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Viewpoint

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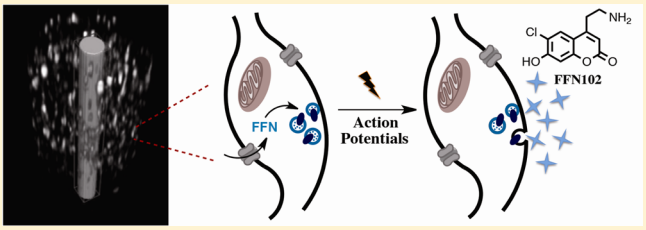
Visualizing Neurotransmitter Secretion at Individual Synapses

Dalibor Sames,*[,†] Matthew Dunn,[†] Richard J. Karpowicz, Jr.,[†] and David Sulzer*[,‡]

[†]Department of Chemistry, Columbia University, 3000 Broadway, New York, New York 10027, United States

[‡]Departments of Neurology, Psychiatry, and Pharmacology, Columbia University Medical Center, New York, New York 10032, United States

ABSTRACT: To advance understanding of the brain, the ability to measure both nerve cell electrical spiking and chemical neurotransmission with high spatial resolution is required. In comparison to the development of voltage sensors and Ca^{2+} indicator dyes over the past several decades, high resolution imaging of neurotransmitter (NT) release at single synapses has not been possible. In this Viewpoint, we discuss two recent developments toward this goal, namely, the design of fluorescent false neurotransmitters (FFNs) and optical neurotransmitter sensors.



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During the formative years of modern neuroscience in the first decades of the 20th century, a central question was whether nerve cells communicate via chemical or electrical means. This question fueled a vigorous debate between adherents of the two opposing theories, also known as the debate of "the soups and the sparks". Today, it is well-known that the vast majority of synapses are chemical synapses, relying on the release of neurotransmitters. Thus, the brain function is fundamentally about *both* soups *and* sparks, or in other words, secretions and spikes. In comparison to measuring the electrical activity of individual neurons and their processes with electrodes, imaging neurotransmitter secretion in the brain with high spatial resolution has for decades remained challenging. In this Viewpoint, we discuss recent advances toward this goal, visualization of neurotransmitter release in brain tissue with the spatial resolution of individual synapses, and suggest future directions, challenges, and impact on neuroscience.

Direct measurement of neurotransmitters in the brain has traditionally been achieved via microdialysis or voltammetry, where the former provides superior chemical selectivity while the latter enables superior temporal resolution. For example, the favorable redox potentials of dopamine allow for direct amperometric measurements of the release and reuptake of this neurotransmitter using carbon fiber microelectrodes. A wealth of information about the dynamics of dopamine release and reuptake in the brain (*ex vivo* and in freely behaving animals), its modulation of other synaptic inputs, and its importance in motor and motivational aspects of behavior has been gained through the use of fast scan cyclic voltammetry. Similar types of studies are feasible for nonelectroactive neurotransmitters using enzyme-coupled electrodes.

Spatial resolution of existing neurochemical measurements, however, is limited by the size of the probes. For example, in the striatum, a carbon fiber microelectrode, which is considerably smaller than a microdialysis probe, might detect dopamine release from hundreds of presynaptic release sites (Figure 1). The number of presynaptic elements assayed also depends on the extent of dopamine overflow from individual presynaptic terminals and its reuptake. Only neurotransmitter molecules that diffuse away from their release sites to the electrode are detected, a phenomenon often labeled as "volume" or "social" neurotransmission. We therefore need to consider two major modes of neurotransmission when discussing NT release imaging: (1) point-to-point or "private" transmission within synapses and (2) extrasynaptic or "social" transmission.

To reach the spatial resolution required for discerning activity at individual presynaptic varicosities (~1 μm in size), scientists have turned to fluorescence microscopy. The development of calcium and voltage sensors, be they synthetic small molecules or proteins, represents a major advance in neuroscience and the imaging of these fundamental parameters (Ca^{2+} concentrations and membrane voltage changes) with high spatial resolution (single cells and synapses) and high temporal resolution (ms-s) is feasible in the living brain tissue. Despite the importance of these approaches, they do not however measure NT release. Furthermore, the arrival of an action potential and the subsequent rise in presynaptic calcium concentrations do not necessarily lead to exocytotic NT release, as neurotransmitter release at individual presynaptic elements is an intermittent, probabilistic event (perhaps because the number of synaptic vesicles poised for release is relatively low).

