution. In this study, the signals of phosphatidylcholine species cationized with sodium and potassium were investigated. Sodium and potassium concentrations in ischemic brain regions differed from control regions in the same tissue. Matrix effects were compensated efficiently by normalizing the signals of endogenous phosphatidylcholine to the signals of internal standards.[74] Twenty-two phosphatidylcholine species in seven different brain regions were quantified and nano-DESI enabled a lipidomics shotgun approach to quantify endogenous phospholipids by incorporating standards in the nano-DESI solvent.

Nano-DESI was also used to image drugs and small molecules in brain sections by effectively accounting for the matrix effects. For example, nano-DESI imaging was used to map the spatial distribution of nicotine in rat brain tissue by adding deuterated nicotine as a standard.[75] Nicotine was quantified with subfemtomole sensitivity in each brain area even though it was low abundance. Small molecule neurotransmitters were also recently imaged in rat brain tissue.[76] Glutamate, GABA and acetylcholine were accurately mapped and quantified by doping the nano-DESI solvent with selected deuterated internal standards. Thus, nano-DESI MS has potential for the study of molecules in biological samples with high spatial resolution and sensitivity, providing enhanced opportunity for investigating the changes and the distributions of analytes related to health and disease.

MS conclusions—Imaging MS is a powerful tool for the detection and visualization of molecular species in tissues and cells, including proteins, lipids, and neurotransmitters. MALDI is mainly used for high molecular weight compounds, such as peptides and proteins, although it has been adapted for lipids and small molecule neurotransmitters. DESI is an ambient MS technique useful for drug, lipid, and small molecule neurotransmitter imaging. SIMS is best suited for small molecules, such as lipids and metabolites, but provides higher spatial resolutions. These MS techniques provide spatial information of molecules of interests with high specificity and the main advantage of MS is always the chemical identification that can be made, particularly with tandem MS detectors. Challenges associated with matrix effects, sample preparation, and data acquisition still need to be fully addressed. For example, deuterated standards are helping improve quantitation but those standards are expensive, difficult to work with, and may be impractical for certain applications. MS imaging is currently best for *in vitro* studies of tissue samples. However, newer applications, such as defining tumor borders with DESI, could push MS imaging into more *in vivo* applications.

Imaging conclusions and perspectives

Advances in imaging have provided a variety of tools to assess chemical change in the brain. These techniques include fluorescent indicators, fMRI, PET and MS. Fluorescence has been a very popular technique because it has both high temporal and spatial resolutions compared with other imaging techniques and does not damage the tissue. GECIs and GEVIs enable subsecond tracking of calcium and membrane changes, but exciting developments in NeuroSensors and FFNs are allowing real time tracking of neurotransmitter function for the

first time. fMRI is a non-invasive technique, making it favored for functional imaging of brain activity in human. Recent advances are pushing fMRI past the traditional BOLD measurements into the molecular range, and will allow better determination of molecular changes associated with brain activity. PET also can be used for whole brain imaging, and the recent combination with new genetic techniques, such as DREADD, allows imaging of the effect of turning off specific receptors in the brain. Indeed, many of the recent advances in genetics are useful in combination with imaging methods because they facilitate either specific insertion of probes into given neurons or control of different cells by light (optogenetics) or chemical activation (DREADD). MS imaging provides a wealth of chemical information from a tissue sample for the detection of proteins, lipids, peptides, metabolites, small molecules, and drugs with high spatial resolution. Ongoing challenges for MS include expanding the array of compounds detected, increasing the speed of measurements to acquire large images faster, and developing accurate calibration methods. Each imaging technique has advantages and disadvantages and all approaches reviewed here would benefit from multiplexing, which could provide a better understanding of brain activity and the resultant chemical changes.

Sampling Methods, Separations, Microfluidics

For some neurochemical assays, a sample is taken and a separation is performed to analyze compounds. The greatest advantage of separations is that multiple analytes can be monitored simultaneously. Research is typically focused on extracellular levels of neurochemicals and how those levels change over the course of minutes to hours. Sampling methods are also important, as the method used to take the sample can influence the neurotransmitters detected. This section will cover a few different sampling techniques, such as microdialysis and push-pull perfusion, and methods commonly used to separate neurochemicals, including chromatography, capillary electrophoresis and microchip electrophoresis. Microfluidic platforms to grow neurons and integrate analyses are also discussed.

Sampling Methods

Microdialysis—Microdialysis is the most common technique for sample collection prior to separation. Microdialysis probes consist of an inlet tube and an outlet tube in a single shaft. Perfusate flows through the inlet tube to the tip of the probe, which is covered with a semi-permeable membrane. Small molecules diffuse in and out of the probe while larger molecules, such as proteins, are blocked. The perfusate is collected and subsequently analyzed, usually with a separation technique. Major research areas in microdialysis currently include preventing damage from probe insertion, decreasing the size of the probe, developing advanced calibration methods, and segmenting the outlet flow into droplets to improve the temporal resolution.

Most microdialysis probes are 200–400 μm in diameter and implantation in the brain leaves a noticeable wound track in the tissue. Implantation also generates a rapid immune response, which can impact the types and concentration of neurochemicals measured in the extracellular space. The Michael group has been characterizing and working to combat the immune response due to probe implantation in rats. Inserting a microelectrode near a

microdialysis probe reveals that electrically stimulated dopamine was eradicated near the probe due to damage.[77] However, administering dexamethasone via retrodialysis recovered about 25% of the evoked dopamine response (Fig 4). Additionally giving a 20 mg/kg dose (i.p.) of nomifensine further improved the dopamine response to 80% of its pre-implantation response. Dexamethasone also resulted in strong tyrosine hydroxylase labelling recovery, indicating that the implantation did not do permanent damage to dopaminergic terminals.[78] This was later proven by allowing the rat to recover for 5 days after probe implantation.[79] Microglial activation is one of the fastest immune responses to the brain injury caused by the microdialysis probe, although this only happens within about 300 μm of the injury site. Dexamethasone decreased the distance of microglial activation down to about 200 μm, making the most significant impact 50–175 μm away from the probe. It also dramatically decreased the T-stage morphology of microglia in this range, implying that microglia are less likely to congregate around the injury.[79,80]

Decreasing the size of the sampling probe is another way to minimize tissue damage. The Kennedy group has recently microfabricated smaller microdialysis probes that were 160 μm wide and 45 μm thick.[81] Probes were microfabricated from silicon and the porous membrane consisted of an anodic aluminum oxide layer over an etched polysilicon layer connected to the perfusion channels. The probes were batch fabricated with 50–70 successful probes per wafer and the porous membrane was structurally stable over the open microfluidic channels. While there was significantly less tissue damage with this microfabricated probe, the analyte recovery was less than 10% for most of the analytes tested because of the small size. However, it was still able to detect changes in analyte concentrations during amphetamine administration and the percent change detected by the microfabricated probe matched those of the traditional microdialysis probe. Microfabricated probes can be much more reproducible than handmade probes and provide an easy route to customized probe designs.

The Kennedy group also developed a method for the calibration of microdialysis measurements *in vivo*.[82] A stable-isotope labeled (SIL) neurotransmitter was included in the perfusate to perform retrodialysis. Any SIL that did not cross the probe membrane was collected with endogenous neurotransmitter that crossed the membrane in the dialysate and was subsequently derivatized and analyzed by ultrahigh performance liquid chromatography (UPLC)-MS. The ratio of SIL recovered to SIL perfused is inversely proportional to the ratio of endogenous neurotransmitter collected to neurotransmitter in the extracellular space. This correlation allows actual extracellular concentrations to be calculated independent of probe efficiency and flow rate.

Another interesting development in microdialysis is to directly deposit cells on the probe membrane. Bowser's group has deposited astrocytes directly on the microdialysis probe and the proximity of the cells to the probe means that released analytes were not as severely diluted by diffusion. This limited diffusion has allowed for a sampling time as little as 20 seconds.[83]

With online microdialysis, the collected dialysate is flowed through a capillary, often for meters, before it is analyzed. During this flow, molecules can diffuse laterally, thus decreasing the temporal resolution of the measurements. In order to combat lateral diffusion,

one strategy is to segment the flow into droplets directly after collection. Aqueous droplets of dialysate are segmented with a hydrophobic oil, like perfluorodecalin. Once segmented, these drops can flow long distances and there will not be any spreading due to lateral diffusion. For example, 32 nL droplets were made of dialysate and acetylcholine concentrations were measured in each droplet with a detection limit of 5 nM.[84] A similar strategy is used for clinical microdialysis in humans, where the detector must be placed a few meters away from the bedside.[85] Advances in both minimizing tissue damage and increasing the temporal resolution of microdialysis will make it more useful for both preclinical and clinical applications.

Push-pull perfusion—Another sampling technique that has recently received attention is low-flow, push-pull perfusion sampling (LFPS). LFPS uses probes that are slightly smaller than microdialysis probes and in turn, causes less tissue damage. However, the absence of a size selective membrane allows enzymes and other macromolecules to be collected as well, which may require additional sample manipulation prior to separation and analysis. LFPS has been used to investigate sex differences in amino acid concentrations in the mouse brain with detection limits around 190 nM.[86,87] A new form of this probe is the push-pull theta pipette which has a diameter as small as 1.2 μm. Sample was pulled through one barrel of the theta pipette while perfluorodecalin was pushed through the other. Similar to Kennedy's segmented flow method, this creates alternating plugs of sample and perfluorodecalin in the first barrel with controlled volumes. Once several sub-nL plugs are collected, the theta pipette was removed and the samples were analyzed via electrospray ionization (ESI)-MS.[88]

Sampling conclusions—Sampling methods have been recently improved by focussing on improving the temporal resolution and reducing the damage of the implanted probes. Microdialysis continues to be a workhorse method for sampling from the brain because it is easily coupled to many separation and detection techniques. The newer studies that segment the microdialysis flow into droplets will dramatically improve the time resolution because no diffusion takes place on the way to the detector. This droplet method has been applied to both microdialysis and push-pull perfusion, but one challenge is that it requires detection strategies that are not contaminated by the hydrophobic oils. Segmented flow has been coupled with biosensors and ESI but not yet classical separations like HPLC and CE because the volumes are small. Work on reducing the probe size will also diminish the microglia activation and immune response. Coupled with pharmacological strategies such as dexamethasone, future studies with microdialysis might not be as clouded by the concerns of tissue damage. LFPS probes have also been minimized and the low flow rates minimize damage. The goal of sampling continues to be to collect a sample as rapidly and with as little damage as possible in order to get a good idea of the chemicals present in the brain. These methods are routinely coupled with separations, as described in the following section.

Separations

Liquid chromatography—Chromatographic techniques separate analytes based on their interactions with a mobile phase and a stationary phase. Depending on the compounds of interest, separations are based on factors such as polarity, boiling point, size, and/or sterics. Many of the significant advancements to chromatography in neuroscience are related to

increasing its temporal resolution to enable coupling to online microdialysis. Traditionally, microdialysis samples were analyzed offline; that is, samples are collected from the microdialysis probe and stored until analysis was carried out on a separate instrument. While storage allows for sample processing between collection and detection, low flow rates generate small volumes and it is difficult to work with less than 1 μL offline. Online analysis often requires that smaller sample volumes be tested rapidly, and thus requires techniques with improved temporal resolution and sensitivity.

The Weber group developed fast chromatographic separations of dopamine using capillary liquid chromatography (cLC) with electrochemical detection (EC). Operating at a higher temperature (48°C) allows for a faster elution and separations on the minute time scale.[89,90] This chromatography method had a lower detection limit (0.15 nM) than fast scan cyclic voltammetry for *in vivo* dopamine transients (12 nM)[91] and a 1 minute temporal resolution, but the temporal resolution was still not high enough to characterize individual transients, as many happened within a few seconds of each other. The method allows for rapid monitoring of basal changes in dopamine. A similar study on serotonin also pushed the temporal resolution down to 1 minute.[92] These rapid chromatography techniques are beginning to be applied to measure real biological changes, for example changes in serotonin due to circadian rhythms[90] or the estrous cycle.[93] These studies had 2–3 minutes temporal resolution and measured serotonin changes over the course of up to 18 hours. Rapid chromatography is growing as a way to analyze microdialysis samples on a fast time scale and one advantage is that it can be performed using commercial instrumentation.

A major advancement in HPLC is the incorporation of temperature-assisted on-column solute focusing (TASF). Temperature at the capillary inlet is kept low during the injection (−7.5°C) then quickly raised during the separation. This maximizes gradient compression, focusing the analytes and combating volume overload. Over the last couple years TASF instrumentation and procedures have been optimized for sensitivity, peak resolution, and retention time using tunable UV detection. TASF also works for isocratic or gradient elution and can be performed on small molecules or peptides.[94,95]

Recent HPLC studies use a wide variety of detection methods for the quantification of neurotransmitters. HPLC-EC has been used to measure dopamine, serotonin, epinephrine, norepinephrine, and their precursors and metabolites in rodent brain homogenates with LODs in the low nM range.[96,97] An HPLC-MS method separated 17 total neurotransmitters (primarily amino acids and monoamines) from zebrafish homogenates across 8 time points of their life cycle and achieved low to sub-ng/mL LODs for all of the analytes.[98] Possibly the largest number of neurochemicals quantified with a single separation procedure was an HPLC tandem mass spectrometry (MS/MS) method developed by the Kennedy group.[99] Analytes were derivatized with benzoyl chlroride and 70 different neurologically relevant compounds were monitored simultaneously (Fig 5). They analyzed rat microdialysate, human cerebrospinal fluid, human serum, *Drosophila* heads and *Drosophila* bodies. All but four of the compounds were above the LOD in at least one of the samples and the LOD was in the low to sub-nanomolar range for most of the compounds. The derivatization procedure only requires seconds to react at room temperature, and the reactants are less prone to

degradation. Thus, HPLC is very useful for measuring complicated mixtures and getting a good idea of chemical interactions.

LC-MS has been routinely used for neuropeptides, but a major problem is poor recovery from the sample collection and preparation stages. Sample loss is due to strong electrostatic interaction between the peptides and hydrophobic surfaces, such as sample vials or the microdialysis probe itself. Coating the microdialysis tubing and membrane with polyethyleneimine (PEI) as well as increasing the molecular weight cutoff of the membrane improved the detection limits of neuropeptides by more than an order of magnitude [100] Adding an organic solvent (acetonitrile) to the dialysate improved recovery as well. These had a combined LOD improvement of up to 800-fold, though those values are highly structure dependent as this treatment favors positively charged peptides. Future work could focus on improving recovery for anions.

Chromatography, primarily HPLC or UPLC, remains one of the popular techniques employed in neuroscience research. HPLC has more reliable and reproducible results than capillary electrophoresis and can easily be coupled to a variety of detectors.[92,101] Commercial equipment can be used; for example the 1 minute separations by the Andrews group were performed using commercial equipment.[93] Derivatization of small molecules with benzoyl chloride allows many compounds to be detected simultaneously. EC and MS detection predominate since they are sensitive enough to detect physiological concentrations and require little sample preparation. UV detection is used in neuroscience research, though typically for drug metabolism studies.[102] LC does have the drawback of requiring larger sample volumes than CE, but that has been combatted by the implementation of cLC and coupling it to microdialysis online.[94,95]

Capillary electrophoresis—Capillary electrophoresis (CE) is a technique that separates molecules based on their electrophoretic mobility, which is primarily determined by their charge and size. CE is naturally suited as a separation method for neurochemistry due to its rapid separations, high resolving power, amenability to small sample volumes, and easy coupling to a wide variety of detection techniques.[103] An excellent review of CE advancements in neuroscience was recently published by Crego and Marina, which includes a chart of detection limits of various samples using a wide variety of detection methods.[104] This section will primarily highlight studies published after that review and techniques where the CE field is advancing.

Laser-induced fluorescence (LIF) has long been the most sensitive detection method for CE, but it suffers from requiring time consuming derivatization processes. One new fluorophore that helped combat this obstacle was 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-F), which had a highly efficient reaction time of 5 minutes with primary and secondary amines.[105] Other fluorophores were designed to improve the sensitivity of a specific analyte include 2,4,6-trinitrobenzenesulfonic acid (TNBS) for the derivatization of adenosine and inosine or 1,3,5,7-tetramethyul-8-(*N*-hydroxysuccinimidyl butyric ester) difluoroboradiaza-*S*-indicene (TMBB-Su) for neurotransmitter amino acids.[101,106] Micellar electrokinetic chromatography (MEKC) is a form of CE that is frequently used because a surfactant is added to separate neutral molecules. After derivatization with TMBB-Su, glycine, alanine,

γ-aminobutyric acid, taurine, glutamine, glutamic acid, and aspartic acid were cleanly separated using a cationic surfactant with MEKC.[106] A primary advantage of CE separations is their speed, and CE-LIF separations can be performed on the 10 second time frame. For example, salamander retinas were prepared and dopamine, D-serine, L-serine, phosphoethanolamine, glutathione, 2-aminoadipic acid (internal standard), glutamate, and L-aspartate were separated using microdialysis with an online derivatization for CE-LIF. In order to achieve 10 second temporal resolution, the injections were overlapped such that the final 5 analytes from the first injection would elute during the second file. This eliminated the 7 seconds of baseline at the start of the electropherogram due to transport down the capillary upon injection.[107]

While fluorescence detection is commonly used with CE due to its sensitivity, it frequently requires costly or time-consuming derivatization steps.[97] Deep-ultraviolet excitation is a way to avoid this obstacle as many neurologically relevant analytes will fluoresce under short wavelengths (~224nm).[103] However, it has not been incorporated frequently due to high levels of scattering on the capillary, which dramatically lowers the sensitivity. A new elliptical fluorescence collection cell was demonstrated to minimize Rayleigh scattering, focus emitted photons on the detector, and increase the collector efficiency. LODs for tyrosine and serotonin with this elliptical cell were 30 and 4 nM, respectively. Another alternative to LIF is pressure assisted large-volume sample stacking with capacitive coupled contactless conductivity detection. Capacitive coupled contactless conductivity detection had detection limits for GABA, glycine, and glutamate of about 10 nM for calibrations and 30 nM in real samples. This method is less expensive than CE-LIF though not quite as sensitive.[108]

Enantiomeric separations have been a recent focus in neuroscience, particularly since many amino acids serve as, or are metabolic precursors to, neurotransmitters. One study used HP-β-cyclodextran and M-β-cyclodextran to achieve baseline separation on all enantiomers in the dopamine metabolic pathway, with exception of L-DOPA and D-DOPA, using MS detection.[109] However, the LOD for most of the analytes (40–150 nM) were higher than the expected concentrations in the rat plasma sample used.

CE is sensitive enough to detect neurotransmitter content in a single fly brain. The Ewing group incorporated freeze-drying as a sample preparatory step for analysis of fly neural tissue using MEKC-EC.[110] Freeze-drying allowed for faster dissections, less time for sample degradation, and was more amenable to pooling samples to concentrate the analytes of interest. They quantified how much of a drug (e.g methylphenidate) was in the brain after feeding different doses with CE-ESI-MS.[111] Metabolite structures suggest methylphenidate is broken down by different pathways in *Drosophila* than in mammals. Our group has employed CE with fast-scan cyclic voltammetry (FSCV) detection to determine the neurotransmitter content of a single fruit fly brain. We characterized changes in neurotransmitter content of fruit flies between life stages, and explored genetic mutations to investigate metabolic processes such as the β-alanylation of dopamine and histamine.[112,113] While not as sensitive as LIF, FSCV is a powerful detection technique for CE because the resulting voltammograms allow for analyte identification, while still being less expensive than MS.

