Little Strokes Fill Big Oaks: A Simple In Vivo Stain of Brain Cells

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DOI 10.1016/j.neuron.2007.03.001

High-resolution functional imaging of neural activity in vivo relies on appropriate labeling methods. In this issue of *Neuron*, Nagayama et al. introduce a simple procedure for staining subsets of neurons with organic calcium indicator dyes via local electroporation. Neuronal populations are sparsely labeled, preserving the ability to resolve calcium signals in dendrites and synaptic structures.

An old saying among neuroscientists states, "The gain in brain is mainly in the stain." This notion clearly expresses the fundamental importance of staining methods for neuroscientific research. Labeling techniques have been and continue to be the crucial step for both neuroanatomical analysis using histological methods and modern functional analysis using activity-dependent markers. For example, the major insights gained at the end of the 19th century into the organization of the nervous system largely relied on the development of the silver chromate staining technique by Camillo Golgi. The Golgi method stains only a small fraction of cells but with high contrast. This sparseness of the stain turned out to be a major advantage because it permitted identification of individual cells and subcellular elements within the dense neural tissue and thus fostered ideas about how neuronal circuits might be organized.

Nowadays, neuroscientists still face the old problem of how to stain the brain when they strive for functional measurements of neural circuit activity in living animals. Meanwhile, key technologies are at hand with deep-tissue two-photon microscopy (Helmchen and Denk, 2005) and a large toolbox of activity-dependent fluorescent probes—containing both "traditional" organic dyes such as commonly used calcium indicators (Tsien, 1989) and more recent activity-dependent fluorescent proteins (Miyawaki, 2005; Knöpfel et al., 2006). Besides the sensitivity of the functional markers, the mode of dye delivery remains a critical issue. A number of labeling techniques are available—ranging from physical methods to genetic means—which differ, however, with respect to their efficiency, specificity, and simplicity.

Functional in vivo two-photon imaging mainly relies on fluorescent indicators reporting intracellular calcium concentration. The most common modes of applying the traditional organic calcium indicators are intracellular filling of individual cells via recording electrodes (Svoboda et al., 1997) and bulk-loading of membrane permeable acetoxy-methyl (AM)-ester forms of the indicator (Stosiek et al., 2003). The latter method labels populations of neurons but also leads to a diffuse stain of the neuropil with all its axonal, dendritic, and glial components (Kerr et al., 2005). The pros and cons of these approaches are evident: single-cell loading permits high-resolution investigation of dendritic signaling down to the synapse level, while bulk loading is well suited for investigating population activity, albeit without the option to identify subcellular compartments with high contrast (Figures 1A and 1B).

In this issue of *Neuron*, Wei Chen's group presents yet another approach to deliver organic indicators to neurons (Nagayama et al., 2007). This novel method results in an intermediate, Golgi-like sparse labeling of neuronal populations. They filled a micropipette with a micrometer-sized tip with high concentrations (5%–10% w/v) of calcium indicators (either as dextran conjugates or in their salt form) and inserted the pipette into the brain of anesthetized mice. Through the same pipette, they then applied more than one thousand mild electrical pulses (~5 μA for 25 ms) over a period of 10 min. Surprisingly, using this procedure the dye deposited in the extracellular space was taken up by a subset of neurons in the vicinity of the stimulation site, and these neurons were crisply labeled with calcium indicator following washout of the extracellular stain within 1–2 hr (Figure 1C). Because dye uptake critically depended on electrical stimulation, the most likely explanation for this observation is that each electrical pulse caused a transient permeabilization of nearby cell membranes through electroporation (although a potential role of activity-dependent uptake at synapses remains possible). The formation of nonselective membrane pores leads to the repeated delivery of tiny extracellular droplets of highly concentrated calcium indicator to the cytosol of the affected cells. The accumulation of many droplets in these cells and subsequent diffusional equilibration throughout their dendritic trees result in sufficiently high intracellular dye concentrations for in vivo imaging (estimate around 20 μM). The whole procedure is reminiscent of in vivo juxtacellular labeling of neurons with biocytin (Pinault, 1996).