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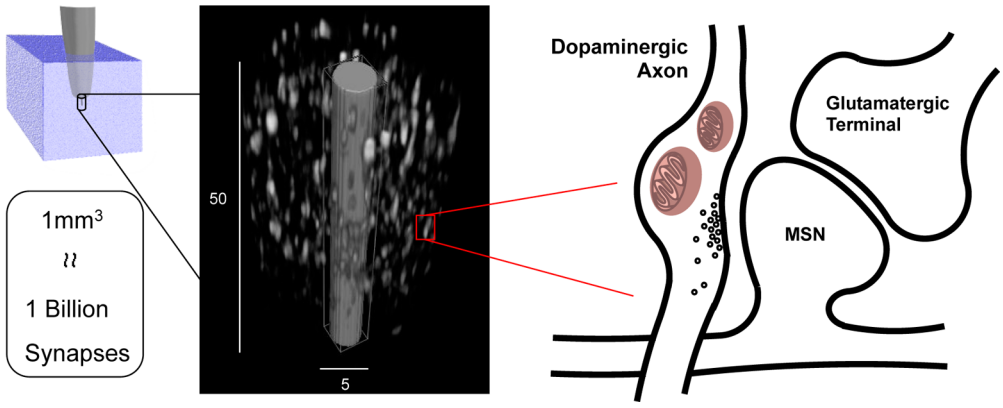

Figure 1. Voltammetric measurement of NTs provides excellent kinetic data, but lacks the spatial resolution required for examining NT secretion from single synapses in intact CNS tissue. It is often estimated that 1 mm^3 of gray matter contains more than one billion synapses. In the rodent striatum, 1 mm^3 contains 10^8 dopaminergic synapses, and thus, a carbon fiber microelectrode of 5 μm in diameter × 50 μm in length detects transmitter released from hundreds of presynaptic varicosities. The central panel visually illustrates this point with an assemblage of a 3D-image of dopaminergic presynaptic sites in living mouse striatum labeled with the fluorescent false neurotransmitter, FFN102. An illustration of a microelectrode represented by the cylinder of the indicated dimensions (scale bars indicate μm) is included for perspective. The oval shape of the presynaptic varicosities is a consequence of the lower spatial resolution of two-photon imaging on the *z*-axis. The right panel is a representation of a striatal dopaminergic presynaptic release site, which is often close to excitatory synaptic inputs to dendritic spines of medium spiny neurons (MSN). The small black circles represent dopaminergic synaptic vesicles and the larger brown structures are axonal mitochondria.

Visualization of exocytosis at active presynaptic varicosities is enabled by the endocytic dyes (for example, the compound FM-1-43), which visualize recycling membranes, or by pH-dependent pHluorin proteins linked to synaptic vesicle proteins. The most common synaptic proteins labeled by pHluorins are synaptobrevin and the vesicular glutamate transporter, VGLUT1. Application of these probes has advanced understanding of presynaptic modulation and plasticity for excitatory and modulatory synaptic inputs; however, even these probes do not directly report on NT release. Further, with endocytic dyes, delivery of hundreds of pulses is typically required to achieve sufficient dye loading and destaining of presynaptic elements in the CNS, limiting the examination of physiologically relevant conditions and, in many cases, likely inducing synaptic plasticity that alters the system being studied.

The direct optical measurement of NT release therefore requires either fluorescent labeling of synaptic vesicle content or placing a fluorogenic NT sensor at or near synapses. With regard to the first approach, our laboratories have pursued the design of fluorescent tracers of NTs, which we termed "fluorescent false neurotransmitters" (FFNs). Focusing initially on the dopaminergic system, we designed FFNs as fluorescent substrates for VMAT2 (vesicular monoamine transporter 2), to load synaptic vesicles and enable visualization of exocytosis. The most recent generation of FFNs were designed as dual DAT (dopamine transporter) and VMAT2 substrates, ensuring high selectivity for dopamine axonal processes via DAT-mediated uptake into dopamine neurons, as well as synaptic vesicle loading mediated by VMAT2 transport (Figure 2).[1] Electrical stimulation results in exocytosis and release of dopamine along with the FFN agent, affording the "FFN destaining" trace for each varicosity as a measure of release kinetics (Figure 2A). Although this approach reached the goal of monitoring dopamine release at individual synapses, destaining requires a high number of pulses (>100), which does not emulate physiologically relevant burst firing of dopamine neurons (in rodents *in vivo*, ~5 pulse trains at 20–25 Hz).

To address this problem, we developed pH-responsive FFNs (e.g., FFN102) that are partially quenched in the acidic lumen of synaptic vesicles and afford a fluorescence increase upon exocytotic release ("FFN flashes", Figure 2B).[1] Ongoing experiments suggest that "flashing FFNs" will enable optical imaging of NT release stimulated by physiologically relevant short pulse sequences. Current and future work focuses on improving the flash signal, characterizing the release and uptake kinetics of specific FFNs, determining how these parameters relate to those for dopamine, and finally developing FFNs for NTs other than dopamine.