Microchip electrophoresis (MCE) is a primary application of microfluidics in neuroscience research and one of the most popular separation techniques for neurotransmitters. It compliments microdialysis and push-pull perfusion sampling techniques, particularly when a high temporal resolution separation is required. MCE accommodates smaller sample sizes than HPLC or CE and the chip can be engineered to include online sample processing such as derivatization for LIF detection. Most detection methods that are typically used with CE have been coupled to MCE as well, including LIF, EC[114], and MS[115]. The Liu group has expanded upon their previous work on MCE-MS.[116] The chip was redesigned to include on-chip stimulation of PC-12 cells with KCl or with acute ethanol stimulation.[117] Aspartate, glutamate, dopamine, and serotonin were simultaneously measured over several minutes and each showed a different release profile, suggesting that the analytes have separate vesicular pools within the neuron.

Separations conclusions—In conclusion, the temporal resolution of separations is greatly improving. Chromatography can now performed, with the aid of higher temperatures, on the 1 minute time scale. CE-LIF can measure amino acid neurotransmitters on a 10–15 second time scale. Derivatization methods are often used for both methods, to enable simultaneous detection of many molecules. Increasing the temporal resolution of the method therefore requires faster derivatization techniques. EC and MS detection techniques are used with both HPLC and CE while LIF is also popular for CE. The detector choice is obviously influenced by the molecules of interest and the main advantage of separations continues to be the ability to separate many things at once. Microchip electrophoresis allows for minimization and automation of not only the separation procedure, but sample treatment and detection as well. Advances in microfluidics as an integrated platform for analysis are covered in the next section.

Microfluidics

The application of microfluidics in neuroscience research stretches beyond that of microchip electrophoresis. Several devices have been designed to allow axons of cultured neurons to traverse a channel between 2 separated chambers.[118,119] This feature, along with microfluidics' strong synergy with imaging techniques, has allowed researchers to investigate neuronal cell differentiation and migration[120] as well as the transport of vesicles, their contents, and relevant synaptic proteins.[121]

Microfluidic devices for neuronal growth—The strategy of having axons traverse a channel to a separate chamber has recently been advanced in a few ways. One device used carbon-black polydimethylsiloxane-PDMS to make each chamber light isolated such that somas or axons could be optogenetically stimulated separately without concerns of scattered light affecting the opposing chamber. That study showed that axonal myelination can be triggered optogenetically whether the soma receives the stimulation or the axons receive the stimulation.[122] Another design incorporated detachable PDMS layers to accommodate whole-cell patch clamp experiments.[123] The Huang group also developed a chip that applies chemical gradients of varying slopes to a series of microgrooves containing axons to study axonal development.[124] The axon-in-channel technique is exciting in the microfluidics field as the advances described above provide a unique platform to investigate questions about

axonal development and neurotransmission.[124] These advances in microfluidic engineering are also exciting because they not only accommodate techniques such as electrophysiology and optogenetics, but they open the door to the implementation of other techniques as well. The removable components of a PDMS chip could make it easier not only for patch clamp probes to access the cell, but also for other probes such as biosensors or microelectrodes. Additionally, the light isolated chambers of a microfluidic device could enable far more complex neural circuitry experiments with optogenetic stimulation. Another device has been developed along these lines, with cultured neurons and astrocytes in a grid of separated compartments, although instead of optogenetics, they used glutamatergic stimulation.[125] Ultimately, these microfluidic devices set up the framework to assemble unique neural networks to better understand how such circuits function.

Microfluidic platforms coupled to other techniques—The Sweedler group developed a simple microfluidic capillary that contains a neuron culture near its tip. A KCl solution was pulled into the capillary to trigger neuronal depolarization and the releasates were continually pulled to a particle-embedded monolithic capillary which would trap neuropeptides for subsequent MALDI-MS analysis.[58] The method allowed for facile coupling to liquid chromatography or ESI-MS. The study also discovered that polyimide is very compatible for growing neuronal cultures.

Microfluidics have also been used to advance brain slice experiments. A PDMS add-on component for a brain slice perfusion chamber was implemented to gain better control of oxygen delivery to the brain slice. Because PDMS is permeable to many gases, they used PDMS to deliver varying concentrations of oxygen below the slice. Hypoxic conditions could be applied to the slice with 500 μm spatial resolution and in a shorter time frame than buffer perfusion alone.[126] This approach was also used to aide in dye delivery to the brain slice for calcium imaging. The device's efficiency of oxygen delivery improved tissue viability, allowing for longer dye-loading times of up to three hours.[127]

The Boutelle group devised a microfluidic chip coupled online to microdialysis that incorporated an ion selective electrode for potassium and a glucose biosensor.[128] K^+ and glucose were detected in tandem to track the passage of a spreading depolarization wave imposed by a traumatic brain injury. The K^+ signal was used to determine the initiation of an spreading depolarization wave and the glucose signal was used to track metabolic activity with high temporal resolution. Thus, microdialysis sampling and microfluidic detection are useful for human treatment, to inform doctors of brain neurochemical changes in trauma patients.

Microfluidics conclusions—Microfluidics is an incredibly broad field, even in the application of neuroscience. Typically, microfluidic devices are implemented to integrate multiple processes; for example, the axon-in-channel technique can be integrated with optogenetic stimulation, electrophysiological measurements, or imaging. Researchers can control the growth of the axon such that somatic and axonal processes can be studied in a segregated manner. Microfluidic devices can also be designed to improve the experiments, such as the PDMS devices that deliver oxygen to brain slices with high spatial resolution.[127] Chips with removable components could be used in the future to integrate even more

techniques on one device or integrate devices to other instruments, such as MS. Microfluidic devices are inexpensive, increasingly adaptable to a variety of experimental needs, and amenable to small sample sizes.

Sampling, separations, and microfluidics conclusions and perspectives

Separations are frequently necessary in analytical neurochemistry because of the complexity of the sample. The advances made to sampling techniques have improved both the temporal resolution of the separation procedures and the viability of the tissue being sampled. Ultimately, the goal in microdialysis research is to make the sampling probe as small as possible without losing recovery efficiency. Fortunately, the application of dexamethasone alleviates some of the damage caused by these probes and enables longer term experiments to be performed.[80] Push-pull perfusion is an appealing alternative to microdialysis due to its size, but it frequently requires additional sample treatment steps prior to analysis and the small probes can clog. LC and CE methods have improved in both speed and resolving power. CE is faster than LC and has a higher resolving power. However, CE is not as reproducible as LC and also has trouble separating neutral compounds, although MEKC has helped combat this. Kennedy's LC-MS/MS method with benzoyl chloride derivatization, is possibly the most powerful separation demonstrated for neurochemistry, as over 70 different neurologically relevant compounds were separated, quantified, and identified in 17 minutes.[99] Separations can be performed on microfluidic devices or microfluidic devices can be used as a platform for neuronal growth or as a brain slice chamber. Microfluidic electrophoresis can accommodate smaller sample volumes than LC or CE and small microfluidic devices are used to produce the segmented flow that prevents diffusion of samples after collection. Separations and microfluidic devices enable measurements of the chemical complexity of brain samples. While they are typically not on as rapid a time scale as imaging or electrochemistry, the wealth of chemical information that can be obtained simultaneously makes them one of the most popular techniques for neuroscience research.

Electrochemical Detection of Neurotransmitters

Various electrochemical techniques have been applied to monitor neurotransmitter release in living tissues including amperometry, cyclic voltammetry and potential pulse methods. Detection of neurotransmitters is generally through oxidation or reduction of the target species at the electrode surface. The corresponding currents generated provide a quantitative measure of dynamic chemical changes. Biosensors can be made, incorporating a selective sensing element to recognize the analyte and create an electroactive signal, but those are outside the scope of this review. Here, we focus on three areas of electrochemical techniques for neuroscience: (1) ongoing investigations and technique development to understand the exocytosis at single synapses, (2) *in vivo* neurochemical monitoring using fast-scan cyclic voltammetry (FSCV) in rodent and nonrodent species, and (3) new electrode fabrication methods for *in vivo* neurochemical monitoring.

Electrochemical detection of exocytosis

Exocytosis is a complex biochemical process, occurring in many different types of cells. Understanding exocytosis is vital to understanding the process of chemical intercellular

communication. During exocytosis, biochemical messengers such as neurotransmitters, hormones, or neuropeptides, which are stored in vesicles, are secreted after the vesicle fuses to the cell membrane and releases its contents.[129] Research on the mechanisms controlling the initiation of exocytosis was recognized recently with the 2013 Nobel prize in physiology and medicine, awarded to James E. Rothman, Randy W. Schekman and Thomas C. Südhof.[130–132] The concentration of neurochemicals is high in a single vesicle, but the small size of a vesicle means the absolute amount of neurotransmitter released is on the order of femto to zeptomoles. Hence, measuring exocytosis requires highly sensitive techniques with good temporal resolution. While fluorescent imaging techniques have been used with the aid of fluorescent intravesicular markers,[133] the traditional technique for probing exocytosis is amperometry. In this section, we discuss the most recent advances in the field of exocytosis measurements using electrochemistry, including new evidence about the amount of neurotransmitter released per event. We further discuss new technical advances in microfabrication techniques of arrays that allow to the monitoring of multiple exocytotic measurements simultaneously.

Exocytosis at different cell types—Electrochemical techniques such as amperometry and cyclic voltammetry have been widely used to study vesicular exocytosis with millisecond time resolution. Electrochemical monitoring of neurochemicals using carbon-fiber microelectrodes (CFMEs) is a powerful way to selectively measure released chemicals at the single cell level.[134] The microelectrode is typically positioned on top of a cell to accurately measure released electroactive species without diffusion away from the cell surface. The micron-sized CFMEs fits well with traditional cell models such as PC12 cells, chromaffin cells, or neurons but new research pushing to smaller cells such as platelets requires smaller electrodes.[135–137] The most popular electrochemical method is amperometry, where the working electrode is held at a constant potential to oxidize the molecules of interest, and the oxidation current is measured as a function of time. Spikes of current are measured that correspond to release from a single vesicle and they can be integrated to determine the number of molecules released per vesicle.[138] Amperometry does not provide much information about the identity of the substance detected, but cyclic voltammetry can be used to provide a cyclic voltammogram fingerprint of the molecule detected.[139] Several reviews have documented using electrochemical techniques to understand exocytosis.[140–142]

Single-cell amperometry has been used to examine vesicular release from a variety of cell types. Catecholamine secretion from chromaffin and PC12 cells has been studied to investigate different modes of exocytosis.[143] Fig.6 shows a schematic of the microelectrode setup for single cell amperometry, the mechanisms of release, and a traditional amperometric spike.[142] A precise analysis and processing of the amperometric peak parameters, involving a cluster analysis and Gaussian fitting, revealed the presence of three different clusters corresponding to 3 different exocytotic modes. The major mode was apparent full distention that accounted for 70% of the released catecholamine, where the vesicle was fully integrated into the membrane. The other two modes, partial membrane distention and kiss-and-run, accounted for 20% and 10% of the released catecholamine content, respectively. Recently, the exocytotic release of serotonin from human carcinoid

BON cells, a cell line derived from a metastatic human carcinoid tumor of the pancreas, was measured for the first time and compared to events at chromaffin cells.[144] Amperometric spike amplitudes for serotonin from BON cells were low although BON cells contain similar dense-core vesicles (200–300 nm diameter) to chromaffin cells. The low amount of serotonin secretion indicates that different cell types release different amounts of neurotransmitter, despite the similar size of the vesicles. The Haynes group investigated the exocytosis of platelet granule contents using amperometry to study the quantal secretion behavior of serotonin release from single rabbit platelets.[145–147] The role of dynamin-related protein-1 on fusion pore stability was examined by monitoring full fusion events and control events. Blocking dynamin-related protein-1 inhibits platelet granule exocytosis and impairs the fusion pore stability.[135] Endogenous serotonin release from human blood platelets was studied using FSCV.[139] Real-time serotonin release from thrombin-stimulated human platelet suspensions was similar to single platelet measurements. The method gives insight into the fundamental platelet secretion process and may replace the current gold standard [^{14}C]-serotonin assay for serotonin measurements because it is much faster and avoids radioactivity.

Single-cell amperometry and electrochemical cytometry—Traditionally, the dogma in the field has been that amperometric spikes were due to full fusion and release of the full contents of a vesicle. But in the past decade, several research groups have presented evidence that amperometric spikes may not be due to full release and that most of the time the fusion pore closes before all the vesicle content is expelled.[148–151] To quantify the amount of neurotransmitter in a vesicle, the Ewing group developed a new microfluidic device with electrochemical detection, called electrochemical cytometry.[152] This is a hybrid microfluidic-capillary electrophoresis-electrochemical detection platform that separates individual vesicles by capillary electrophoresis so that vesicles elute one at a time from the channel. An electrode at the end detects neurotransmitters from each vesicle as they break in contact with it. Multiple transients were recorded, each corresponding to the total catecholamine content of a single vesicle. The total content measured with cytometry was significantly higher than the amount of catecholamine released during exocytosis, indicating that exocytosis does not result in complete expulsion of transmitters in a vesicle. In a different study, PC12 cell vesicles released only 40 % of their total catecholamine content, indicating that the pore closes again before all the vesicular content has been released, and that the vesicle is then most often recycled and releases again.[153] A very recent study by the Amatore group on quantal release of neurotransmitters confirms that under normal exocytosis conditions, the initial release is not the total content.[154] Their amperometric results indicate that vesicles release at most half of the initial loading of the neurotransmitters during control exocytotic events, in agreement with the Ewing group, although they used a different methodological approach.[153] Partial release was further supported by studying the roles of dynamin[155] and actin[156] in the exocytotic activity of PC12 cells. Amperometric spike analysis revealed that inhibition of dynamin leads to shorter and smaller spikes because of a narrow and short-lived fusion pore. The action of dynamin in PC12 studies agreed with studies of its action in platelets.[135] Tampering with the actin sub-membrane cytoskeleton polymerization/depolymerization revealed that actin is involved in regulating exocytosis, by mediating the constriction of the pore. The Amatore group also

recently reported a theoretical model to understand the partial mode of neurotransmitter release during exocytosis.[157,158] Thus, through new techniques, careful analysis of data, and theoretical modeling, a paradigm shift is occurring in the exocytosis field from the idea that vesicles release their full contents to the idea that the release is only 30–50% of the content before the fusion pore closes. Electrochemistry continues to provide new insights into the mechanisms of exocytosis signaling.

Microelectrode arrays for quantal exocytosis—In the past decade, due to advancement in microfabrication procedures, researchers have increasing developed microelectrode array (MEA) systems as an alternative to single CFMEs, particularly in the electrochemical study of cellular release.[159,160] MEAs offer many advantages such as high spatial resolution and simultaneous recordings of exocytotic events from single cells or cell clusters using individual microelectrodes in the array. The Gillis group fabricated an MEA with 36 parallel gold ultramicroelectrodes with 20 μm diameter wells/electrodes.[161] Each planar electrode can be used to electropermeabilize a single chromaffin cell and also to measure the neurotransmitter release during exocytosis. Very recently, an MEA of 60 circular microelectrodes with a diameter of 20 μm modified with poly L-lysine and polydopamine was designed for monitoring dopamine release from single PC12 cell.[162] Modification of the electrode surface with a biocompatible polymer improved the sensitivity for dopamine release.

MEAs are often integrated into microfluidic devices to allow on-chip detection of exocytosis. For example, the Gillis group has also developed a microfluidic cell trapping device to measure single cell exocytosis.[163] A PDMS device was used to trap the cells and exocytosis measurements of single cells or clusters of chromaffin cells were measured using an indium tin oxide MEA. Another recent study reported a microfluidic device for real-time, quantitative chemical investigation of dynamic mechanisms during exocytosis at artificial cells-on-paper samples.[164] PC12 cells were spotted on a patch of filter paper, and dopamine release was measured electrochemically. The results are similar to data reported for single cell experiments. Since carbon-fiber microelectrodes cannot be easily integrated on to the chip using microfabrication methods, the Vakushenko group described the fabrication of a disposable microdevice containing printed carbon microelectrodes.[165] Screen-printed carbon paste electrodes modified with PDMS films were successfully used to monitor the dopamine release from PC12 cells. The Wang group developed a thin-film ultramicroelectrode array with 16 to 36 square microelectrodes in the MEAs of and an electrode size of 2–4 μm.[166] Catecholamine containing PC12 cells were cultured directly on the chip based platform to facilitate the parallel measurements of release events amperometrically. MEA based systems enable the measurements of multiple events simultaneously, facilitating insight into coordinated mechanisms of release. MEAs also increase throughput compared to existing methods.

Exocytosis conclusions

Electrochemical techniques are the predominant method to measure neurotransmitter release at single cells. Single cell amperometry is useful for studying mechanisms of catecholamine release and events have been compared in a variety of different cells. Much recent work has

centered around the question of whether vesicles release their full content. The dogma had always been that each amperometric spike represented full fusion, but recent evidence shows most of the time the fusion pore closes before all the vesicle content is expelled. This new finding of partial release was made possible by technique development; the independent technique of electrochemical cytometry was needed to accurately measure vesicular content. The finding was also solidified using theory and modeling of the fusion pore dynamics.[167] Most of the current cytometry work has been on dopamine in PC12 cells so future studies should explore whether this partial release occurs in other cell models such as platelets or pancreatic beta cells. Moreover, this technique could be expanded to understand the factors that affect the fraction of release and the processes that control these phenomena. New advances in MEAs are multiplexing the amperometric measurements, allowing simultaneous measurements of exocytosis from different cells or different points on the same cells. These MEAs are easily integrated into microfluidic devices, providing platforms for cell growth or trapping and potential integration with techniques beyond electrochemistry in the future.

Fast-scan Cyclic Voltammetry (FSCV) detection of neurotransmitters and neuromodulators in the brain

Electrochemistry is one of the most widely used techniques for monitoring neurochemical changes in the brain due to its high spatial resolution and its ability to monitor chemical changes in real-time. Ralph Adams and coworkers, in the early 1970s, implanted graphite paste microelectrodes in the rat brain and demonstrated that electrochemistry could be used to measure changes in electroactive compounds in intact animals for the first time.[168] Wightman and coworkers popularized FSCV at carbon-fiber microelectrodes to measure electroactive neurotransmitters, such as dopamine, norepinephrine, epinephrine, serotonin, histamine and adenosine.[169] FSCV at microelectrodes facilitates high temporal resolution monitoring of release and uptake of neurochemicals on sub-second timescales. In this section, we focus on recent advancements in FSCV for neurochemical measurements, including technical advances in FSCV waveform modification strategies. We highlight new studies pushing electrochemical studies into smaller organisms, including zebrafish and *Drosophila melanogaster*.

FSCV for dopamine—In FSCV, a triangular waveform is applied to a microelectrode at a high scan rate and the electroactive species is rapidly oxidized and reduced at the electrode surface. As an example, the most common waveform for dopamine detection is scanning from −0.4 V to +1.3 V at a scan rate of 400 V/s every 100 milliseconds. Large background currents are subtracted out to provide a resulting cyclic voltammogram of Faradaic currents that is a signature fingerprint to identify the species detected. Corresponding peak currents are converted into concentrations using calibration factors obtained from known standard concentrations. The FSCV waveforms can be tuned for other compounds by altering the potential limits and scan rates.