Electroporation is widely used for delivery of small molecules to the cytosol. For example, electroporation of DNA and RNA is a common method

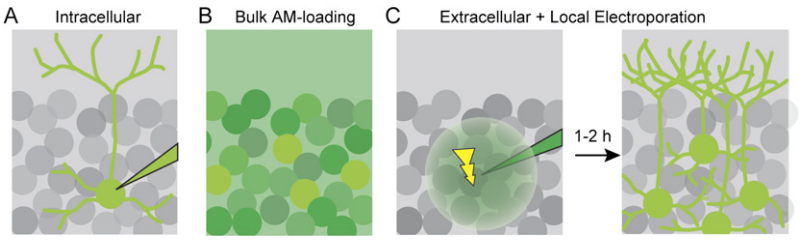

Figure 1. Different Protocols for Loading Organic Calcium Indicator Dyes In Vivo
(A) Single-cell loading via intracellular recording electrodes results in high contrast. Similarly, targeted single-cell electroporation can be used to label one to a few cells with high contrast.
(B) Bulk loading of membrane-permeable dyes results in population loading but with diffuse background.
(C) In the novel method described by Nagayama et al. in this issue of *Neuron*, dextran-conjugates or the salt form of calcium indicators are delivered to a confined brain region through a glass pipette (left). Repetitive electrical stimulation causes dye uptake into neuronal processes by local electroporation. Following extracellular washout of the dye and intracellular diffusion, subsets of neurons are labeled with high contrast.

to transfect cells with specific genes, both in vitro and in vivo (Haas et al., 2001; Rathenberg et al., 2003). In addition, fluorescent dyes have been electroporated into cells, including more recently calcium indicator dyes (Teruel et al., 1999; Bonnot et al., 2005; Nevian and Helmchen, 2007). What is specific about the new approach is that it neither uses large area electrodes for bulk electroporation (Teruel et al., 1999; Bonnot et al., 2005) nor does it specifically target individual neurons, as it is possible in vitro and in vivo (Nevian and Helmchen, 2007). Instead, it is based on the presumption that membranes of dendritic and axonal processes passing through a confined region surrounding the electroporation pipette are permeabilized. The affected region has an estimated diameter of only a few tens of microns, which poses an interesting boundary condition on the labeled network. Depending on the circuit organization in the targeted brain region, networks with different morphological characteristics are loaded. For example, in the cerebellar cortex, local electroporation stained parallel fibers and Purkinje cells in a highly confined manner. In the neocortex, the distribution of labeled pyramidal cells was more dispersed, consistent with the dendritic field span of basal dendrites; and in the olfactory bulb widely distributed populations of mitral cells were filled, presumably because any given region is crisscrossed by many of their long secondary dendrites. Although one could expect that uptake into different cell types should be unspecific within the electroporation region, glial cells were not labeled for unknown reasons.

An important issue with electroporation is cell viability as the application of a strong electric field near a lipid bilayer transiently opens nonselective pores and thus leads to a breakdown of the membrane potential. Nagayama et al. (2007) demonstrate that evoked field potential in the olfactory bulb were not affected by the loading procedure, indicating that circuit responses are not disrupted. Consistently, monitoring the electroporation process directly using whole-cell recordings, we found that neurons recover rapidly and with high viability rate (Nevian and Helmchen, 2007). Furthermore, the biophysical properties of the cells, like input resistance, resting membrane potential, and action potential waveform were unaltered. Taken together with the observation of calcium transients with normal shape in a number of studies, these findings indicate that electroporation is a rather gentle staining method.

