Imaging cortical electrical stimulation *in vivo*: fast intrinsic optical signal versus voltage-sensitive dyes

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We applied high-temporal-resolution optical imaging utilizing both the fast intrinsic optical signal (fIOS) and voltage-sensitive dyes (VSDs) to observe the spatiotemporal characteristics of rat somatosensory cortex during electrical stimulation. We find that changes in both the fIOS and VSD signals occur rapidly (<30 ms) after the stimulus is applied, suggesting that both membrane depolarization and transmembrane ion movement occur shortly after the stimulus, preceding the more gradual physiological changes in oxygen consumption revealed by the slower component of the intrinsic optical signal. We find that the VSD signal spreads through a much larger area of cortex than the fIOS.

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In voltage-sensitive dye (VSD) imaging, dye molecules embedded in the cell membrane fluoresce proportionally to changes in the transmembrane potential difference. In contrast, the IOS reveals various activity-related optical changes in neural tissue, depending on the wavelength of the incident light [1]. For wavelengths in the green range, a drop in reflectance reveals an increase in blood volume; for longer wavelengths (600–630 nm), the signal indicates the oxygenation state of hemoglobin. For red and near-infrared light, the signal is primarily a result of light scattering and cell swelling [1]; the fast component of the IOS (fIOS) occurs in this wavelength range [2].

If the fIOS is to be used as a stand-in for neural activity, it is critical to investigate precisely how the fIOS overlaps spatially and temporally with the VSD signal. Here, we investigate the cortical response to direct electrical stimulation *in vivo* using the fIOS, in comparison with VSD imaging under identical conditions. We investigate two VSDs, RH-795, which has been used for a number of years in *in vivo* neuroimaging studies, and RH-1691, one of the new class of so-called "blue dyes," which is less sensitive to contamination from the hemodynamic components of the intrinsic signal [3].

Imaging was performed on 12 adult rats (Sprague-Dawley; weight 250–350 g; age 2–3 months), initially anesthetized with ketamine (90 mg/kg, i.p.) and xylazine (2.0 mg/kg, i.p.) and sustained with urethane (1.25 gm/kg, i.p.) and furosemide sulfate (0.1 mg/kg, i.m.). Temperature was kept at 37°C with a heating blanket (Harvard Apparatus). The heart rate was monitored using a small-animal Capnograph (SurgiVet) and maintained stable during the experiment.

After the animal was fixed in a stereotaxic frame, the cranium above the left somatosensory cortex was removed using a dental drill. A chamber made of dental cement (5–7 mm i.d.; height 1–2 mm) was constructed above the hole in the skull. The dura within the area of the chamber was removed, and the brain was covered with Ringer's solution. For VSD imaging, the dyes RH-795 (Molecular Probes, 0.6 mg/ml in Ringer's) or RH-1691 (Optical Imaging, 0.6 mg/ml in Ringer's) were applied to the exposed cortex for 60 min. After staining, the cortex was washed with dye-free Ringer's for 10 min. The chamber was filled with 0.3% agar and sealed with a cover glass.

A tungsten filament lamp (12 V, 100 W, Zeiss) was used for illumination. The light passed through a heat filter (Zeiss) and a 546±30 nm (for RH-795) or 630±20 nm (for RH-1691) interference filter (Edmund Optics) and a dichroic mirror (650DRLP, Optical Imaging Inc.). For fIOS imaging, the brain was illuminated at a wavelength of 694 nm±10 nm. A 16-bit CCD camera (Cascade 512B, Roper Scientific) was focused 0.3–0.4 mm below the cortical surface. Imaging was performed through a tandem configuration of two 50 mm lenses, at a frame rate of 100 Hz, and recorded using Metamorph software (Molecular Devices) on a Windows XP platform.

A bipolar tungsten electrode MX216TW(VT1) (FHC Inc.) was introduced into the cortex at a 0.3–0.4 mm depth. A 100 μA stimulus was generated by an S-48 stimulator (Grass Instruments) and consisted of a 10 ms train of 0.2 ms pulses at 300 Hz.

To reduce noise, optical signals from both imaging modalities were averaged over 30 trials in each animal, with a 35 s interval between trials. After averaging, percentage change in the averaged reflectance [$\Delta R/R$ (%), fIOS] or fluorescence [$\Delta F/F$ (%), VSD] was calculated using custom-written software (Matlab, The Mathworks). VSD imaging was performed in six rats, three with RH-795 and three with RH-1691; fIOS imaging was performed in seven rats; one of these was also used in the RH-795 group.