Dopamine has been the primary molecule of interest for FSCV studies both *in vitro* and *in vivo*. FSCV in animal models, from brain slices, to anesthetized animals, to freely-moving animals, has been useful to understand the regulatory mechanisms controlling dopamine release and uptake and how these processes are related to disease states. A recent study in

brain slices showed how large doses of cocaine change dopamine uptake and release.[170] Stimulated dopamine release decreased in coronal brain slices of the nucleus accumbens (NAc) core, indicating reduced dopamine release and uptake following the self-administration of cocaine. FSCV was also used to measure evoked dopamine release in the core or shell of the NAc in mice.[171] The effect of kappa opioid receptor activation on cocaine reward and dopamine levels was tested in a time-dependent manner in reward-based learning. Advancement of FSCV application for studies in freely moving animals has been a major accomplishment, especially in the areas of drug addiction and reward-based learning. Dopamine signals were studied recently in freely moving rats in NAc core and shell to determine the role of dopamine in motivation and reinforcement learning.[172] Spontaneous dopamine fluctuations were recorded in freely moving rats exploring the test chamber and mean amplitude of the spontaneous transients was higher in NAc shell than in core. Pharmacological studies showed similar results of increased spontaneous transients after inhibiting dopamine uptake with cocaine. Thus, FSCV is useful for studying dopamine release in freely moving rats, and is useful for understanding the mechanisms of both stimulated and spontaneous dopamine release. Paired with pharmacology, electrochemical detection is enabling a better understanding for drug mechanisms and behaviorally evoked dopamine.

The Wightman group used a combined electrochemical/electrophysiological (echem/ephys) method coupled to iontophoresis to study pre- and postsynaptic activity of dopamine in awake rats.[173] The probe consisted of a 4 barrel assembly; one barrel housed the carbon-fiber microelectrode while the other barrels were used to introduce the drugs of interest by ionotophoresis. During echem/ephys experiments, the traditional dopamine waveform was used to measure dopamine changes but the waveform was applied only at 5 Hz, half the normal frequency, to allow ~180 milliseconds of electrophysiological recordings between the scans. Introduction of D1 and D2 dopamine receptor antagonists to NAc neurons changed the firing rate of medium spiny neurons both immediately and on a prolonged time scale. These early results demonstrate the utility of echem/ephys measurements in understanding both action potential firing and neurochemistry. Future studies can be expanded to behavioral studies.

FSCV studies have also been expanded recently into humans. By exploiting the routine surgical implantation of neuroprosthetic devices for deep-brain stimulation (DBS) in the treatment of Parkinson’s disease, the Kishida group recorded dopamine measurements with FSCV in human striatum for first time.[174] This study was proof-of-principle and conducted in a single human subject, who suffered from late-stage Parkinson’s disease and was undergoing elective surgery for DBS-electrode implantation into the subthalamic nucleus. Dopamine measurements were performed at a CFME, with the overall implanted electrode dimensions were matched to the DBS electrode. Another study in humans measured dopamine signals while the subject was engaged in a behavioral task, providing preliminary evidence that sub-second fluctuations in dopamine concentrations in the human striatum can cause prediction error signals.[175] As an invasive technique, FSCV is only used when another probe will be inserted or brain surgery performed. However, it has the potential to provide insight into the mechanisms of deep-brain stimulation and why it is effective. The small size of the electrode provides no more damage than a normal DBS probe.

Changes to traditional FSCV waveform to detect other compounds—Traditional FSCV waveforms for dopamine are not suitable to detect many electroactive neurochemicals, either due to limitations of the applied potential range or fouling of the electrode. Thus, many researchers are modifying FSCV waveforms to enlarge the classes of neurochemicals that can be detected. Examples of molecules that have recently been targeted include adenosine, oxygen, hydrogen peroxide, histamine, and peptides.

Our group has developed an FSCV method for adenosine by modifying the anodic limits of the traditional dopamine waveform, raising the voltage to +1.45 V.[176] Adenosine is a purine signaling molecule formed from the degradation of ATP. Spontaneous adenosine transients were detected for the first time *in vivo*, with a characteristic electrochemical signature of a primary oxidation peak at +1.4V and a second oxidation peak at +1.0 V in subsequent scans.[177] Recently the Budygin group also reported spontaneous adenosine release in rat striatum and motor cortex.[178] Although they used the same waveform, there is a discrepancy in the data as they did not observe a secondary oxidation peak. Their spontaneous transients were at much higher frequencies than previously reported and they also detected adenosine efflux in response to a brief tail pinch. Adenosine detection was also conducted in human seizure patients, since extracellular adenosine levels are known to rise during seizure.[179] Since traditional methods such as microdialysis cannot provide high temporal measurements, FSCV was used to detect adenosine in humans for the first time. This study was performed on epilepsy subjects who had been scheduled for an anterior temporal lobectomy, by placing the 5 μm CFME onto the cortical surface. The peak adenosine signal typically occurred after seizure termination, demonstrating that FSCV can be used to determine the role of adenosine in epileptic patients.

More elaborate changes to the waveform have been made to increase selectivity and allow multiplexed analyte detection. We developed a modified sawhorse waveform to discriminate between adenosine and two major interferents: H_2O_2 and ATP, which oxidize at similar potentials.[180] By holding the electrode at a switching potential of +1.35 V for 1 millisecond during the anodic scan, adenosine was successfully distinguished from ATP and hydrogen peroxide. More recently, we developed an FSCV adenosine/oxygen waveform to detect adenosine and oxygen changes simultaneously *in vivo* in the rat caudate-putamen.[181] This study modified previous waveforms that had extended the anodic limits to −1.4 V to detect oxygen reduction and dopamine oxidation.[182] For adenosine and oxygen, the electrode was scanned from 0 V to +1.45 V, then to −1.4 V and back to 0 V every 100 millseconds at a scan rate of 450 V/s. Simultaneous monitoring revealed that oxygen changes accompanied adenosine transients one-third of the time, and that the concentration of adenosine and evoked oxygen changes are correlated. This study, along with codetection of dopamine and adenosine,[183] show that FSCV can be used to study the fast neuromodulatory properties of adenosine.

FSCV has also been expanded to monitor reactive oxygen species. The Sombers group recently quantified both the generation of H_2O_2 and its modulation of dopamine dynamics in the dorsal striatum of an intact animal.[184] They developed a waveform for hydrogen peroxide that allows it to be detected selectively on the cathodic scan. Validation that the measured signal was H_2O_2 was performed by local microinfusion of H_2O_2 as well as a

glutathione peroxidase inhibitor that inhibits mitochondrial function, leading to increased levels of endogenous H_2O_2. Many electrochemical sensors detect hydrogen peroxide amperometrically, particularly biosensors where the H_2O_2 is a product of an enzymatic reaction, but this is the first approach to use voltammetry for H_2O_2 detection. FSCV is particularly useful *in vivo*, where amperometry is not selective enough to discriminate H_2O_2 from interferents.

The Hashemi group has developed a new waveform for histamine detection. They scanned from −0.7 V to 1.1 V, resting at −0.5 V, at a scan rate of 600 V/s. An oxidation peak for histamine was observed at around +0.3 V.[185] By applying this waveform, histamine was selectively detected in mouse premammillary nucleus. Although the results showed detection of histamine *in vitro* and *in vivo*, the mechanism of histamine oxidation is unclear. Most other studies have observed histamine at higher potentials, around 1.1 V, and so there is a discrepancy between this new waveform and older studies. More work is needed to elucidate the oxidation mechanisms of histamine.

Electroactive moieties present in peptide neurotransmitters were also exploited by applying modified FSCV waveform. Peptides are electroactive if they contain tryptophan, tyrosine, or cysteine residues. In collaboration with the Moenter group, our lab detected gonadotropin-releasing hormone release in mouse brain slices, where it acts as a neuromodulator.[186] FSCV has also been employed for the detection of methionine-enkephalin (M-ENK), a small opioid peptide.[187] Small opioid peptides are involved in reward processing, drug addiction, and pain perception.[188] M-ENK has an electroactive moiety of tyrosine, which, similar to tryptophan in gonadotropin-releasing hormone release, can cause fouling on the electrode surface. Thus the Sombers group developed a sawhorse waveform for M-ENK that reduces the fouling by using different scan rates for different segments of the scan and holding at the anodic limit for 3 milliseconds to clean the electrode surface.[187] Selectivity against other tyrosine-containing peptides was achieved with the oxidation peak for M-ENK occurring at +1.0 V. Since the oxidation peaks for catecholamines occur at more negative potentials, this novel approach can be applied for the simultaneous detection of catecholamines and M-ENK in living rat adrenal tissue.

Modification of the FSCV waveform is beneficial for detecting a variety of neurochemicals. Some compounds, such as adenosine and hydrogen peroxide, require extended anodic limits. Other compounds, such as peptides, look better at sawhorse waveforms, where the potential is held at the anodic limit for a few seconds. Extending the cathodic limit below −0.8 V enables oxygen detection, which can be detected simultaneously with either catecholamines or adenosine. Thus, modified waveform approaches have proven to be very successful for tuning the selectivity of microelectrodes for different neurotransmitters. Continued testing of these modified waveforms *in vivo* is necessary to ensure that redox potentials and responses to interferents are the same in the brain as in *in vitro* calibrations.

Neurotransmitter measurements in nonrodent models—Most of the *in vivo* electrochemical neurotransmitter studies have been performed in rodent models, such as mice or rats. This work has provided a fundamental understanding on neurotransmitter release in brain. In the last few years, FSCV measurements have been expanded to different

model organisms, including the fruit fly, songbird, zebrafish brain, and salamander. Many of these newer model organisms are smaller than rodents, which presents a technical challenge and motivates future developments of smaller probes.

Drosophila melanogaster, more commonly known as fruit fly, has become a popular alternative model organism because of its short lifespan and ease of genetic manipulations.[110] The Ewing group performed electrochemical studies in adult *Drosophila* brain for the first time, with exogenous application of dopamine to investigate the changes in the dopamine uptake in the protocerebral anterior medial region.[189] They further studied the effect of cocaine to block the dopamine transporter. In a recent study, they tested methylphenidate as a competitive inhibitor of the dopamine transporter and possible treatment for cocaine addiction.[190] Flies were fed with different concentrations of methylphenidate, and 15 mM methylphenidate effectively inhibited the action of cocaine. Our group has studied *Drosophila* larvae and monitored endogenous monoamine release from an isolated ventral nerve cord. Release was stimulated using optogenetics, with a light activated cation channel expressed in specific neurons. Stimulated, endogenous dopamine release was measured using FSCV that has similar concentrations to stimulated release in mammals, 810 ± 60 nM.[191] Similar measurements expressing the channel only in serotonergic terminals results in 280–640 nM serotonin detected from 2 to 30 seconds stimulations.[192] Clearance of exogenously applied dopamine has also been studied in the isolated ventral nerve cord.[193]

While fruit flies have neurotransmitters such as dopamine and serotonin that are similar to mammals, they also utilize some different neurotransmitters such as the phenolamines octopamine and tyramine. Octopamine release was detected in the ventral nerve cord of *Drosophila larvae* for the first time by optimizing the FSCV waveform to reduce octopamine fouling.[194] Stimulated octopamine release was compared with those obtained from puffing octopamine into ventral nerve cord (Fig 7). Octopamine release was further validated by pharmacological manipulations; the octopamine synthesis inhibitor disulfiram decreased release by 80%. The Ewing group reported octopamine exocytosis events at individual varicosities in the *Drosophila* larval by placing microelectrode at a neuromuscular junction.[195] Taking advantage of the peripheral localization of the neuromuscular junction to facilitate electrode access, octopamine release from target cells was monitored by amperometry after optogenetic stimulation. This method was able to estimate the content of the vesicles as well as to monitor changes after pharmacological manipulation. These electrochemical methods are enabling the fruit fly to be used as a neurochemical model for the first time. Future studies could examine genetic *Drosophila* models of diseases and the process of aging on neurotransmitter release. The challenge of fruit flies is selectively implanting the electrode in a discrete region, and this challenge is pushing the development of functional nanoelectrodes for *in vivo* electrochemistry.

Zebrafish share a similar anatomy to other vertebrates, including humans, and are a widely accepted model organism for behavioral genetics and neuroscience.[196] Voltammetric measurements of serotonin were made *in vivo* in the intestine of intact zebrafish embryos during early development.[197] Recently, FSCV recordings were performed in zebrafish brain for the first time.[198] Simultaneous releases of dopamine and histamine was measured after

stimulation of the telencephalon in sagittal slices of the adult zebrafish. Dopamine release was further validated pharmacologically using a selective dopamine reuptake inhibitor. In another exotic study, dopamine release in locomotor networks was recently measured in the salamander brainstem. These studies target how cell activation triggered locomotion.[199] FSCV has also been applied to an avian model system for the first time, to measure catecholamine release in the striatum of the European starling.[200] Dopamine-and-adenosine-related phosphotase-32 was used as a marker of dopamine regions in the starling striatum, to facilitate electrode placement, and the signal amplitudes were similar to rats. As starlings are seasonal breeders and show stark differences in their hormonal and behavioral profiles, they are a useful model to study seasonal differences in the dopamine system. Taken together, these studies show that other small model systems are of interest to the neuroscience community and the FSCV measurements are useful for nonrodent model systems.

Fast-scan adsorption voltammetry (FSCAV)—FSCV has been used to study fast changes in electroactive neurotransmitters but is not useful for studying basal levels because it is a background-subtracted technique. The Heien group developed a complimentary technique, fast-scan controlled-adsorption voltammetry (FSCAV), which enables direct measurements of absolute concentrations *in vitro*.[201] FSCAV measurements are conducted first by applying a traditional dopamine waveform at a very high speed of 1200 V/s at 100 Hz to reduce the amount of dopamine adsorbed to the electrode. Then the waveform is switched to a constant potential, typically at −0.4 V, for a holding time to allow new dopamine to adsorb. The FSCV waveform is then applied again to oxidize the amount of dopamine adsorbed onto the electrode surface. The background current generated during this step is removed through deconvolution techniques using an electrode response function determined in a buffer solution. The results showed a sensitivity of 81 ± 11 nA/μM for dopamine and a LOD of 3.7 ± 0.5 nM, which is sufficient for *in vivo* use. Initial FSCAV studies in the mouse striatum reported basal dopamine levels close to 100 nM.[202] FSCAV and FSCV can be alternated at the same electrode to allow monitoring of both slow and fast changes in dopamine at the same location.

FSCV conclusions—FSCV is one of the most powerful and widely used techniques to measure neurochemicals in brain, because of its high sensitivity and ability to measure chemical release on sub-second timescales. Dopamine continues to be studied and FSCV is a good technique to elucidate the functional role of dopamine in drug addiction or reward based learning in freely moving animals. FSCV at microelectrodes is also amenable for dopamine measurements in other model organisms, including the small brains of the fruit fly or zebrafish. Moving beyond dopamine, FSCV is being expanded to many other classes of molecules, primarily through the use of modified waveforms. While adenosine has been more extensively studied in vivo, much of this work is still in the developmental stage and will require more in vivo validation of the techniques. Modified waveforms have proven to be successful not only in selective detection of individual neurotransmitters but also in differentiating multiple analytes as well. FSCAV is enabling basal measurements and can be combined with FSCB to provide a piture of tonic and phasic dopamine changes. Altogether, FSCV remains a popular and useful technique for real-time in vivo neurotransmitter measurements.

Advances in microelectrodes for *in vivo* Applications

The carbon-fiber microelectrode was introduced in the 1970's and it has been popular for *in vivo* electrochemistry ever since because of its small diameter, ease of use, and biocompatibility.[203] Traditionally, most research on improving electrochemical detection has focused on CFME surface modification techniques to enhance the sensitivity, selectivity and kinetic properties through chemical or electrochemical pretreatments or coating with polymer films.[204–206] However, in recent years, research is shifting towards making improved microelectrodes from completely different materials, including diamond, carbon nanomaterials, and polymers. There is also a push towards making electrodes smaller, allowing measurements from single synapses or in smaller organisms.

Carbon nanotube based electrodes—Carbon nanotubes (CNTs) have been explored for electrochemical studies because of their electrocatalytic activity, high mechanical strength, high aspect ratio, and excellent electrical conductivity. [207–209] CNT-modified CFMEs for neurotransmitter measurements have improved electron-transfer kinetics and sensitivity for adsorption-controlled species, such as dopamine. CNT-modified electrodes were first achieved through dip coating carbon fibers in a CNT-Nafion suspension.[210] However, this method suffered from poor reproducibility. Modifying carbon-fiber disk microelectrodes with single-walled CNTs forests via chemical self-assembly produced aligned CNTs that had exposed the end groups to the analyte.[211] Aligned CNT forest-modified electrodes had a 36-fold increase in sensitivity for dopamine, while maintaining a similar response times compared to bare electrodes. The procedure for self-assembly is tedious and requires many steps, so direct growth of CNTs on substrates is advantageous. The Mao group grew vertically aligned CNTs on CFMEs and used them for *in vivo* monitoring of ascorbate with high selectivity and reproducibility.[212] Electrochemical pretreatment of these electrodes in 1.0 M NaOH prior to ascorbate testing opened the nanotube tips, which significantly increased the electron transfer for ascorbate. Although these electrodes proved to be promising for ascorbate detection, more studies are needed of their ability for long-term measurements *in vivo* and their performance at higher scan rates for other neurochemical measurements. Our group has also demonstrated that aligned CNTs can be grown on both carbon fibers and metal wires and used for the detection of dopamine with FSCV.[213] The advantages of direct growth of nanomaterials are that the electrodes can be batch fabricated and the surface modification is more reproducible.

Carbon nanotube yarns (CNTYs) were recently utilized as an electrode material for high speed measurements with FSCV. CNTYs are carbon nanotube fibers formed from multiwalled CNTs via spinning methods; CNTYs with micrometer diameters can be obtained by adjusting the size of the CNTs and spinning angle.[214] One advantage of CNTYs is that they are commercially available and can be fabricated into microelectrodes using similar methods to carbon fibers. Carbon nanotube yarn microelectrodes (CNTYMEs) exhibited a number of interesting electrochemical properties such as low background currents, enhanced current density, and faster electron transfer kinetics. CNTYMEs also showed enhanced chemical discrimination along with improved sensitivity and selectivity compared to traditional CFMEs.[215] CNT yarns have dopamine oxidation currents that are independent of the FSCV waveform application frequency. Traditionally, large decreases in

signal are observed at CFMEs with increasing waveform frequency due to less time for dopamine to adsorb. CNTYMEs can be used at very high scan rates and waveform application frequencies, while maintaining their sensitivity, and dopamine could be detected on a 2 milliseconds time scale.[216] In a recent report, CNTY microelectrodes were laser treated to increase the surface area and oxygen containing functional groups on the surface, which increased the number of adsorption sites for dopamine.[217] During *in vivo* studies, these electrodes maintained high sensitivity for dopamine detection, even after applying a 5-fold faster scan repetition frequency than conventionally applied. Overall, these findings indicate that CNTYMEs exhibit high sensitivity at high repetition rates (Fig 8). Although initial studies in brain slices and *in vivo* showed a proof of concept for use of these electrodes, the performance of these electrodes *in vivo* is not yet fully characterized.