The local electroporation technique has several advantages: (1) the procedure is simple and functional staining is obtained within a few hours. (2) Dextran-conjugated and salt forms of calcium indicators work equally well, making a wide range of indicators with different spectral properties and Ca^{2+} affinities applicable. (3) The Golgi-like staining of only a fraction of cells with low background permits calcium imaging from subcellular compartments. Nagayama et al. (2007) impressively demonstrate this potential of the method by recording calcium transients in axonal boutons and single dendritic spines in vivo. Multicolor labeling should even allow for simultaneous imaging of pre- and postsynaptic calcium dynamics at synaptic contacts (Nevian and Helmchen, 2007). (4) Cellular signals within the labeled subpopulation may be interpreted in terms of the circuit organization. For example, Nagayama et al. (2007) measured odor-evoked responses in mitral cell populations and could assign each cell to its respective glomerulus. (5) The method can easily be combined with various other labeling and recording techniques. Since any small molecule can be delivered by electroporation, coelectroporation of Ca^{2+} indicator with other fluorophores or DNA is feasible.

An obvious disadvantage of the local electroporation method is that cell labeling is rather unspecific unless the structure is highly ordered. Targeted electroporation could help to achieve labeling of better-defined subpopulations (Nevian and Helmchen, 2007). Alternatively, genetically encoded calcium indicators (GECIs) are promising tools, as their expression can be targeted to specific cell types. In fact, GECIs with improved sensitivity and functional expression in transgenic animals become now available (Heim et al., 2007). The combination of GFP expression and electroporation of organic indicators (e.g., in two different colors) might also be useful, as only those cells could be picked for analysis that belong to a specific cell class. On the network level, such approaches might help to elucidate the functional role of cell subtypes in the circuit. Another ambiguity is in how far the new method can link network dynamics and connectivity. Even with sparse labeling and the possibility to resolve dendrites and axons, light-microscopic reconstruction of the synaptic connectivity pattern seems difficult, if not impossible.

In summary, this novel staining method further expands the repertoire of functional labeling techniques for high-resolution imaging. It fills a niche

between single cell loading, targeted filling of a few cells, and staining of complete populations using bulk loading. The use of all these techniques—alone or in combination—will provide important steps toward the functional characterization of local neural circuits in the brain. Moreover, the simple application to living animals might facilitate novel imaging approaches for optical recordings of neural activity in awake, freely behaving animals.

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In the Eye of the Beholder: Visual Experience and Categories in the Human Brain

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DOI 10.1016/j.neuron.2007.03.003

How does experience change representations of visual objects in the brain? Do cortical object representations reflect category membership? In this issue of *Neuron*, Jiang et al. show that category training leads to sharpening of neural responses in high-level visual cortex; in contrast, category boundaries may be represented only in prefrontal cortex.

In 350 BC, Aristotle asked: "What is there"? His answer is given in the title of his book: *Categories*. Ever since, philosophers and scientists have asked how we carve the world into distinct categories.

Two millennia later, neuroscientists have begun to tackle this question with methods Aristotle could not have dreamt of. Using single-cell recording and functional magnetic resonance imaging (fMRI), they can ask: what are the neural mechanisms that underlie categorization, and visual perception in general? The first steps in answering this question were made when, in the 1950s, David Hubel and Torsten Wiesel began to elucidate the response properties of single neurons in early visual cortical areas. Later, scientists shifted their focus to more high-level visual areas, such as inferotemporal cortex, where Bruce et al. (1981) discovered single neurons that respond selectively to complex object categories like faces and hands. Most recently, researchers have tackled the central question of how cortical object representations arise in the first place and specifically how they may be shaped by experience (Op de Beeck et al., 2006; Baker et al., 2002).

In this issue of *Neuron*, Jiang et al. (2007) report a study in which they combine the old question about the nature of categories with the contemporary neuroscientists' question about the origin of cortical object representations. Inspired by previous electrophysiological studies in monkeys (Freedman et al., 2001), they ask: what are the neural mechanisms that underlie the formation of visual categories through experience? Specifically, does training sharpen neural object representations? Further, is neural sensitivity higher to differences between stimuli belonging to different categories compared to stimuli belonging to the same category?

In the new study, human participants were trained for an average of 5 hr to discriminate between two types of cars. The cars came from a morphed