In Fig. 1, we qualitatively compare fIOS and VSD imaging from the same animal. Panel A shows the imaged area superimposed on the exposed cortex; panel B shows the time course of the fIOS and the

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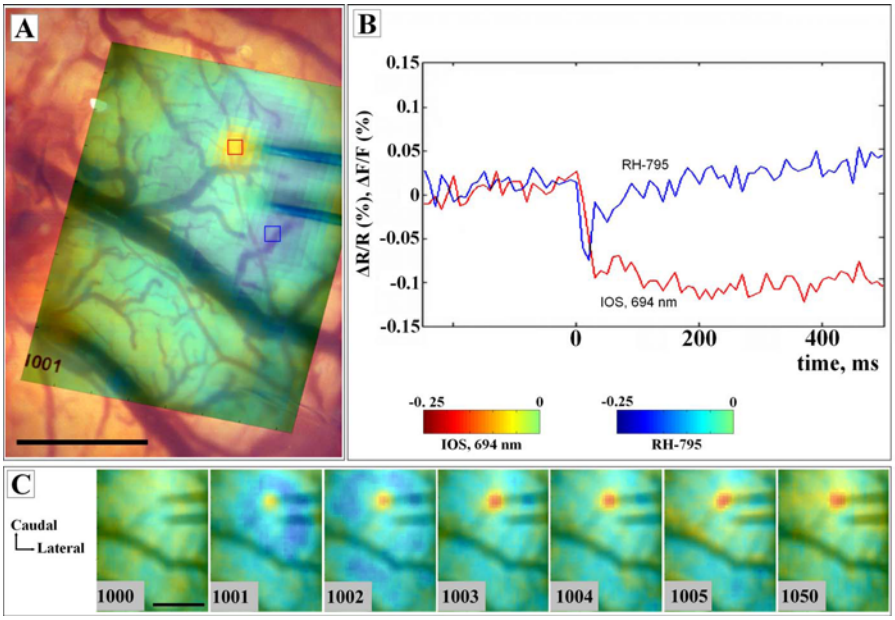

Fig. 1. (Color online) A, superimposition of the region of recording on a color image of the cortical surface. B, VSD (top curve), fIOS (bottom curve). In both cases (VSD and fIOS), the signal is averaged over 30 trials. C, images of the cortex before and after stimulus onset; numbers indicate camera frame number; time interval between frames is 10 ms; frame 1000 coincides with time $t=0$ ms in 1B. The fIOS signal (left color scale) is superimposed on the image of the cortex, as is the VSD signal (right color scale). Calibration bar shows 1 mm.

VSD (RH-795) signals in the red and blue (gray curve and black curve, respectively) regions of interest (100 μm $\times$ 100 μm), respectively. Note that the fIOS occurs in a small region in the immediate vicinity of one of the stimulating electrodes (cathode). In contrast, the VSD signal occurs in a targetlike pattern, spreading outward from the electrode.

With RH-795 (as with RH-1691), a fluorescence change was observed in the frame immediately following the stimulation onset, reaching a maximum at 10–20 ms after stimulation onset and then decaying during the subsequent frames. The fIOS likewise appeared in the frame immediately after stimulus onset and increased sharply in the first 10–20 ms before reaching a plateau and gradually returning toward baseline (after 500 ms, not shown in the figure).

Figures 2A and 2B show the time course of the spread of the RH-795 signal at various locations. The numbers of the traces in Fig. 2A correspond to the numbers of the 100 $\times$ 100 μm regions of interest in Fig. 2B. Note the fast signal onset (<10 ms); in this rat, a faster frame rate than usual was employed

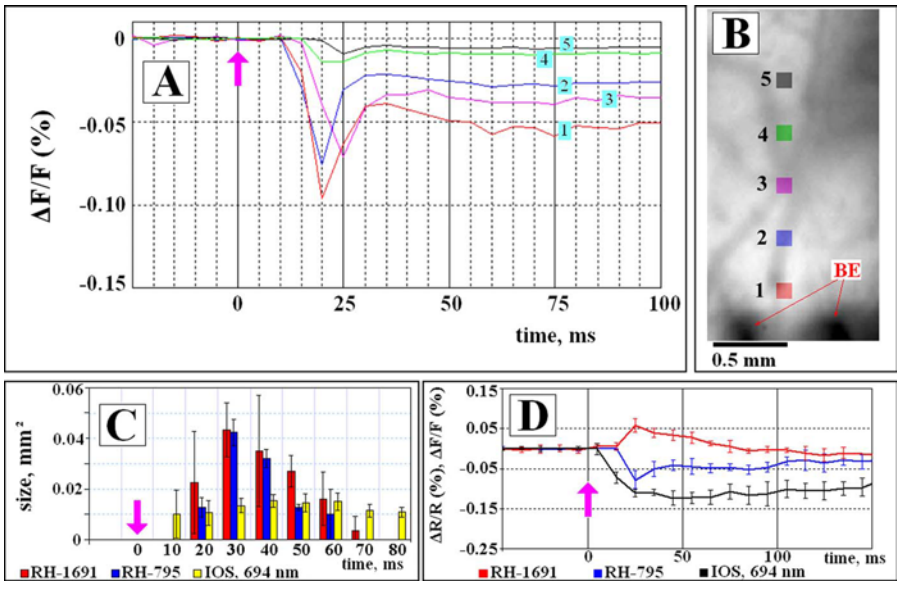

Fig. 2. (Color online) A, time course of RH-795 response at the five locations depicted in B. The numbers and colors in A correspond to those in B; the bipolar electrode is labeled BE. C, areas activated by electrical stimulation. D, signal time course in a 100 $\times$ 100 μm region of interest near the stimulation electrodes, averaged over all rats imaged. In all panels, arrow indicates stimulus onset. In panels C and D, error bars show standard deviation over all rats imaged.