CNT fibers have also been made by wet spinning techniques and then made into fibers. CNT fiber microelectrodes, made from the fibers obtained via wet spinning CNTs in poly(vinyl alcohol)(PVA) exhibited efficient electrochemical response with high resistance against dopamine fouling.[218] Fibers made with polyethyleneimine, instead of PVA, have enhanced sensitivity to dopamine.[219] In addition, these polyethyleneimine-CNT fibers had less fouling for serotonin and its metabolite 5-hydroxyindoleacetic acid. A carbon nanofiber based nanoelectrode array was recently developed and integrated with Wireless Instantaneous Neurotransmitter Concentration Sensor System (WINCS).[220] Voltammetry was used to detect individual neurotransmitters simultaneously in a mixture of dopamine, serotonin and ascorbic acid. These array based systems can be valuable to simultaneously detect and resolve individual neurotransmitters in a mixture. The main advantage of CNT fiber and yarn electrodes is that they can be fabricated in a similar manner to CFMEs. Their size is similar to CFMEs, although the surface chemistry may be different. Future studies that correlate surface properties with electrochemical performance may better explain the enhanced electrochemical properties of certain nanomaterials.

Polymer microelectrodes—Nafion, a perfluorinated ion-exchange polymer is one of the most widely used electrode coatings and is effective at repelling negatively charged interferents such as ascorbic acid while preconcentrating cationic neurotransmitters such as dopamine. For example, Nafion-coated CFMEs were selective in measuring 3,4-dihydroxyphenyl-l-alanine (L-DOPA) augmented dopamine release *in vivo*, while maintaining a rapid electrode response time.[221] As L-DOPA is the standard drug for the treatment of Parkinson's disease, systemic L-DOPA doses were administered and dopamine dynamics measured in rat striatum. However, the doses of L-DOPA that caused dopamine release were larger than clinically relevant doses, which did not affect electrically evoked dopamine release. The Heien group developed a poly(3,4-ethylenedioxythiophene) (PEDOT) and Nafion composite modified CFME to measure dopamine transient events *in vivo*.[222] PEDOT:Nafion-coated CFMEs showed multifold increase in sensitivity and selectivity and comparable temporal response and mechanical stability to unmodified CFMEs. Biofouling was tested by implanting the electrodes in rat brain and PEDOT:Nafion-coated CFMEs lost only 9 ± 5% of the precalibration sensitivity, despite being implanted for 5.5 h longer than the uncoated fibers. The same group recently developed a different type of polymer based thin microelectrode using high conductivity, ultralow capacitance

PEDOT:Tosylate.[223] These microelectrodes were formed by depositing PEDOT:tosylate films onto TOPAS 5013L substrates through vapor-phase deposition and then the films were patterned and etched into electrodes. Dopamine was evaluated as a test compound and PEDOT:tosylate electrodes showed higher sensitivity to reduction than oxidation. Although these electrodes are novel, extensive analytical characterization is required to explore applications for other neurochemicals and their viability *in vivo*. There is much research occurring in the flexible electronics industry and materials, such as PEDOT, that are being developed there may be useful as sensors for the electrochemical community.

Nanoelectrodes for neurotransmitter detection—One of the holy grails of neuroscience research is to be able to measure neurochemicals in a single synapse. For electrochemical detection, that requires an electrode with a tip diameter on the nanometer scale. For nanoelectrodes based on carbon fibers, flame or electrochemical etching is typically used to reduce the diameter.[224] Carbon fiber nanoelectrodes (CFNEs) were recently prepared that can fit inside a synapse. CFNEs with a tip diameter about 100 nm and length less than 1 μm were prepared by flame-etching the micrometer sized carbon fibers encased in nanopipettes.[167] These nanoelectrodes facilitated direct monitoring of the kinetics of neurotransmitter exocytosis flux from inside an individual, artificial synapse. These are the first direct electrochemical measurements from a synapse. Future studies in more intact tissues will give a better idea of the synaptic concentrations of neurotransmitters. Our group reported the use of carbon nanopipette electrodes (CNPEs), which are fabricated without the use of a carbon fiber.[225] CNPEs were prepared through chemical vapor deposition by depositing carbon on the inside of a pulled quartz pipette. The quartz was then etched to reveal an average tip diameter of 250 nm and an exposed carbon length between 5 and 175 μm. This method is amenable to batch fabrication, compared to etched CFNEs which need to be prepared individually. CNPEs were implanted in *Drosophila* larvae and dopamine release was measured in tissue. With the many advances in nanolithography that are being made, pushing electrodes to the nanoscale promises to be an important line of research in the future. The problem with nanoelectrodes is that sensitivity scales with area, so concomitant exploration of new materials and coatings is necessary to develop truly functional nanoelectrodes that are useful for tissue or single synapse measurements.

Boron doped diamond electrodes—An alternative to carbon-fiber microelectrodes is conductive diamond, which has advantages of a wide potential window, stable background currents, and low fouling. Boron has been most commonly used because of its low charge carrier activation energy and the compatibility of its covalent radius with that of the carbon atom. The Swain group first demonstrated that diamond microelectrodes can be used for electrochemical detection of neurochemicals in biological samples. In a recent study, BDD films were grown onto Pt wire using microwave-assisted chemical vapor deposition. They demonstrated that pretreatment did not increase surface roughness or detachment of the diamond coating from the Pt wire substrate.[226] Diamond microelectrodes are resistant to fouling by serotonin and they are now routinely used to measure serotonin release in the enteric nervous system.[227] Recently thin-film diamond electrodes have been used for FSCV measurements. BDD films were grown on Si substrates or on tungsten thin rods using chemical vapor deposition [228] *In vitro* measurements of dopamine using FSCV resulted in

oxidation and reduction peaks at +0.8 V and −0.2 V, respectively; thus, BDD electrodes exhibited a consistent positive shift of 0.2 V for oxidation. While kinetics might need to be improved, the sensitivity and linear range were similar to CFMEs. Future studies may try to improve the doping procedure, as boron not incorporated into the lattice or other impurities may accumulate at grain boundaries. The ability to make thin film diamond electrodes broadens the potential applications of BDD electrodes and they could be incorporated into microfluidic devices or combined with other flexible, implantable materials.

Advances in microelectrodes conclusions

CFMEs have been used for decades as neurochemical sensors *in vivo*. Research into modifications of CFMEs continues, with recent studies exploring Nafion coatings, CNT coatings, and growing carbon nanomaterials directly on CFMEs. Evaluating the effects of coatings, particularly of CNTs, is difficult because you must deconvolute the response of the CNT and the CFME. CNT yarn and fiber mircoelectrodes provide an excellent opportunity to study the electrochemistry of the pure CNT material. Some CNT yarns are available commercially and they can be fabricated into microelectrodes using similar procedures as carbon fibers. However, their use *in vivo* has only been recently demonstrated and their robustness or resistance to biofouling has not been tested. On the other hand, electrodes modified with biocompatible ion-exchange polymers have proven to be very promising in minimizing the biofouling. Development of nanoelectrodes provided a breakthrough: electrochemical measurements inside a single synapse. Advances in nanolithography could be applied in the future to electrochemistry to produce smaller, robust nanoelectrodes that can be implanted in small spaces. Finally, research to move the field away from the traditional carbon electrodes is continuing, with polymer PEDOT electrodes being demonstrated for FSCV detection. While this technology is in its early stages, expanding the range of electrode materials has the potential to perhaps expand the array of neurochemicals that can be measured.

Electrochemistry conclusions and perspectives

Electrochemistry continues to be a popular tool for understanding neurochemical changes in the brain. While many of the techniques have been around for a while, they are being constantly improved and expanded to provide new knowledge about brain signaling. One example of that is the new understanding of exocytosis being due to partial release. This discovery was the result of newer chemical cytometry methods for measuring vesicular content as well as modeling and interpretation of traditional amperometric data. FSCV continues to be popular for *in vivo* measurements, and the field is expanding from measuring dopamine to many other small molecules by improving both the waveform and the electrode materials. While electrochemistry will never be a highly multiplexed technique, waveform changes have facilitated detection of 2–3 analytes simultaneously, and these are the fastest measurements of concurrent neurochemical changes. Improvements in electrode development, such as pushing electrodes to the nanoscale, will also facilitate a new research thrust into smaller model organisms such as fruit flies and zebra fish. Electrochemistry is also a good technique to combine with other measurements. Recent efforts by the Wightman group combined FSCV with electrophysiology and iontophoretic drug delivery and more efforts at echem/ephys integration will follow. Overall, electrochemistry continues to be a

robust technique to provide a real-time picture of neurotransmission and continues to be one of the highest spatial and temporal resolution techniques for neurotransmitter measurements.

Conclusions and Future Directions

The field of neuroscience is expanding tremendously as is the subfield of researchers developing analytical tools to measure neurotransmitters. While the emphasis in neuroscience has traditionally been on the “sparks” of action potentials and cell firing, there is now a growing understanding that the it is necessary to understand the “soup”, that is the underlying chemistry, in order to understand function. The brain is complex because it operates on different length scales, from single cells, to regional networks, to the whole brain output. Measuring brain chemistry is complex because changes occur across different time scales, from millisecond synaptic communication to changes in lipids that occur in minutes to hours, to circadian changes that vary over the time course of days.[93,167,229] Finally, the actual chemicals to measure in the brain are complex: ranging from molecular oxygen, to small molecule neurotransmitters, to peptides, and larger proteins and lipids. Examining methods using those lenses reveals that no method will be able to measure all chemicals across all time and length scales. Instead, a toolbox is needed with many different tools and the grand future challenge is to integrate more methods to better understand the coordinated changes of different molecules on different time and length scales in the brain.

Measuring chemistry in the brain: Integrating across different length scales

The techniques used to measure brain chemicals range from small scale, single synapse measurements, to tools that measure regional changes on the order of microns, to large scale measurements of whole brain imaging. On the small scale, the Amatore group recently published electrochemical measurements from a single synapse, which has been a goal of the exocytosis community.[230] The continued challenge for electrochemistry is making small, but functional microelectrodes, that can reach small places such as a single synapse or a specific cell type in a slice, or a small brain region of the fruit fly. True, nanometer sized probes that are robust enough to implant routinely in tissue remain elusive. An integration of nanotechnology and neuroscience is on the horizon; nanolithography techniques will be used to fabricate small sensors and sampling probes reproducibly. A good example of this is the lithographically fabricated microdialysis probe from the Kennedy group, that while not nanosized now, could be miniaturized even further in the future.[81] Batch fabrication of PEDOT electrodes or carbon nanomaterial electrodes might yield practical methods for nanoelectrode fabrication. On the other hand, fluorescence imaging experiments do not require the fabrication of small sensors, but have the ability to localize molecules and image them in different environments. The new NeuroSensor compounds that bind to monoamines in vesicles or the FFNs are also a strategy that should yield new information about exocytosis and concentrations in the synapse on the nanometer scale.[28,33] Thus, the prospects for understanding neurochemistry on the nanometer scale are bright, but much more work needs to be done to optimize sensitivity, time resolution, and spatial resolution.

It is equally challenging to step back on the length scale and understand coordinated cellular actions and regional variations in neurochemistry. The mass spectrometry imaging

techniques are particularly good at looking at difference in lipids or proteins in different brain regions or even within a given region. DESI is useful because it is less destructive and does not require a matrix, like MALDI does.[65] SIMS typically provides the best spatial resolution of the mass spectrometry methods. Fluorescent techniques such as GECIs or GEVIs give information about cell firing and how cells may fire in concert to work together in a regional manner. Direct electrochemical or sampling methods typically measure more on this length scale as well. Microdialysis probes are typically a couple hundred microns wide and long, and electrodes are on the order of 10 μm wide, but hundreds of microns long; thus they measure not from a single synapse, but from multiple cells and synapses in a given region. While many techniques have been developed to understand neurochemistry when looking at the brain on a micron dimension scale, the challenge then becomes to integrate the information from the different tools on similar samples. for *in vivo* measurements there is a brain "atlas",[231] but exactly where measurements are taken and aligning measurements taken with different techniques remains elusive. Therefore, on this length scale, new efforts to create a more well-defined brain atlas that allows coordination of results could result in a better picture of brain signaling.

The largest length scale is whole brain imaging, simply looking at how different structures react to the same behavioral task or disease. BOLD fMRI and PET scanning are the most developed techniques that image the whole brain and are routinely used on humans today. fMRI has traditionally imaged oxygen changes in the brain,[38] but new advances in molecular MRI[232] are allowing a better understanding of some specific chemical changes. PET uses radioactive ligands to look at receptor binding and is limited by the type and effectiveness of the PET ligands.[47] Thus, for PET there continues to be a synthetic chemistry challenge to make newer ligands, while for fMRI, there continues to be a challenge to expand its reach. As of now, imaging will continue to lead the way for whole brain measurements and there is not any clear competition for fMRI or PET in providing whole brain, integrated chemical information.

Measuring chemistry in the brain: Integrating across different time scales

Understanding the dynamics of chemical changes in the brain is critical to understanding brain signaling and function. Often, the word dynamic implies fast, and exocytosis is a fast, millisecond scale process of chemical signaling in the brain. However, other brain changes may be on a slower scale and it is important to understand their dynamics too. Protein expression may change over a period of minutes. Lipids may rearrange or be expressed differently in membranes over a period of minutes to hours. Even neurochemicals have different signaling modes; for example, neurotransmitters are traditionally thought of as fast-acting synaptic neurotransmitters, but neuromodulators and neurohormones act on slower time scales. In addition, basal levels of neurotransmitters can vary due to disease or even circadian rhythms. Thus, it is important to have methods that measure across a variety of time scales.

Electrochemical methods are typically the fastest neurotransmitter measurements in the brain. Amperometric measurements of exocytosis are typically performed every 1–2 millseconds,[195] and FSCV measurements in the extracellular space on the order of 10

milliseconds.[217] Fluorescence imaging techniques, particularly those measuring changes in calcium or voltage, can also achieve millisecond time resolution.[3] Thus, electrochemistry and fluorescence are best suited for studying the dynamics of exocytosis and rapid clearance of neurotransmitters. On a slightly longer time scale, FSCAV has been developed to look at basal changes in monoamines, such as dopamine and serotonin, on the 10 seconds time scale.[201] Microdialysis or push-pull perfusion samples are typically analyzed on the minute time scale. While 10 minutes is traditional, faster CE and HPLC separations are pushing that time scale now close to the 10–60 seconds time frame.[107] However, long term implantation of probes to measure for days at a time is still an ongoing issue. Microdialysis causes damage upon implantation, but the Michael group has shown that perfusing with dexamethasone can alleviate that damage and facilitate long term implantation.[80,79] Chronic implantation of microelectrodes is now routinely performed,[233] but ironically those electrodes work best after being implanted for 1–2 weeks so there is still much to be learned about how to measure on the rapid time scale for days or weeks at a time.

Imaging techniques can be rapid, such as fluorescence, but also can take longer to get a complete picture if the measurement is slower, as with mass spectrometry imaging. To study dynamics of neurotransmitters, fluorescent techniques that make multiple measurements in a second are very useful. The NeuroSensor compounds, QDs, and FFNs can be used to measure dynamics of release and uptake, although the time resolution is not as fast as amperometry. fMRI and PET are also typically taken on the minute time scale. Dynamic changes in blood flow can be measured, but the time scale is typically minutes to hours. PET is not used for dynamic information as often, but receptor binding over time can be imaged. These techniques are good for *in vivo* mapping but do not provide real-time images of rapid changes. The mass spectrometry techniques are typically much slower, but provide more chemical information. Imaging a brain slice can take hours. Right now, mass spectrometry imaging is not routinely being used in a repeated fashion to look at dynamic measurements in the same sample. However, future studies in brain slices could be done to visualize peptide or lipid changes over the time course of hours. The speed of these measurements can also be improved if single ion monitoring is done and general improvements in time resolution for mass spectrometry will greatly influence the field of neuroscience applications.

Measuring chemistry in the brain: Chemical diversity

An ideal technique in the brain would measure every chemical with good spatial and temporal resolution. In reality, there is far to go in understanding the interplay of different neurochemicals and how they affect each other. Understanding how neurochemical changes integrate to cause function is still a great unsolved mystery of neuroscience. The key to solving this mystery will be either techniques that can measure multiple compounds or an integration of different techniques that measure more than any one of them could on its own.

Traditional techniques monitor one compound at a time. Electrochemistry, GECIs, and fMRI are all examples of techniques that have been used very successfully, but typically measure only one compound, such as dopamine, calcium, or oxygen. Efforts to expand electrochemistry to measure multiple compounds, such as dopamine and adenosine, or

oxygen and neurotransmitters, are underway but the reality is they are not optimal for large scale multiplexing.[183,181] However, multiplexing sensors with different sensing elements is a way around this problem. For example, the Andrews and Weiss groups have developed field-effect based sensors that can be tailored to different neurotransmitters by changing the binding aptamer.[234] Making an implantable sensor with arrays of these FETs would be useful for measuring multiple compounds simultaneously. Imaging techniques and electrochemistry can also be combined: for example, measurements of cell calcium by GECIs could be made while measuring neurochemical changes with electrochemistry. Multiplexed fluorescence imaging is typically limited by the number and wavelength of available fluorophores and spectral overlap between them. Thus, electrochemistry and imaging are predominantly good for measuring a few compounds at a time.

Sampling techniques paired with separations and MS are powerful techniques that are able to detect multiple compounds in one experiment. There are tradeoffs, spatial resolution and temporal resolution discussed above, but the power to understand the dynamics of multiple chemicals will be important to get a better picture of how neurochemicals interact. Separations can measure many compounds in one experiment, although they typically have to be of one class. A recent separation with LC-MS/MS can detect 70 small molecule neurochemicals in a 17 minute chromatogram, showing the power of the separation.[99] MS-MS detection provides a high amount of chemical information and aids with identifying species by their fragmentation patterns. MS continues to be the dominant technique for neuropeptidomics, and typical experiments identify hundreds of peptides from a single sample.[57] The challenge is to understand which peptides are important for chemical signaling and behaviors or diseases of interest. Very few experiments have attempted to integrate the more chemically rich experiments, such as microdialysis/HPLC or MS, with techniques such as imaging or electrochemistry. Microfluidic platforms for growing cells or tissues may be able to help with the integration, as they could offer integrated electrodes, with channels for fluid sampling or direct MS output.[128] The classes of molecules that are best for each technique, small molecules for electrochemistry, peptides/proteins/lipids for MS, oxygen/calcium for imaging make comparing the outputs of the different techniques a challenge. Nevertheless, a future push to index and integrate techniques will provide a better picture of the complex chemical changes in the brain.

Measuring chemistry in the brain: Final conclusions

The papers reviewed in this article show that the field of analytical neurochemistry is alive and well. There are many new techniques being developed or refined for new uses. The field continues to struggle with integrating chemical information across length and time scales, but individual techniques are pushing the boundaries for time and length scales, both larger and smaller. Future efforts to combine and integrate techniques to take advantage of different sensitivities or selectivities will also facilitate a more integrated picture of the brain signaling. In addition to chemical knowledge, integration of chemical techniques with traditional electrophysiological techniques that measure cell firing is also important for understanding how electrical signals lead to chemical signaling. In other words, there is a need to understand both the “sparks” and the “soup” to appreciate how they influence each other. Thus, the challenge of understanding chemical changes in the brain is nowhere near

solved, but the current techniques provide a glimpse of the complex changes that make up our thoughts.

Acknowledgments

The Venton lab is supported by NIH (R01MH085159, R21DA037584, R01NS076875).

Biographies

Mallikarjunarao Ganesana obtained his M.Sc degree in chemistry from Nagarjuna University, India in 2005 and PhD degree in Analytical Chemistry from Clarkson University, NY in 2012. While at Clarkson, his research was focused on the development of electrochemical enzymatic biosensors for clinical applications. He is currently working as postdoctoral research associate at University of Virginia. His current research interests include electrochemical detection of neurotransmitters and their roles during ischemic brain injury.