FFNs are novel probes that enable imaging of both the microanatomy and the functional parameters of presynaptic varicosities by simply incubating brain tissue with the probe, with no need for prior chemical or genetic tissue manipulation. FFNs report on the function of relevant NT transporters and enable measurement of NT release kinetics at individual release sites (point-to-point transmission imaging) by measuring either the released FFN (flashing) or the remaining FFN (destaining). However, FFNs as surrogate markers of vesicular content do not enable measurement of NTs upon release.

The second major approach to high resolution imaging of NT release relies on the use of fluorogenic sensors that directly measure extracellular NTs. Several optical protein sensors for glutamate have been developed and one class of these sensors, based on a protein-fluorophore hybrid design, known as EOS (for glutamate (E) optical sensors), have been applied to imaging glutamate release in rodent cerebellar, hippocampal, and cortical slices, as well as in the cortex *in vivo*.[2] Molecules of EOS were anchored in the extracellular space via biotin-streptavidin linkage by sequential tissue alkylation and sensor injection. It was demonstrated that the great majority of the sensors were situated extrasynaptically and that glutamate release induced by a few electrical pulses could be measured with high spatial resolution. Further, the extracellular concentration of glutamate could be obtained by numerical deconvolution of the fluorescent signals. This approach provided direct evidence for glutamate volume transmission, where several pulses (2–5) lead to glutamate spikes of 2–9 μM

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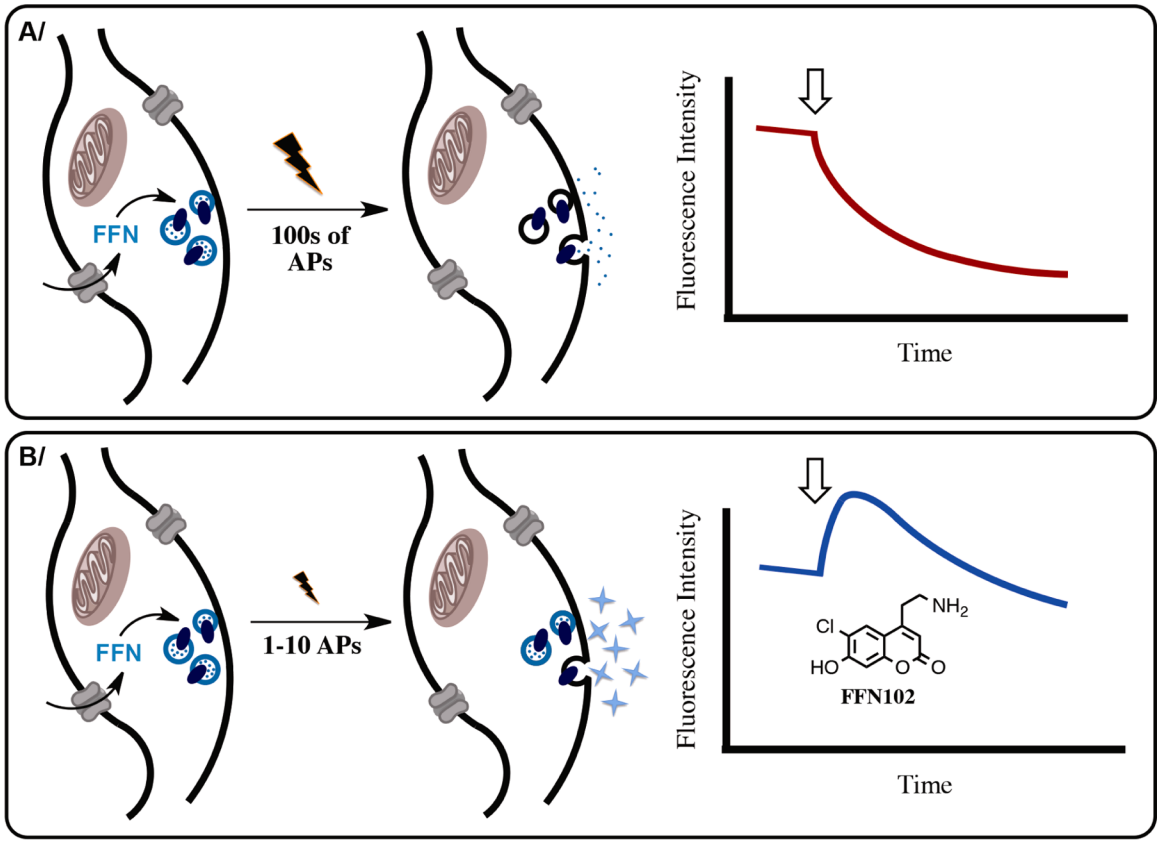