(200 Hz) that allowed for a measurement of latency that could not be obtained in other animals in the study. Both the time of signal onset and the signal attenuation increase with distance from the electrode.

The activated area, shown in Fig. 2C, was determined by the number of activated pixels exhibiting a drop in reflectance greater than 0.05% for the fIOS signal, a drop in fluorescence greater than 0.05% for RH-795, and an increase in fluorescence greater than 0.05% for RH-1691. The choice of a 0.05% threshold was motivated by the fact that this is approximately half the dynamic range of all three signals. Activated pixels were converted to area in square millimeters. Figure 2D shows the averaged signal time courses.

As is evident from Fig. 2C, the fIOS area of activation did not spread significantly after the first 10 ms, while the VSD area increased and then dropped rapidly. The fIOS signal appeared before the VSD; at 10 ms following the stimulus, the fIOS signal covered an area that was larger than the VSD area, though this difference was not quite statistically significant ($p=0.098$). By 30 ms, both dyes showed areas larger than the fIOS area with $p<0.001$ (ANOVA with Student-Newman-Keuls post-test), and similarly at 40 ms poststimulus, with $p<0.05$.

We observe a rapid response to direct electrical stimulation using both fIOS and VSD imaging. The two imaging methods, however, reveal quite different spatial patterns of activity. The fast IOS response occurs only in the vicinity of one of the two electrodes. Indeed, it is well known that, in bipolar stimulation, activation occurs at lower currents at the cathode, since (positive) current flows from the anode toward the cathode, thus leading initially to depolarization at the cathode rather than the anode [4]. A study from several decades ago may provide insight into the spatial extent of the excited area and its relationship to the exciting current. Stoney *et al.* [5] found that the threshold gradient across neurons ($\Delta V/\Delta r$) is constant and is related to the applied current as

$$\frac{\Delta V}{\Delta r}=\frac{\mathrm{d}V}{\mathrm{d}r}=\frac{is}{4\pi r^2}=K, \tag{1}$$

where i is the applied current, s is the specific resistivity of the medium, and r is the distance from the current source to the cell. Thus, the relationship between a particular neuron's threshold current and the neuron's distance from the tip of the stimulating electrode can be expressed as $i=kr^2$, where $k=4\pi/s$ [5,6]. Stoney *et al.* found that, for mammalian cortical tissue, the constant k has a value of $\sim$1200 μA/mm^2 [5]. In the case of 100 μA current stimulation, as in our experiments, most neurons are excited in a sphere with a radius of at least 288 μm (from the tip of the electrode). This is consistent with the order of magnitude of the activated area we observe. In Fig. 1, note that the light gray spot surrounding the upper square (yellow spot online) near the cathode is approximately twice the size of the upper square (red square online) region of interest (which has dimensions of 100 μm $\times$ 100 μm).

The difference in area encompassed by the VSD and fIOS may well have its explanation in the underlying nature of the two signals. The fIOS is hypothesized to derive from light-scattering changes [2], which also correlate with increases in extracellular potassium [7–9], which accompany neural firing. The fIOS signal, then, may derive from fast, local changes in potassium concentration in the vicinity of the neurons activated by direct electrical stimulation. The gradual drop-off of the signal may relate to slower changes, such as astrocyte swelling in response to potassium uptake [11].

Finally, we suggest several hypotheses as to the "hole" at the center of the VSD imaging signal. First, the strong electrical stimulation at the electrode is likely to result in hyperpolarization, briefly and locally suppressing neural activity immediately following the initial, intense stimulus-generated firing. This might result in masking of the VSD signal in the vicinity of the electrode. Additionally, microstimulation of the neocortex primarily activates pyramidal cell fibers, rather than affecting postsynaptic potentials [6], which are the primary contributors to the VSD signal [11]. Thus, the type of activation occurring in the immediate vicinity of the electrode may be undetectable by VSD imaging, while the fIOS is an ideal imaging technique for imaging the local results of direct electrical activation. In contrast, further from the stimulating electrode, the VSD signal comes into its own, revealing synaptic potentials as the signal spreads away from the electrode, while the fIOS, dependent on massive changes in extracellular K^+, falls silent.

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