Scott T. Lee is a Ph.D. candidate in the Department of Chemistry at the University of Virginia. He received his B.S. degree in Chemistry with a forensics emphasis from Boise State University in 2012. While there, his research covered enzymatic inhibition using computational and spectroscopic techniques. His current research interests include electrochemical detection and characterization of adenosine and histamine and their roles in neurological function.

Ying Wang is a chemistry Ph. D. candidate in the research group of Professor B. Jill Venton at University of Virginia. She completed her B.S. degree in Chemistry from Geneva College in 2013. She annually performed undergraduate research at the University of Pittsburgh, where she studied nanomaterials and their physical properties as well as biochemistry. Her research interests currently lie in investigating the mechanism and function of transient adenosine release in the brain.

B. Jill Venton is a Professor of Chemistry at University of Virginia, with an appointment also in the Neuroscience Graduate Program. She obtained her B.S. in Chemistry in 1998 from University of Delaware and her PhD in 2002 from University of North Carolina Chapel Hill, where she worked under Mark Wightman. After a postdoc with Robert Kennedy and Terry Robinson at University of Michigan, she joined the faculty at Virginia in 2005. She has received numerous awards such as the Eli Lilly Young Analytical Investigator Award, the SEAC Young Investigator award, and a Dreyfus Teacher-Scholar Award. Her research interests include developing new carbon nanomaterial electrodes, making neurotransmitter measurements in *Drosophila*, and studying real-time adenosine changes *in vivo*.

References

1. Valentine, ES. The War of the Soups and the Sparks: The Discovery of Neurotransmitters and the Dispute Over How Nerves Communicate. New York: Columbia University Press; 2006.
2. Mao L. Anal. Chem. 2017
3. Lin MZ, Schnitzer MJ. Nat. Neurosci. 2016; 19(9):1142–1153. [PubMed: 27571193]

4. Berlin S, Carroll EC, Newman ZL, Okada HO, Quinn CM, Kallman B, Rockwell NC, Martin SS, Lagarias JC, Isacoff EY. Nat. Methods. 2015; 12(9):852–858. [PubMed: 26167640]
5. Inoue M, Takeuchi A, Horigane S, Ohkura M, Gengyo-Ando K, Fujii H, Kamijo S, Takemoto-Kimura S, Kano M, Nakai J, Kitamura K, Bito H. Nat. Methods. 2015; 12(1):64–70. [PubMed: 25419959]
6. Akerboom J, Carreras Calderón N, Tian L, Wabnig S, Prigge M, Tolö J, Gordus A, Orger MB, Severi KE, Macklin JJ, Patel R, Pulver SR, Wardill TJ, Fischer E, Schüler C, Chen T-W, Sarkisyan KS, Marvin JS, Bargmann CI, Kim DS, Kügler S, Lagnado L, Hegemann P, Gottschalk A, Schreiter ER, Looger LL. Front. Mol. Neurosci. 2013; 6:2. [PubMed: 23459413]
7. Wu J, Liu L, Matsuda T, Zhao Y, Rebane A, Drobizhev M, Chang Y-F, Araki S, Arai Y, March K, Hughes TE, Sagou K, Miyata T, Nagai T, Li W, Campbell RE. ACS Chem. Neurosci. 2013; 4(6): 963–972. [PubMed: 23452507]
8. Marvin JS, Borghuis BG, Tian L, Cichon J, Harnett MT, Akerboom J, Gordus A, Renninger SL, Chen T-W, Bargmann CI, Orger MB, Schreiter ER, Demb JB, Gan W-B, Hires SA, Looger LL. Nat. Methods. 2013; 10(2):162–170. [PubMed: 23314171]
9. Gong Y, Huang C, Li JZ, Grewe BF, Zhang Y, Eismann S, Schnitzer MJ. Science. 2015; 350(6266): 1361–1366. [PubMed: 26586188]
10. Mishina Y, Mutoh H, Song C, Knöpfel T. Front. Mol. Neurosci. 2014; 7:78. [PubMed: 25324718]
11. St-Pierre F, Marshall JD, Yang Y, Gong Y, Schnitzer MJ, Lin MZ. Nat. Neurosci. 2014; 17(6):884–889. [PubMed: 24755780]
12. Jin L, Han Z, Platisa J, Wooltorton JRA, Cohen LB, Pieribone VA. Neuron. 2012; 75(5):779–785. [PubMed: 22958819]
13. Cao G, Platisa J, Pieribone VA, Raccuglia D, Kunst M, Nitabach MN. Cell. 2013; 154(4):904–913. [PubMed: 23932121]
14. Storace DA, Braubach OR, Jin L, Cohen LB, Sung U. Sci. Rep. 2015; 5:10212. [PubMed: 25970202]
15. Nguyen Q-T, Schroeder LF, Mank M, Muller A, Taylor P, Griesbeck O, Kleinfeld D. Nat. Neurosci. 2010; 13(1):127–132. [PubMed: 20010818]
16. Muller A, Joseph V, Slesinger PA, Kleinfeld D. Nat. Methods. 2014; 11(12):1245–1252. [PubMed: 25344639]
17. Kumar V, Rahbek-Clemmensen T, Billesbølle CB, Jorgensen TN, Gether U, Newman AH. ACS Med. Chem. Lett. 2014; 5(6):696–699. [PubMed: 24944746]
18. Zhang P, Jørgensen TN, Loland CJ, Newman AH. Bioorg. Med. Chem. Lett. 2013; 23(1):323–326. [PubMed: 23168018]
19. Nørager NG, Jensen CB, Rathje M, Andersen J, Madsen KL, Kristensen AS, Strømgaard K. ACS Chem. Biol. 2013; 8(9):2033–2041. [PubMed: 23838571]
20. Yamaura K, Kiyonaka S, Numata T, Inoue R, Hamachi I. Nat. Chem. Biol. 2016; 12(10):822–830. [PubMed: 27526031]
21. Liu S, Shi F, Zhao X, Chen L, Su X. Biosens. Bioelectron. 2013; 47:379–384. [PubMed: 23608539]
22. Shi F, Li Y, Lin Z, Ma D, Su X. Sensors Actuators B Chem. 2015; 220:433–440.
23. Murphy-Royal C, Dupuis JP, Varela JA, Panatier A, Pinson B, Baufreton J, Groc L, Oliet SHR. Nat. Neurosci. 2015; 18(2):219–226. [PubMed: 25581361]
24. Varela JA, Dupuis JP, Etchepare L, Espana A, Cognet L, Groc L. Nat. Commun. 2016; 7:10947. [PubMed: 26971573]
25. Gubernator NG, Zhang H, Staal RGW, Mosharov EV, Pereira DB, Yue M, Balsanek V, Vadola PA, Mukherjee B, Edwards RH, Sulzer D, Sames D. Science. 2009; 324(5933):1441–1444. [PubMed: 19423778]
26. Lee M, Gubernator NG, Sulzer D, Sames D. J. Am. Chem. Soc. 2010; 132(26):8828–8830. [PubMed: 20540519]
27. Rodriguez PC, Pereira DB, Borgkvist A, Wong MY, Barnard C, Sonders MS, Zhang H, Sames D, Sulzer D. Proc. Natl. Acad. Sci. 2013; 110(3):870–875. [PubMed: 23277566]

28. Pereira DB, Schmitz Y, Mészáros J, Merchant P, Hu G, Li S, Henke A, Lizardi-Ortiz JE, Karpowicz RJ, Morgenstern TJ, Sonders MS, Kanter E, Rodriguez PC, Mosharov EV, Sames D, Sulzer D. Nat. Neurosci. 2016; 19(4):578–586. [PubMed: 26900925]
29. Merchant P, Sulzer D, Sames D. Neuropharmacology. 2015; 98:90–94. [PubMed: 25837712]
30. Hettie KS, Liu X, Gillis KD, Glass TE. ACS Chem. Neurosci. 2013; 4(6):918–923. [PubMed: 23527575]
31. Klockow JL, Hettie KS, Glass TE. ACS Chem. Neurosci. 2013; 4(10):1334–1338. [PubMed: 23926946]
32. Hettie KS, Klockow JL, Glass TE. J. Am. Chem. Soc. 2014; 136(13):4877–4880. [PubMed: 24611584]
33. Hettie KS, Glass TE. Chemistry. 2014; 20(52):17488–17499. [PubMed: 25346467]
34. Hettie KS, Glass TE. ACS Chem. Neurosci. 2016; 7(1):21–25. [PubMed: 26521705]
35. Ferris CF, Kulkarni P, Toddes S, Yee J, Kenkel W, Nedelman M. Front. Neurol. 2014; 5:94. [PubMed: 25071696]
36. Ferraro S, Nanetti L, Piacentini S, Mandelli ML, Bertolino N, Ghielmetti F, Epifani F, Nigri A, Taroni F, Bruzzone MG, Di Donato S, Savoiardo M, Mariotti C, Grisoli M. Neurology. 2014; 83(1):65–72. [PubMed: 24898924]
37. Madularu D, Yee JR, Kenkel WM, Moore KA, Kulkarni P, Shams WM, Ferris CF, Brake WG. Psychoneuroendocrinology. 2015; 56:200–212. [PubMed: 25827963]
38. Madularu D, Kulkarni P, Yee JR, Kenkel WM, Shams WM, Ferris CF, Brake WG. Horm. Behav. 2016; 82:56–63. [PubMed: 27154458]
39. Cox RW, Jesmanowicz A, Hyde JS. Magn. Reson. Med. 1995; 33(2):230–236. [PubMed: 7707914]
40. Scharnowski F, Veit R, Zopf R, Studer P, Bock S, Diedrichsen J, Goebel R, Mathiak K, Birbaumer N, Weiskopf N. Biol. Psychol. 2015; 108:85–97. [PubMed: 25796342]
41. Paret C, Ruf M, Gerchen MF, Kluetsch R, Demirakca T, Jungkunz M, Bertsch K, Schmahl C, Ende G. Neuroimage. 2016; 125:182–188. [PubMed: 26481674]
42. Zotev V, Phillips R, Yuan H, Misaki M, Bodurka J. Neuroimage. 2014; 85(3):985–995. [PubMed: 23668969]
43. Shamshiri EA, Tierney TM, Centeno M, St Pier K, Pressler RM, Sharp DJ, Perani S, Cross JH, Carmichael DW. Hum. Brain Mapp. 2016
44. Shapiro MG, Westmeyer GG, Romero PA, Szablowski JO, Küster B, Shah A, Otey CR, Langer R, Arnold FH, Jasanoff A. Nat. Biotechnol. 2010; 28(3):264–270. [PubMed: 20190737]
45. Lee T, Cai LX, Lelyveld VS, Hai A, Jasanoff A. Science (80-.). 2014; 344(6183):533–535.
46. Moussaron A, Vibhute S, Bianchi A, Gündüz S, Kotb S, Sancey L, Motto-Ros V, Rizzitelli S, Crémillieux Y, Lux F, Logothetis NK, Tillement O, Angelovski G. Small. 2015; 11(37):4900–4909. [PubMed: 26179212]
47. Tuominen L, Nummenmaa L, Keltikangas-Järvinen L, Raitakari O, Hietala J. Hum. Brain Mapp. 2014; 35(5):1875–1884. [PubMed: 23671038]
48. Villien M, Wey H-Y, Mandeville JB, Catana C, Polimeni JR, Sander CY, Zürcher NR, Chonde DB, Fowler JS, Rosen BR, Hooker JM. Neuroimage. 2014; 100:192–199. [PubMed: 24936683]
49. Michaelides M, Hurd YL. Neuropsychopharmacology. 2015; 40(1):239–240. [PubMed: 25482170]
50. Michaelides M, Anderson SAR, Ananth M, Smirnov D, Thanos PK, Neumaier JF, Wang G-J, Volkow ND, Hurd YL. J. Clin. Invest. 2013; 123(12):5342–5350. [PubMed: 24231358]
51. Anderson SAR, Michaelides M, Zarnegar P, Ren Y, Fagergren P, Thanos PK, Wang G-J, Bannon M, Neumaier JF, Keller E, Volkow ND, Hurd YL. J. Clin. Invest. 2013; 123(12):5334–5341. [PubMed: 24231353]
52. Urban DJ, Roth BL. Annu. Rev. Pharmacol. Toxicol. 2015; 55(1):399–417. [PubMed: 25292433]
53. Spengler B. Anal. Chem. 2015; 87(1):64–82. [PubMed: 25490190]
54. Caprioli RM, Farmer TB, Gile J. Anal. Chem. 1997; 69(23):4751–4760. [PubMed: 9406525]
55. Hong JH, Kang JW, Kim DK, Baik SH, Kim KH, Shanta SR, Jung JH, Mook-Jung I, Kim KP. J. Lipid Res. 2016; 57(1):36–45. [PubMed: 26538545]

56. Zhang Y, Buchberger A, Muthuvel G, Li L. Proteomics. 2015; 15(23–24):3969–3979. [PubMed: 26475201]
57. Ye H, Wang J, Zhang Z, Jia C, Schmerberg C, Catherman AD, Thomas PM, Kelleher NL, Li L. J. Proteome Res. 2015; 14(11):4776–4791. [PubMed: 26390183]
58. Lee CY, Fan Y, Rubakhin SS, Yoon S, Sweedler JV. Sci. Rep. 2016; 6:26940. [PubMed: 27245782]
59. Pirman DA, Reich RF, Kiss A, Heeren RMA, Yost RA. Anal. Chem. 2013; 85(2):1081–1089. [PubMed: 23214490]
60. Shariatgorji M, Nilsson A, Goodwin RJA, Källback P, Schintu N, Zhang X, Crossman AR, Bezard E, Svenningsson P, Andren PE. Neuron. 2014; 84(4):697–707. [PubMed: 25453841]
61. Lazar AN, Bich C, Panchal M, Desbenoit N, Petit VW, Touboul D, Dauphinot L, Marquer C, Laprévote O, Brunelle A, Duyckaerts C. Acta Neuropathol. 2013; 125(1):133–144. [PubMed: 22956244]
62. Hanrieder J, Malmberg P, Lindberg OR, Fletcher JS, Ewing AG. Anal. Chem. 2013; 85(18):8741–8748. [PubMed: 23947367]
63. Hanrieder J, Ewing AG. Sci. Rep. 2014; 4:942–955.
64. Mohammadi AS, Phan NTN, Fletcher JS, Ewing AG. Anal. Bioanal. Chem. 2016; 408(24):6857–6868. [PubMed: 27549796]
65. Takáts Z, Wiseman JM, Gologan B, Cooks RG. Science. 2004; 306(5695):471–473. [PubMed: 15486296]
66. Shariatgorji M, Strittmatter N, Nilsson A, Källback P, Alvarsson A, Zhang X, Vallianatou T, Svenningsson P, Goodwin RJA, Andren PE. Neuroimage. 2016; 136:129–138. [PubMed: 27155126]
67. Eberlin LS, Norton I, Orringer D, Dunn IF, Liu X, Ide JL, Jarmusch AK, Ligon KL, Jolesz FA, Golby AJ, Santagata S, Agar NYR, Cooks RG. Proc. Natl. Acad. Sci. U. S. A. 2013; 110(5):1611–1616. [PubMed: 23300285]
68. Jarmusch AK, Pirro V, Baird Z, Hattab EM, Cohen-Gadol AA, Cooks RG. Proc. Natl. Acad. Sci. U. S. A. 2016; 113(6):1486–1491. [PubMed: 26787885]
69. Roach PJ, Laskin J, Laskin A. Analyst. 2010; 135(9):2233. [PubMed: 20593081]
70. Laskin J, Heath BS, Roach PJ, Cazares L, Semmes OJ. Anal. Chem. 2012; 84(1):141–148. [PubMed: 22098105]
71. Hsu C-C, White NM, Hayashi M, Lin EC, Poon T, Banerjee I, Chen J, Pfaff SL, Macagno ER, Dorrestein PC. Proc. Natl. Acad. Sci. U. S. A. 2013; 110(37):14855–14860. [PubMed: 23969833]
72. Hsu C-C, Chou P-T, Zare RN. Anal. Chem. 2015; 87(22):11171–11175. [PubMed: 26509582]
73. Lanekoff I, Stevens SL, Stenzel-Poore MP, Laskin J. Analyst. 2014; 139(14):3528–3532. [PubMed: 24802717]
74. Lanekoff I, Thomas M, Laskin J. Anal. Chem. 2014; 86(3):1872–1880. [PubMed: 24428785]
75. Lanekoff I, Thomas M, Carson JP, Smith JN, Timchalk C, Laskin J. Anal. Chem. 2013; 85(2):882–889. [PubMed: 23256596]
76. Bergman H-M, Lundin E, Andersson M, Lanekoff I. Analyst. 2016; 141(12):3686–3695. [PubMed: 26859000]
77. Nesbitt KM, Jaquins-Gerstl A, Skoda EM, Wipf P, Michael AC. Anal. Chem. 2013; 85(17):8173–8179. [PubMed: 23927692]
78. Nesbitt KM, Varner EL, Jaquins-Gerstl A, Michael AC. ACS Chem. Neurosci. 2015; 6(1):163–173. [PubMed: 25491242]
79. Varner EL, Jaquins-Gerstl A, Michael AC. ACS Chem. Neurosci. 2016; 7(6):728–736. [PubMed: 27003503]
80. Kozai TDY, Jaquins-Gerstl AS, Vazquez AL, Michael AC, Cui XT. Biomaterials. 2016; 87:157–169. [PubMed: 26923363]
81. Lee WH, Ngernsutivorakul T, Mabrouk OS, Wong JMT, Dugan CE, Pappas SS, Yoon HJ, Kennedy RT. Anal. Chem. 2016; 88(2):1230–1237. [PubMed: 26727611]
82. Hershey ND, Kennedy RT. ACS Chem. Neurosci. 2013; 4(5):729–736. [PubMed: 23374073]
83. Hogerton AL, Bowser MT. Anal. Chem. 2013; 85(19):9070–9077. [PubMed: 23984889]