Figure 2. Fluorescent false neurotransmitters (FFNs) are novel probes that act as NT tracers, enabling the measurement of kinetics of NT secretion at individual synapses. (A) "Destaining FFNs" label synaptic vesicles (light blue circles) via uptake by vesicular monoamine transporter 2 (VMAT2, dark blue filled ovals) and are released upon exocytosis. The rate of release is determined by measuring the remaining presynaptic FFN (the released FFN presumably diffuses away). (B) "Flashing FFNs" provide an increase in fluorescence upon exocytotic release, which is important for the measurement of NT secretion induced by a few action potentials (APs) (providing analysis under a broader range of physiologically relevant conditions). Flashing was achieved by designing pH-responsive FFNs, such as FFN102. The gray squares represent plasma membrane transporters, dopamine transporters in the case of FFN102, and light blue circles represent synaptic vesicles that express VMAT2.

concentrations that last <50 ms and spread over the area of few micrometers (the width at half the maximal fluorescent signal was ~7 μm; a similar spatial range of volume transmission has been found for dopamine by amperometric recordings). Acetylcholine volume transmission has also been measured by implanting special cells engineered to provide a fluorescent readout in response to M1 muscarinic receptor activation (CNiFERs, cell-based neurotransmitter fluorescent engineered reporters) in brain tissue.[3] One can envision the application of this method for the detection of a myriad of NTs and other signaling molecules, such as ATP.

These studies indicate a great potential for NT sensors, although we note that these particular approaches cannot image point-to-point transmission or determine which specific synaptic release sites secreted NT and contributed to volume transmission. This issue has been addressed by a different NT sensor design, namely, a fully genetically encoded, single-wavelength glutamate sensor based on circularly permuted GFP.[4] This sensor (iGluSnFR) has excellent functional parameters (signal-to-noise ratio, kinetics of response, photostability, high saturation point) and was targeted to neurons via viral transfection. Glutamate release was demonstrated at single dendritic spines in different *ex vivo* experimental systems, as well as *in vivo* in *C. elegans*, fish, and rodents. Glutamate release was also measured at single dentritic spines in mouse motor cortex in relation to forward and reverse running.

The development of genetically encoded glutamate sensors is an advance of high potential impact. An important issue, however, is the selective expression of these sensors. In the current version, expression of iGluSnFR was driven by the synapsin promoter and, thus, the sensor was produced in all neurons. As a result, presynaptic elements and dendritic processes had to be differentiated by the anatomical context and micromorphology of neuronal processes. However, these issues can be addressed by using additional cell markers or by more specific targeting, for example, by using cell-specific promoters, or by fusing the sensor to a protein with selective expression in desired cells and structures.

In the context of neurotransmission, there is growing evidence in support of cotransmission, where release sites secrete not only the cognate NT, but also other NTs and modulators. For example, in addition to glutamate, some excitatory inputs secrete aspartate or zinc ions; some dopamine terminals corelease glutamate, and many different synaptic terminals secrete ATP. Furthermore, considering the possibility of NT release by dendritic sites, neuronal soma and glia, the complexity of the transmitter "soups" surrounding the neuronal wiring becomes apparent. Therefore, the design of optical sensors for NTs and other modulators will continue to be an important endeavor. In fact, we can envision a near future when FFNs provide the anatomical and functional parameters for the release sites, while strategically positioned NT sensors afford

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the spatiotemporal measurement of concentration gradients for NTs (and co-NTs) of interest. Further, integration of FFNs and NT sensors with other indicators, most notably voltage sensors, will enable functional mapping of both the spiking and releasing properties of individual synaptic connections. With these tools in hand, the study of activity and modulation of individual synapses, as well as behavior of large ensembles of synapses, in the context of specific circuits and specific behavior paradigms will be possible. With the connectome mapping well underway in a number of model organisms, *novel imaging tools will advance the study of functional plasticity in the context of complete connectivity maps*.

The metaphor of "the soups and the sparks" used to describe the two camps of scientists in the historical debate over the mode of communication between nerve cells holds an evocatively descriptive quality that captures the two fundamental and inseparable aspects of the brain; complex spiking patterns within immensely intricate neuronal wiring, and all of that bathed in the equally complex and dynamic mixtures of concentration gradients of chemicals. The relationship between spikes and secretions is causal in both directions; spikes lead to secretion of neurotransmitters, and the resulting soups control and modulate spiking behavior. We thus envision that two maps of brain activity,[5] voltage maps and NT concentration gradient patterns, will become available at different levels of resolution (specific synaptic connections, entire circuits). These maps can then be correlated with specific phenotypes associated with motor behavior, mental states, pathological processes, and pharmacological or other treatment interventions, ultimately leading to deeper understanding of the nervous system. The development of novel experimental tools that enhance our abilities to examine key functional parameters of brain circuits will, as they always have, play the central role in this exciting and important endeavor.

AUTHOR INFORMATION

Corresponding Author

*E-mail: ds584@columbia.edu (D. Sames); ds43@columbia.edu (D. Sulzer).

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