84. Song P, Hershey ND, Mabrouk OS, Slaney TR, Kennedy RT. Anal. Chem. 2012; 84(11):4659–4664. [PubMed: 22616788]
85. Papadimitriou KI, Wang C, Rogers ML, Gowers SAN, Leong CL, Boutelle MG, Drakakis EM. Front. Hum. Neurosci. 2016; 10:212. [PubMed: 27242477]
86. Ojeda-Torres G, Williams L, Featherstone DE, Shippy SA. Analyst. 2015; 140(19):6563–6570. [PubMed: 26299259]
87. Borra S, McCullagh EA, Featherstone DE, Baker PM, Ragozzino ME, Shippy SA. Anal. methods. 2014; 6(9):2916–2922.
88. Saha-Shah A, Green CM, Abraham DH, Baker LA. Analyst. 2016; 141(6):1958–1965. [PubMed: 26907673]
89. Gu H, Varner EL, Groskreutz SR, Michael AC, Weber SG. Anal. Chem. 2015; 87(12):6088–6094. [PubMed: 25970591]
90. Yang H, Thompson AB, McIntosh BJ, Altieri SC, Andrews AM. ACS Chem. Neurosci. 2013; 4(5): 790–798. [PubMed: 23614776]
91. Rodeberg NT, Johnson JA, Bucher ES, Wightman RM. ACS Chem. Neurosci. 2016 acschemneuro. 6b00142.
92. Zhang J, Jaquins-Gersti A, Nesbitt KM, Rutan SC, Michael AC, Weber SG. Anal. Chem. 2013; 85(20):9889–9897. [PubMed: 24020786]
93. Yang H, Sampson MM, Senturk D, Andrews AM. ACS Chem. Neurosci. 2015; 6(8):1487–1501. [PubMed: 26167657]
94. Groskreutz SR, Horner AR, Weber SG. J. Chromatogr. A. 2015; 1405:133–139. [PubMed: 26091787]
95. Wilson RE, Groskreutz SR, Weber SG. Anal. Chem. 2016; 88(10) acs.analchem.5b04793.
96. Schou-Pedersen AMV, Hansen SN, Tveden-Nyborg P, Lykkesfeldt J. J. Chromatogr. B. 2016; 1028:222–230.
97. Zhang L, Yang J.qing, Luo Y, Shang J.chuan, Jiang X.hui. J. Pharm. Biomed. Anal. 2016; 118:41–51. [PubMed: 26512998]
98. Santos-Fandila A, Vázquez E, Barranco A, Zafra-Gómez A, Navalón A, Rueda R, Ramírez M. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 2015; 1001:191–201.
99. Wong JMT, Malec PA, Mabrouk OS, Ro J, Dus M, Kennedy RT. J. Chromatogr. A. 2016; 1446:78–90. [PubMed: 27083258]
100. Zhou Y, Wong JMT, Mabrouk OS, Kennedy RT. Anal. Chem. 2015; 87(19):9802–9809. [PubMed: 26351736]
101. Stephen TKL, Guillemette KL, Green TK. Anal. Chem. 2016; 88(15):7777–7785. [PubMed: 27314490]
102. Serralheiro A, Alves G, Fortuna A, Rocha M, Falcão A. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 2013; 925:1–9.
103. Dailey AC, Garnier N, Rubakhin SS, Sweedler AJ. Anal. Bioanal. Chem. 2014; 405(8):2451–2459.
104. Sánchez-López E, Montealegre C, Crego AL, Marina ML. TrAC - Trends in Analytical Chemistry. 2015:82–99.
105. Casado M, Molero M, Sierra C, García-Cazorla A, Ormazabal A, Artuch R. Electrophoresis. 2014; 35(8):1181–1187. [PubMed: 24338894]
106. Xiong XJ, Guo XF, Ge XX, Wang H, Zhang HS. J. Sep. Sci. 2013; 36(19):3264–3269. [PubMed: 23894032]
107. O'Brien KB, Esguerra M, Miller RF, Bowser MT. Anal. Chem. 2004; 76(17):5069–5074. [PubMed: 15373444]
108. Tůma P, Šustková-Fišerová M, Opekar F, Pavlíček V, Málková K. J. Chromatogr. A. 2013; 1303:94–99. [PubMed: 23866123]
109. Sánchez-lópez E, Marcos A, Ambrosio E, Luisa M, Crego AL. J. Chromatogr. A. 2016; 1467:372–382. [PubMed: 27371023]
110. Berglund EC, Kuklinski NJ, Karagündüz E, Ucar K, Hanrieder J, Ewing AG. Anal. Chem. 2013; 85(5):2841–2846. [PubMed: 23387977]

111. Phan NTN, Hanrieder J, Berglund EC, Ewing AG. Anal. Chem. 2013; 85(17):8448–8454. [PubMed: 23915325]
112. Denno ME, Privman E, Venton BJ. ACS Chem. Neurosci. 2015; 6(1):117–123. [PubMed: 25437353]
113. Denno ME, Privman E, Borman RP, Wolin DC, Venton BJ. ACS Chem. Neurosci. 2016; 7(3): 407–414. [PubMed: 26765065]
114. Scott DE, Willis SD, Gabbert S, Johnson D, Naylor E, Janle EM, Krichevsky JE, Lunte CE, Lunte SM. Analyst. 2015; 140(11):3820–3829. [PubMed: 25697221]
115. Zeng J, Kuang H, Hu C, Shi X, Yan M, Xu L, Wang L, Xu C, Xu G. Environ. Sci. Technol. 2013; 47(13):7457–7465. [PubMed: 23746042]
116. Li X, Xiao D, Ou XM, McCullm C, Liu YM. J. Chromatogr. A. 2013; 1318:251–256. [PubMed: 24354006]
117. Li X, Hu H, Zhao S, Liu Y-M. Anal. Chem. 2016; 88(10):5338–5344. [PubMed: 27111409]
118. Renault R, Sukenik N, Descroix S, Malaquin L, Viovy JL, Peyrin JM, Bottani S, Monceau P, Moses E, Vignes M. PLoS One. 2015; 10(4)
119. Ren Y, Huang SH, Mosser S, Heuschkel MO, Bertsch A, Fraering PC, Chen JJJ, Renaud P. Micromachines. 2015; 6(12):1923–1934.
120. Lee N, Park JW, Kim HJ, Yeon JH, Kwon J, Ko JJ, Oh SH, Kim HS, Kim a, Han BS, Lee SC, Jeon NL, Song J. Mol Cells. 2014; 37(6):497–502. [PubMed: 24938227]
121. Sgro AE, Bajjalieh SM, Chiu DT. ACS Chem. Neurosci. 2013; 4(2):277–284. [PubMed: 23421679]
122. Lee HU, Nag S, Blasiak A, Jin Y, Thakor NV, Yang IH. ACS Chem. Neurosci. 2016; 7(10):1317–1324. [PubMed: 27570883]
123. Jokinen V, Sakha P, Suvanto P, Rivera C, Franssila S, Lauri SE, Huttunen HJ. J. Neurosci. Methods. 2013; 212(2):276–282. [PubMed: 23124090]
124. Xiao RR, Wang L, Zhang L, Liu YN, Yu XL, Huang WH. Anal. Chem. 2014; 86(23):11649–11656. [PubMed: 25381866]
125. Gao Y, Broussard J, Haque A, Revzin A, Lin T. Microsystems Nanoeng. 2016; 2:15045. (August 2015).
126. Mauleon G, Fall CP, Eddington DT. PLoS One. 2012; 7(8)
127. Mauleon G, Lo JF, Peterson BL, Fall CP, Eddington DT. J. Neurosci. Methods. 2013; 216(2):110–117. [PubMed: 23608309]
128. Rogers ML, Feuerstein D, Leong CL, Takagaki M, Niu X, Graf R, Boutelle MG. ACS Chem. Neurosci. 2013; 4(5):799–807. [PubMed: 23574576]
129. Jahn R, Fasshauer D. Nature. 2012; 490(7419):201–207. [PubMed: 23060190]
130. McNew JA, Parlati F, Fukuda R, Johnston RJ, Paz K, Paumet F, Sollner TH, Rothman JE. Nature. 2000; 407(6801):153–159. [PubMed: 11001046]
131. Kaiser CA, Schekman R. Cell. 1990; 61(4):723–733. [PubMed: 2188733]
132. Südhof TC, Rizo J. Cold Spring Harb. Perspect. Biol. 2011; 3(12) 10.1101/cshperspect.a005637 a005637.
133. Keighron JD, Ewing AG, Cans A-S. Analyst. 2012; 137(8):1755–1763. [PubMed: 22343677]
134. Kita JM, Wightman RM. Curr. Opin. Chem. Biol. 2008; 12(5):491–496. [PubMed: 18675377]
135. Koseoglu S, Dilks JR, Peters CG, Fitch-Tewfik JL, Fadel NA, Jasuja R, Italiano JE, Haynes CL, Flaumenhaft R. Arterioscler. Thromb. Vasc. Biol. 2013; 33(3):481. LP-488. [PubMed: 23288151]
136. Zhu Q, Yamakuchi M, Ture S, de la Luz Garcia-Hernandez M, Ko KA, Modjeski KL, LoMonaco MB, Johnson AD, O'Donnell CJ, Takai Y, Morrell CN, Lowenstein CJ. J. Clin. Invest. 2014; 124(10):4503–4516. [PubMed: 25244095]
137. Eckly A, Rinckel J-Y, Proamer F, Ulas N, Joshi S, Whiteheart SW, Gachet C. Blood. 2016
138. Wightman RM, Jankowski JA, Kennedy RT, Kawagoe KT, Schroeder TJ, Leszczyszyn DJ, Near JA, Diliberto EJ, Viveros OH. Proc. Natl. Acad. Sci. 1991; 88(23):10754–10758. [PubMed: 1961743]
139. Ge S, Woo E, White JG, Haynes CL. Anal. Chem. 2011; 83(7):2598–2604. [PubMed: 21384903]

140. Amatore C, Arbault S, Guille M, Lemaître F. Chem. Rev. 2008; 108(7):2585–2621. [PubMed: 18620370]
141. Mellander L, Cans A-S, Ewing AG. ChemPhysChem. 2010; 11(13):2756–2763. [PubMed: 20737529]
142. Lemaître F, Guille Collignon M, Amatore C. Electrochim. Acta. 2014; 140:457–466.
143. van Kempen GTH, vanderLeest HT, van den Berg RJ, Eilers P, Westerink RHS. Biophys. J. 2011; 100(4):968–977. [PubMed: 21320441]
144. Meunier A, Bretou M, Darchen F, Guille Collignon M, Lemaître F, Amatore C. Electrochim. Acta. 2014; 126:74–80.
145. Ge S, Wittenberg NJ, Haynes CL. Biochemistry. 2008; 47(27):7020–7024. [PubMed: 18557631]
146. Ge S, White JG, Haynes CL. Anal. Chem. 2009; 81(8):2935–2943. [PubMed: 19364141]
147. Ge S, White JG, Haynes CL. ACS Chem. Biol. 2010; 5(9):819–828. [PubMed: 20590163]
148. Omiatek DM, Bressler AJ, Cans A-S, Andrews AM, Heien ML, Ewing AG. Sci. Rep. 2013; 3:1447. [PubMed: 23486177]
149. Li X, Majdi S, Dunevall J, Fathali H, Ewing AG. Angew. Chemie Int. Ed. 2015; 54(41):11978–11982.
150. Dunevall J, Fathali H, Najafinobar N, Lovric J, Wigström J, Cans A-S, Ewing AG. J. Am. Chem. Soc. 2015; 137(13):4344–4346. [PubMed: 25811247]
151. Ren L, Mellander LJ, Keighron J, Cans A-S, Kurczy ME, Svir I, Oleinick A, Amatore C, Ewing AG. Q. Rev. Biophys. 2016; 49:e12. [PubMed: 27659043]
152. Li X, Dunevall J, Ewing AG. Acc. Chem. Res. 2016
153. Omiatek DM, Dong Y, Heien ML, Ewing AG. ACS Chem. Neurosci. 2010; 1(3):234–245. [PubMed: 20368748]
154. Hu R, Ren B, Lin C-J, Oleinick A, Svir I, Tian Z-Q, Amatore C. J. Electrochem. Soc. 2016; 163(9):H853–H865.
155. Trouillon R, Ewing AG. ChemPhysChem. 2013; 14(10):2295–2301. [PubMed: 23824748]
156. Trouillon R, Ewing AG. ACS Chem. Biol. 2014; 9(3):812–820. [PubMed: 24400601]
157. Oleinick A, Lemaitre F, Collignon MG, Svir I, Amatore C. Faraday Discuss. 2013; 164(0):33–55. [PubMed: 24466657]
158. Oleinick A, Hu R, Ren B, Tian Z-Q, Svir I, Amatore C. J. Electrochem. Soc. 2016; 163(4):H3014–H3024.
159. Spegel C, Heiskanen A, Pedersen S, Emneus J, Ruzgas T, Taboryski R. Lab Chip. 2008; 8(2): 323–329. [PubMed: 18231673]
160. Meunier A, Fulcrand R, Darchen F, Guille Collignon M, Lemaître F, Amatore C. Biophys. Chem. 2012; 162:14–21. [PubMed: 22257976]
161. Ghosh J, Liu X, Gillis KD. Lab Chip. 2013; 13(11):2083–2090. [PubMed: 23598689]
162. Wang L, Xu S-W, Xu H-R, Song Y-L, Liu J-T, Luo J-P, Cai X-X. Chinese Chem. Lett. 2016; 27(5):738–744.
163. Gao C, Sun X, Gillis KD. Biomed. Microdevices. 2013; 15(3):445–451. [PubMed: 23329291]
164. Trouillon R, Gijs MAM. RSC Adv. 2016; 6(37):31069–31073.
165. Yakushenko A, Schnitker J, Wolfrum B. Anal. Chem. 2012; 84(10):4613–4617. [PubMed: 22509770]
166. Wang J, Trouillon R, Lin Y, Svensson MI, Ewing AG. Anal. Chem. 2013; 85(11):5600–5608. [PubMed: 23627439]
167. Li Y-T, Zhang S-H, Wang L, Xiao R-R, Liu W, Zhang X-W, Zhou Z, Amatore C, Huang W-H. Angew. Chemie Int. Ed. 2014; 53(46):12456–12460.
168. Kissinger PT, Hart JB, Adams RN. Brain Res. 1973; 55(1):209–213. [PubMed: 4145914]
169. Bucher ES, Wightman RM. Annu. Rev. Anal. Chem. (Palo Alto. Calif). 2015; 8:239–261. [PubMed: 25939038]
170. Calipari ES, Ferris MJ, Jones SR. J. Neurochem. 2014; 128(2):224–232. [PubMed: 24102293]
171. Ehrich JM, Phillips PEM, Chavkin C. Neuropsychopharmacology. 2014; 39(13):3036–3048. [PubMed: 24971603]

172. Dreyer JK, Vander Weele CM, Lovic V, Aragona BJ. J. Neurosci. 2016; 36(1):98–112. [PubMed: 26740653]
173. Belle AM, Owesson-White C, Herr NR, Carelli RM, Wightman RM. ACS Chem. Neurosci. 2013; 4(5):761–771. [PubMed: 23480099]
174. Kishida KT, Sandberg SG, Lohrenz T, Comair YG, Sáez I, Phillips PEM, Montague PR. PLoS One. 2011; 6(8):e23291. [PubMed: 21829726]
175. Kishida KT, Saez I, Lohrenz T, Witcher MR, Laxton AW, Tatter SB, White JP, Ellis TL, Phillips PEM, Montague PR. Proc. Natl. Acad. Sci. 2016; 113(1):200–205. [PubMed: 26598677]
176. Nguyen MD, Lee ST, Ross AE, Ryals M, Choudhry VI, Venton BJ. PLoS One. 2014; 9(1):e87165. [PubMed: 24494035]
177. Ross AE, Nguyen MD, Privman E, Venton BJ. J. Neurochem. 2014; 130(1):50–60. [PubMed: 24606335]
178. Adamah-Biassi EB, Almonte AG, Blagovechtchenski E, Grinevich VP, Weiner JL, Bonin KD, Budygin EA. J. Neurosci. Methods. 2015; 256:56–62. [PubMed: 26310181]
179. Van Gompel JJ, Bower MR, Worrell GA, Stead M, Chang S-Y, Goerss SJ, Kim I, Bennet KE, Meyer FB, Marsh WR, Blaha CD, Lee KH. Epilepsia. 2014; 55(2):233–244. [PubMed: 24483230]
180. Ross AE, Venton BJ. Anal. Chem. 2014; 86(15):7486–7493. [PubMed: 25005825]
181. Wang Y, Venton BJ. J. Neurochem. 2016 n/a-n/a.
182. Venton BJ, Michael DJ, Wightman RM. J. Neurochem. 2003; 84(2):373–381. [PubMed: 12558999]
183. Ross AE, Venton BJ. J. Neurochem. 2015; 132(1):51–60. [PubMed: 25219576]
184. Spanos M, Gras-Najjar J, Letchworth JM, Sanford AL, Toups JV, Sombers LA. ACS Chem. Neurosci. 2013; 4(5):782–789. [PubMed: 23556461]
185. Samaranayake S, Abdalla A, Robke R, Wood KM, Zeqja A, Hashemi P. Analyst. 2015; 140(11): 3759–3765. [PubMed: 25902865]
186. Glanowska KM, Venton BJ, Moenter SM. J. Neurosci. 2012; 32(42):14664. LP-14669. [PubMed: 23077052]
187. Schmidt AC, Dunaway LE, Roberts JG, McCarty GS, Sombers LA. Anal. Chem. 2014; 86(15): 7806–7812. [PubMed: 24967837]
188. Bodnar RJ. Peptides. 2016; 75:18–70. [PubMed: 26551874]
189. Makos MA, Kim Y-C, Han K-A, Heien ML, Ewing AG. Anal. Chem. 2009; 81(5):1848–1854. [PubMed: 19192966]
190. Berglund EC, Makos MA, Keighron JD, Phan N, Heien ML, Ewing AG. ACS Chem. Neurosci. 2013; 4(4):566–574. [PubMed: 23402315]
191. Vickrey TL, Condron B, Venton BJ. Anal. Chem. 2009; 81(22):9306–9313. [PubMed: 19842636]
192. Borue X, Cooper S, Hirsh J, Condron B, Venton BJ. J. Neurosci. Methods. 2009; 179(2):300–308. [PubMed: 19428541]
193. Vickrey TL, Xiao N, Venton BJ. ACS Chem. Neurosci. 2013; 4(5):832–837. [PubMed: 23600464]
194. Pyakurel P, Privman Champaloux E, Venton BJ. ACS Chem. Neurosci. 2016; 7(8):1112–1119. [PubMed: 27326831]
195. Majdi S, Berglund EC, Dunevall J, Oleinick AI, Amatore C, Krantz DE, Ewing AG. Angew. Chemie. 2015; 127(46):13813–13816.
196. Feierstein CE, Portugues R, Orger MB. Neuroscience. 2015; 296:26–38. [PubMed: 25433239]
197. Njagi J, Ball M, Best M, Wallace KN, Andreescu S. Anal. Chem. 2010; 82(5):1822–1830. [PubMed: 20148518]
198. Jones LJ, McCutcheon JE, Young AMJ, Norton WHJ. Front. Behav. Neurosci. 2015; 9:246. [PubMed: 26441575]
199. Ryczko D, Cone JJ, Alpert MH, Goetz L, Auclair F, Dubé C, Parent M, Roitman MF, Alford S, Dubuc R. Proc. Natl. Acad. Sci. 2016; 113(17):E2440–E2449. [PubMed: 27071118]
200. Smith AR, Garris PA, Casto JM. J. Chem. Neuroanat. 2015; 66–67:28–39.

201. Atcherley CW, Laude ND, Parent KL, Heien ML. Langmuir. 2013; 29(48):14885–14892. [PubMed: 24245864]
202. Atcherley CW, Wood KM, Parent KL, Hashemi P, Heien ML. Chem. Commun. (Camb). 2015; 51(12):2235–2238. [PubMed: 25249291]
203. Ponchon JL, Cespuglio R, Gonon F, Jouvet M, Pujol JF. Anal. Chem. 1979; 51(9):1483–1486. [PubMed: 484865]
204. Heien MLAV, Phillips PEM, Stuber GD, Seipel AT, Wightman RM. Analyst. 2003; 128(12): 1413–1419. [PubMed: 14737224]
205. Hermans A, Seipel AT, Miller CE, Wightman RM. Langmuir. 2006; 22(5):1964–1969. [PubMed: 16489775]
206. Ross AE, Venton BJ. Analyst. 2012; 137(13):3045–3051. [PubMed: 22606688]
207. Putzbach W, Ronkainen NJ. Sensors (Basel). 2013; 13(4):4811–4840. [PubMed: 23580051]
208. Barsan MM, Ghica ME, Brett CMA. Anal. Chim. Acta. 2015; 881:1–23. [PubMed: 26041516]
209. Yang C, Denno ME, Pyakurel P, Venton BJ. Anal. Chim. Acta. 2015; 887:17–37. [PubMed: 26320782]
210. Swamy BEK, Venton BJ. Analyst. 2007; 132(9):876–884. [PubMed: 17710262]
211. Xiao N, Venton BJ. Anal. Chem. 2012; 84(18):7816–7822. [PubMed: 22823497]
212. Xiang L, Yu P, Hao J, Zhang M, Zhu L, Dai L, Mao L. Anal. Chem. 2014; 86(8):3909–3914. [PubMed: 24678660]
213. Yang C, Jacobs CB, Nguyen MD, Ganesana M, Zestos AG, Ivanov IN, Puretzky AA, Rouleau CM, Geohegan DB, Venton BJ. Anal. Chem. 2016; 88(1):645–652. [PubMed: 26639609]
214. De Volder MFL, Tawfick SH, Baughman RH, Hart AJ. Science (80-.). 2013; 339(6119):535. LP-539.
215. Schmidt AC, Wang X, Zhu Y, Sombers LA. ACS Nano. 2013; 7(9):7864–7873. [PubMed: 23941323]
216. Jacobs CB, Ivanov IN, Nguyen MD, Zestos AG, Venton BJ. Anal. Chem. 2014; 86(12):5721–5727. [PubMed: 24832571]
217. Yang C, Trikantzopoulos E, Nguyen MD, Jacobs CB, Wang Y, Mahjouri-Samani M, Ivanov IN, Venton BJ. ACS Sensors. 2016; 1(5):508–515. [PubMed: 27430021]
218. Harreither W, Trouillon R, Poulin P, Neri W, Ewing AG, Safina G. Anal. Chem. 2013; 85(15): 7447–7453. [PubMed: 23789970]
219. Zestos AG, Jacobs CB, Trikantzopoulos E, Ross AE, Venton BJ. Anal. Chem. 2014; 86(17): 8568–8575. [PubMed: 25117550]
220. Zhang DA, Rand E, Marsh M, Andrews RJ, Lee KH, Meyyappan M, Koehne JE. Mol. Neurobiol. 2013; 48(2):380–385. [PubMed: 23975638]
221. Qi L, Thomas E, White SH, Smith SK, Lee CA, Wilson LR, Sombers LA. Anal. Chem. 2016; 88(16):8129–8136. [PubMed: 27441547]
222. Vreeland RF, Atcherley CW, Russell WS, Xie JY, Lu D, Laude ND, Porreca F, Heien ML. Anal. Chem. 2015; 87(5):2600–2607. [PubMed: 25692657]
223. Meier AR, Matteucci M, Vreeland RF, Taboryski R, Heien ML. Langmuir. 2016; 32(32):8009–8018. [PubMed: 27483032]
224. Wu W-Z, Huang W-H, Wang W, Wang Z-L, Cheng J-K, Xu T, Zhang R-Y, Chen Y, Liu J. J. Am. Chem. Soc. 2005; 127(25):8914–8915. [PubMed: 15969544]
225. Rees HR, Anderson SE, Privman E, Bau HH, Venton BJ. Anal. Chem. 2015; 87(7):3849–3855. [PubMed: 25711512]
226. Duran B, Brocenschi RF, France M, Galligan JJ, Swain GM. Analyst. 2014; 139(12):3160–3166. [PubMed: 24802953]
227. Patel BA. Neurogastroenterol. Motil. 2014; 26(2):237–246. [PubMed: 24188286]
228. Bennet EK, Lee HK, Kruchowski NJ, Chang S-Y, Marsh PM, Van Orsow AA, Paez A, Manciu SF. Materials. 2013:5726–5741.
229. Rapoport SI. J. Pediatr. 2003; 143(4):S26–S34. [PubMed: 14597911]

230. Li Y-T, Zhang S-H, Wang L, Xiao R-R, Liu W, Zhang X-W, Zhou Z, Amatore C, Huang W-H. Angew. Chemie Int. Ed. 2014; 53(46):12456–12460.

231. Watson C, Paxinos G. Paxinos and Franklin's the Mouse Brain in Stereotaxic Coordinates, Fourth Edition. 2012

232. Bartelle BB, Hai A, Jasanoff A. Brain Mapping: An Encyclopedic Reference. 2015; 1:123–129.

233. Goertz RB, Wanat MJ, Gomez JA, Brown ZJ, Phillips PEM, Paladini CA. Neuropsychopharmacology. 2015; 40(5):1151–1162. [PubMed: 25374094]

234. Kim J, Rim YS, Chen H, Cao HH, Nakatsuka N, Hinton HL, Zhao C, Andrews AM, Yang Y, Weiss PS. ACS Nano. 2015; 9(4):4572–4582. [PubMed: 25798751]

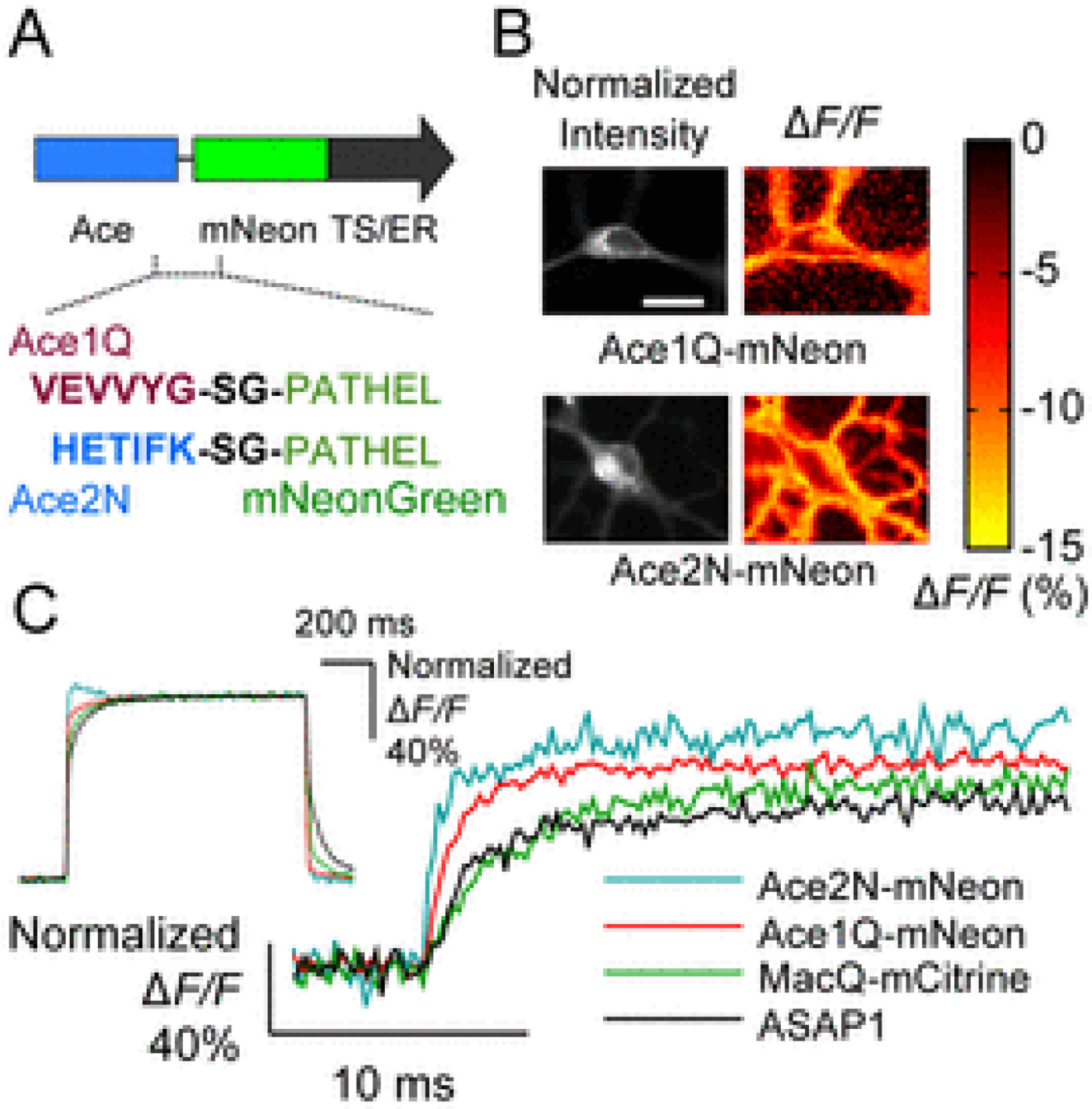

Figure 1.

Ace FRET-opsin sensors report membrane voltage with ~1 millsecond response times. (A) Linker sequences bridging Ace mutants (Ace1Q and Ace2N) to mNeonGreen. Endoplasmic reticulum (ER) export sequence and Golgi export trafficking signal (TS) at the construct's C terminus improves the sensor's membrane localization and hence the signaling dynamic range. (B) Fluorescence signals from neurons expressing Ace1Q-mNeon or Ace2N-mNeon. (Left) Baseline fluorescence emissions from mNeonGreen. (Right) Spatial maps of the fluorescence response (ΔF/F) to a voltage step of approximately 100 mV. Areas of

fluorescence and voltage response were generally co-localized. Scale bar: 20 μm. Illumination intensity: 15 mW·mm^{-2}. (C) Step responses of the Ace sensors, ASAP1 and MacQ-mCitrine in cultured HEK293T cells to +100 mV command voltage steps, normalized to each sensor's maximum (or steady state) ΔF/F response to the command voltage. Ace1Q-mNeon and Ace2N-mNeon sensor responded ~ 5-6-fold faster than that of ASAP1 and MacQ-mCitrine. Illumination intensity: 15–50 mW mm^{-2}. Image frame acquisition rate: 5 kHz. Inset traces were down-sampled to 250 Hz. Reprinted with permission from Gong, Y.; Huang, C.; Li, J. Z.; Grewe, B. F.; Zhang, Y.; Eismann, S.; Schnitzer, M. J. Science 2015, 350 (6266), 1361–1366 (ref 9). Copyright 2015, American Association for the Advancement of Science.

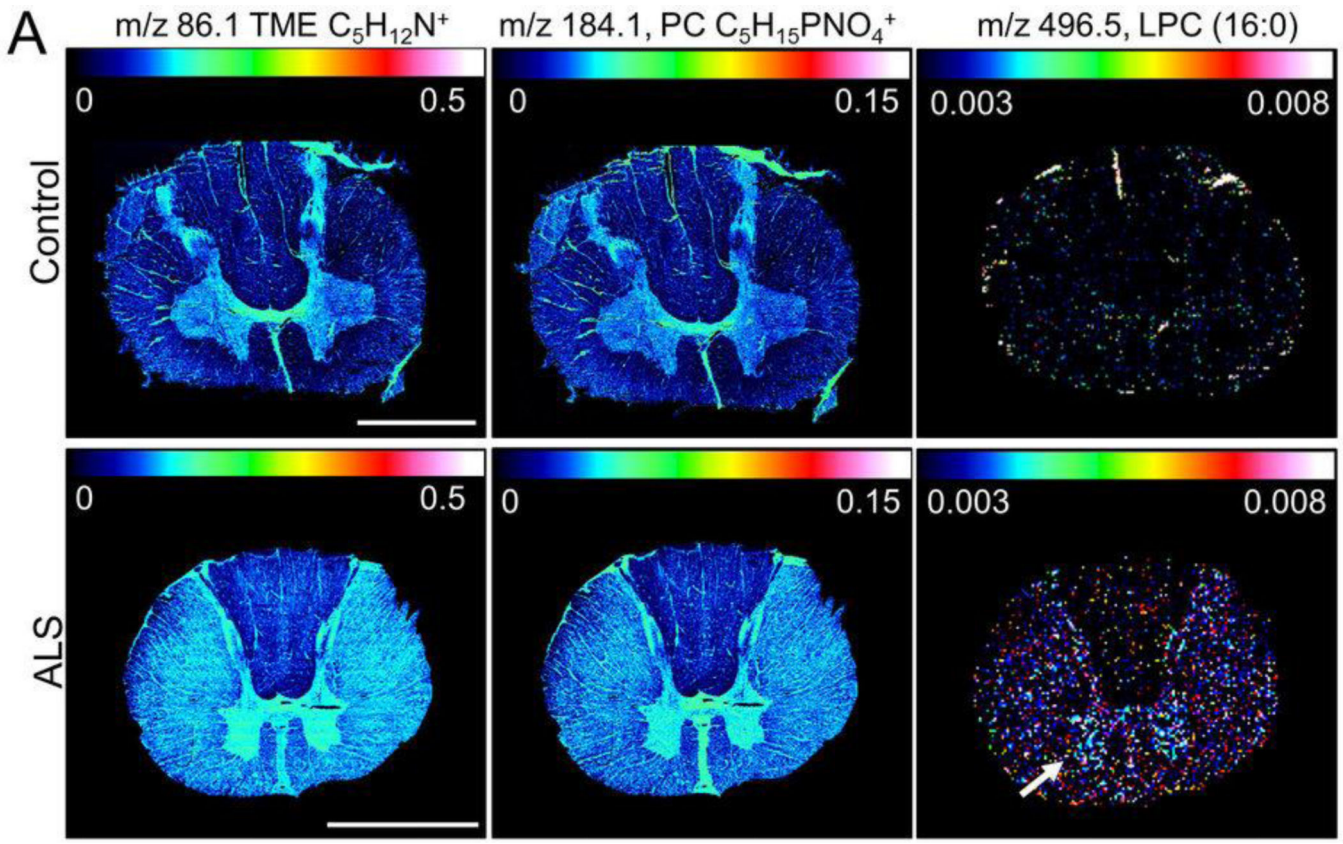

Figure 2.

Single ion images of chemical species elevated in amyotrophic lateral sclerosis spinal cord. Multivariate statistics of regions of interest spectral data using two different multivariate approaches, statistical analysis of microarray data and partial least square discriminant analysis, resulted in detection of Lysophosphatidylcholine as well as its fragments, phosphatidylcholine -headgroup and trimethylethylimine, displaying amyotrophic lateral sclerosis associated increase in the grey matter. Scale bar = 4 mm. Reprinted with permission from Macmillan Publisher Ltd: Spatial Elucidation of Spinal Cord Lipid- and Metabolite- Regulations in Amyotrophic Lateral Sclerosis (ref 63). Copyright 2014.

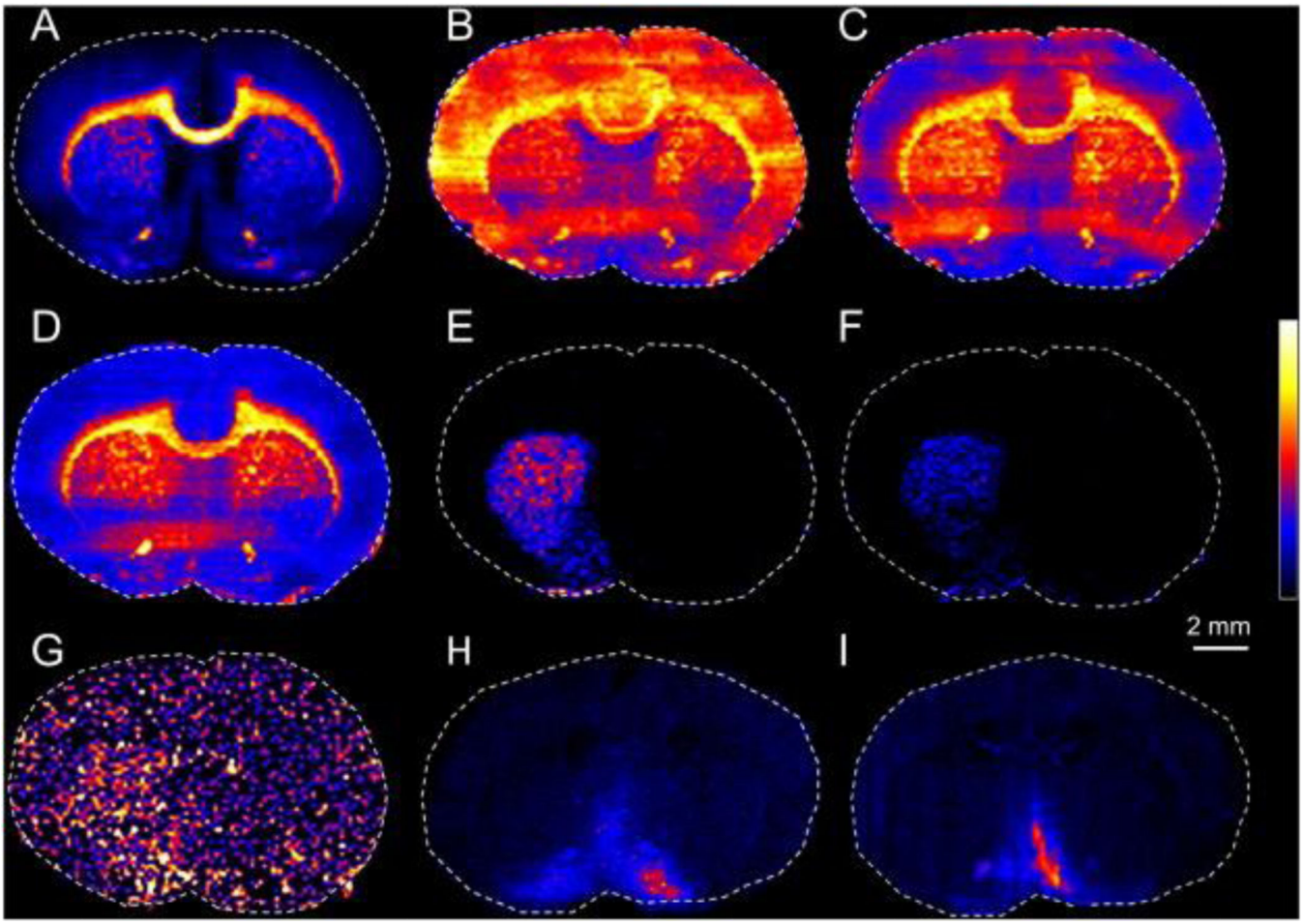

Figure 3.

DESI-MS images of neurotransmitters in coronal rat brain tissue sections. DESI MSI ion intensity maps of (A) adenosine m/z 302.0667, (B) aspartate m/z 132.0304, (C) glutamate m/z 146.0461, (D) glutamine m/z 145.0621, (E) DA m/z 152.0719, (F) D3-DA m/z 155.0908, and (G) DOPAC m/z 167.0352 acquired in negative ionization mode. (H) The GABA molecular ion image m/z 104.0709 and (I) GABA MS/MS product ion image (m/z 87.0445) were acquired in positive ionization mode. Scale bar: 2 mm; spatial resolutions: (A–G, I) 150 μm and (H) 110 μm. Reprinted with permission from Neuroimage, 136, Shariatgorji, M.; Strittmatter, N.; Nilsson, A.; Källback, P.; Alvarsson, A.; Zhang, X.; Vallianatou, T.; Svenningsson, P.; Goodwin, R. J. A.; Andren, P. E., Simultaneous imaging of multiple neurotransmitters and neuroactive substances in the brain by desorption electrospray ionization mass spectrometry, 129–138 (ref 66). Copyright 2016, with permission from Elsevier.

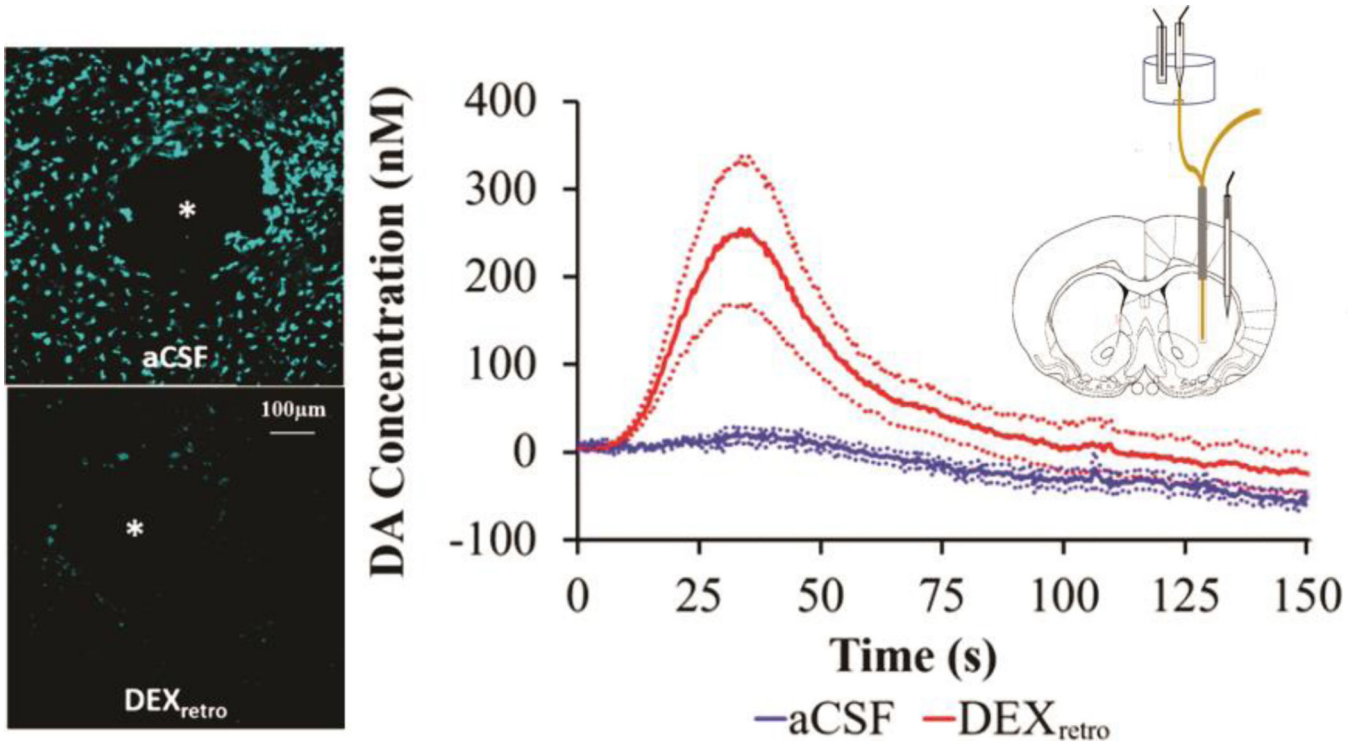

Figure 4.

Administering dexamethasone via retrodialysis diminishes the damage caused by probe implantation. (Left) Immunoreactivity for ED-1 in sections containing the tracks of probes without (top) or with dexamethasone (bottom). (Right) Evoked DA responses (mean ± SEM, n = 6 per group) recorded at the outlet of microdialysis probes 5 days after implantation. Without dexamethasone (blue) the stimulus evoked no response. With dexamethasone (red), the stimulus evoked clear and reproducible responses. Adapted with permission from Varner, E. L.; Jaquins-Gerstl, A.; Michael, A. C. ACS Chem. Neurosci. 2016, 7 (6), 728–736 (ref 79). Copyright 2016, American Chemical Society.

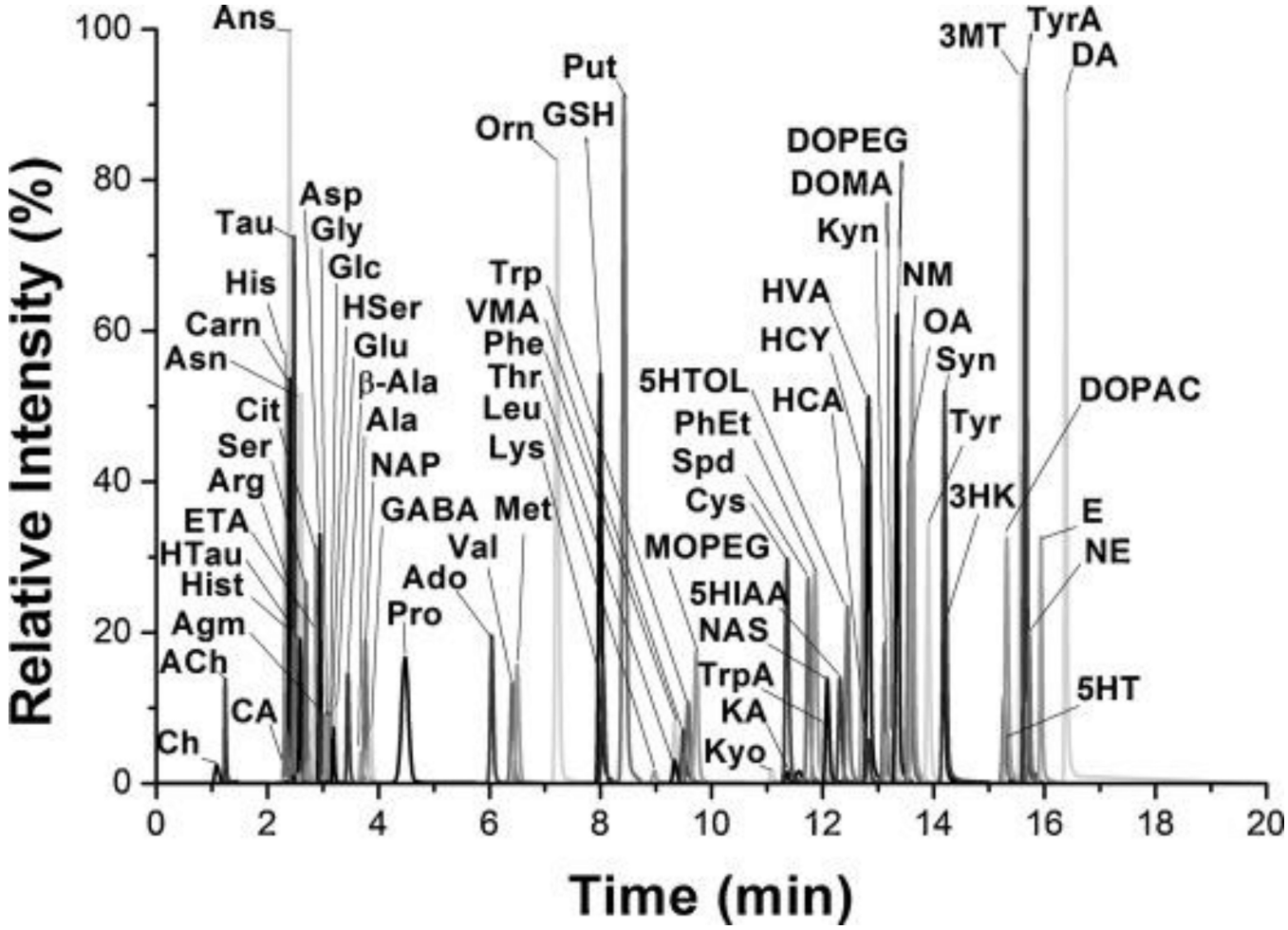

Figure 5.

Reconstructed ion chromatogram of 70 compounds detected in 20 min. Extracted ion chromatograms for each compound at the highest concentration calibration standard run, were normalized to highest intensity and overlaid. Reprinted from Journal of Chromatography A, 1446, Wong, J.-M.T.; Malec, P. A.; Mabrouk,; O.S. Ro, J.; Dus, M.; Kennedy, R.T. Benzoyl chloride derivatization with liquid chromatography-mass spectrometry for targeted metabolomics of neurochemicals in biological samples, pages 78–90 (ref 99). Copyright 2016,with permission from Elsevier

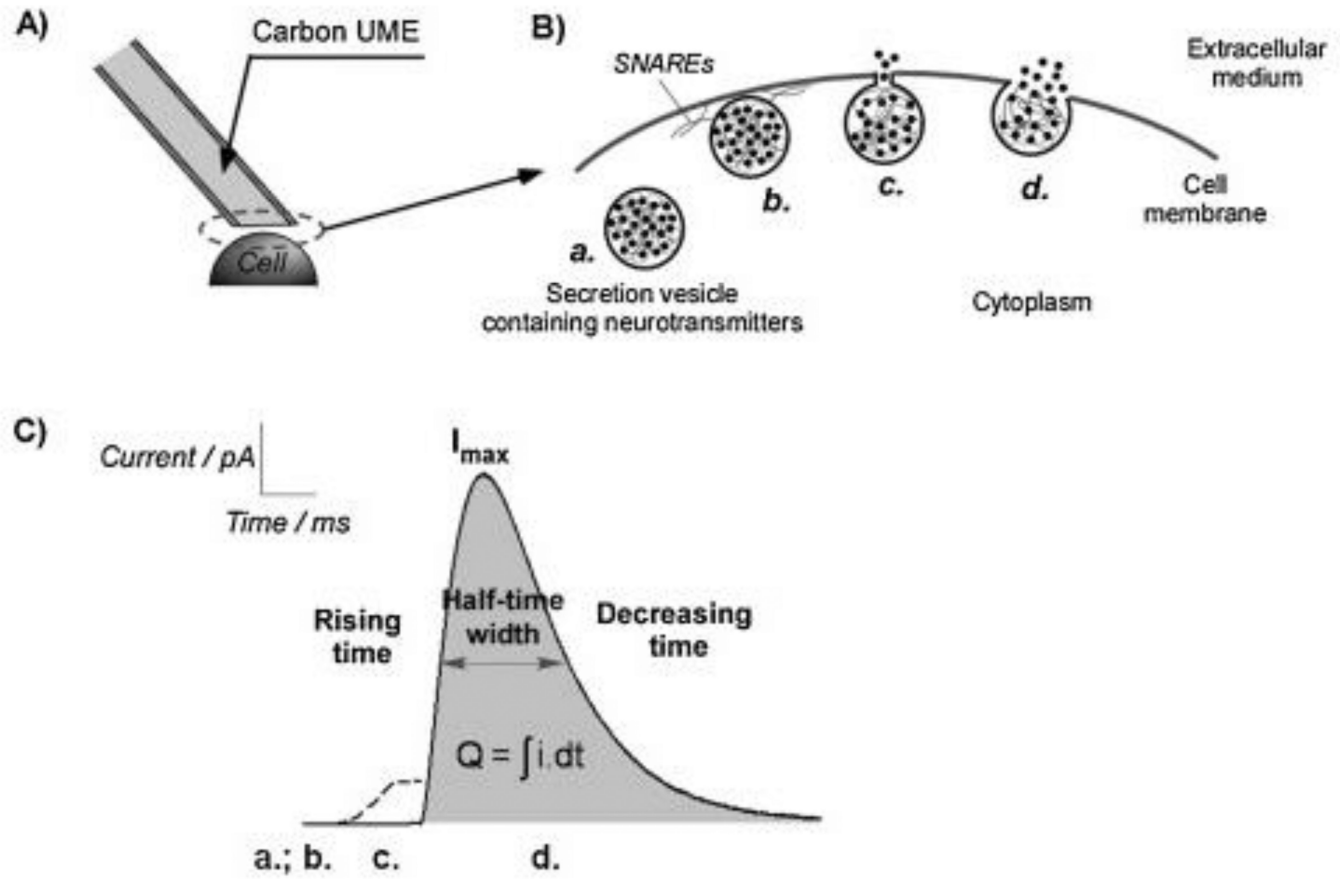

Figure 6.

A) Scheme of the “artificial synapse” configuration. An ultramicroelectrode is positioned in the close vicinity of the emitting cell. If the species released can be oxidized (or reduced) at the electrode surface, amperometry at constant potential allows one to detect a variation of current that features the release in real time. B) Main steps of vesicular exocytosis: after an appropriate stimulation, available vesicles located into the cytoplasm (a.) dock to the cell membrane by the mean of SNAREs assemblies (b.). The subsequent mixing between cell and vesicular membranes induces the formation of a nanometric fusion pore (c.) which can expand and leads to a massive release (d.). C) Typical current spike (correlated to the some steps of exocytosis) recorded by amperometry at a carbon fiber ultramicroelectrode in the “artificial synapse” configuration. The usual extracted parameters (durations, area) are used to decipher the mechanism and its dynamics. Reprinted from Journal of Electrochimica Acta, 140, Frédéric Lemaître, Manon Guille Collignon, Christian Amatore. Recent advances in Electrochemical Detection of Exocytosis, pages 457–466 (ref 142). Copyright 2014, with permission from Elsevier.

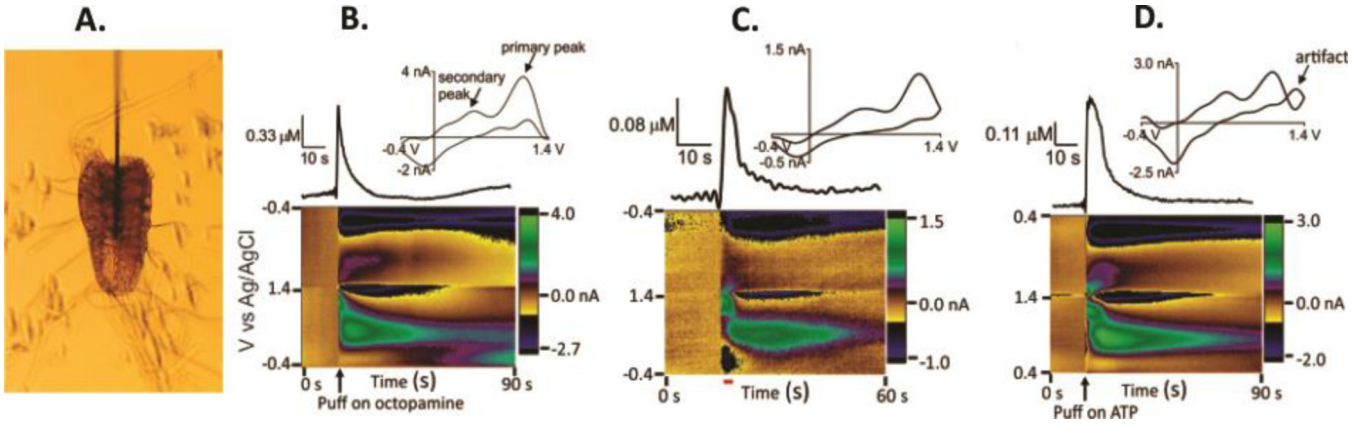

Figure 7.

(A) 7 µm carbon fiber microelectrode inserted into the neuropil of an isolated ventral nerve cord for octopamine measurements. Comparison of puffed-on octopamine and different stimulations. Cyclic voltammograms (top), concentration vs time (center), and color plots (bottom) for (B) octopamine puffed into a larval ventral nerve cord, (C) CsChrimson (red light, 2 s) mediated release, and (D) $P2X_2$ (0.5 pmol of ATP) mediated release in larval ventral nerve cord. All show similar peak characteristics with the primary peak around 1.1 V and secondary peak around 0.5 V with the slower scan waveform. Panels B-D Reproduced from Poojan Pyakurel,; Eve Privman Champaloux,; B. Jill Venton, ACS Chem. Neurosci., 2016, 7 (8), 1112–1119 (ref 193). Copyright 2016, American Chemical Society.

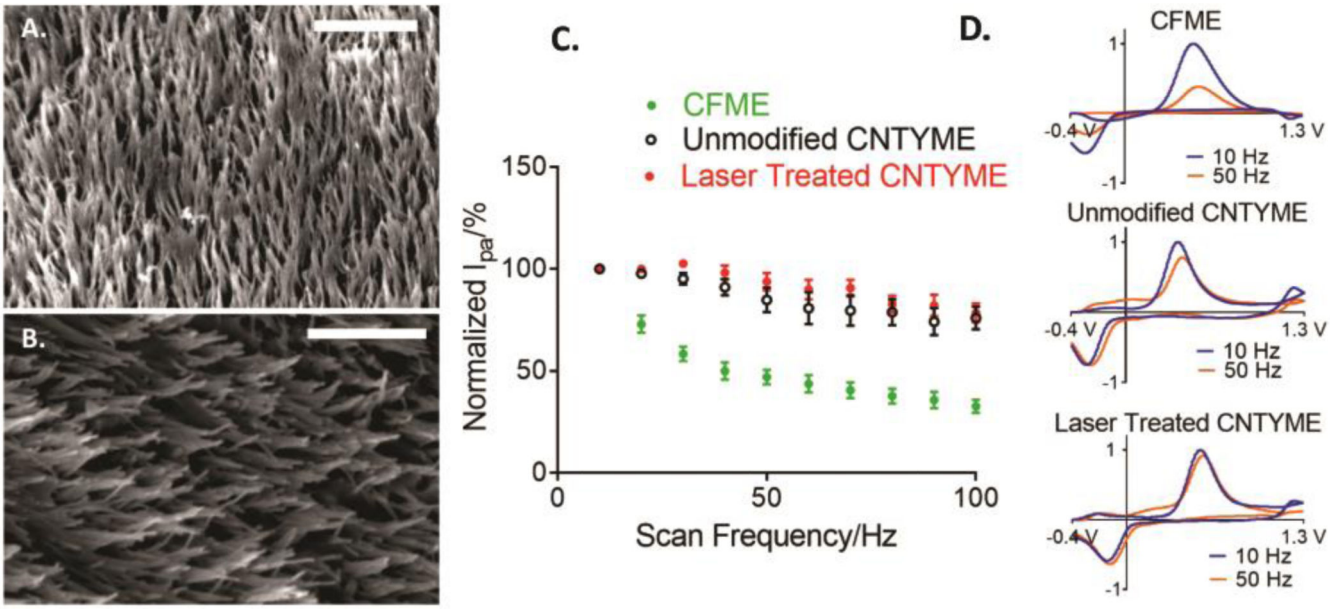

Figure 8.

SEM images of (A) unmodified CNTYME and (B) laser treated CNTYME. Scale bar: 500 nm. Effect of scan repetition frequency for 1 μM dopamine detection at CFMEs, unmodified and laser treated CNTYMEs: (C) Peak oxidation current at CFMEs (green dot, n = 5), unmodified CNTYMEs (black dot, n = 5), and laser treated CNTYMEs (red dot, n = 5) with −0.4 to 1.3 V waveform and scan rate of 400 V/s. Peak currents were normalized to the current at 10 Hz, and error bars represent the standard error of the mean. (D) Example normalized CVs of 1 μM dopamine bolus injection at unmodified CNTYME, laser treated CNTYME, and a CFME at 10 Hz (blue line) and 50 Hz (orange line) scan repetition frequency. CVs are normalized to the 10 Hz signal. Reproduced from Cheng Yang,; Elefterios Trikantzopoulos,; Michael D. Nguyen,; Christopher B. Jacobs,; Ying Wang,; Masoud Mahjouri-Samani,; Ilia N. Ivanov,; B. Jill Venton, ACS Sens., 2016, 1 (5), pp 508–515 (ref 216). Copyright 2016, American Chemical Society.