Combined Voltage and Calcium Epifluorescence Imaging In Vitro and In Vivo Reveals Subthreshold and Suprathreshold Dynamics of Mouse Barrel Cortex

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INTRODUCTION

Many computations in the neocortex are thought to occur on the millisecond timescale within maps composed of cortical columns. Most current techniques to investigate cortical processing offer limited spatial resolution because of the low density of recording electrodes or theoretical difficulties of defining the signal sources. Optical methods, however, offer both sufficient temporal and spatial resolution for real-time analysis of cortical processing.

Two of the most dynamic parameters in the active brain are membrane potential ($V_m$) and intracellular calcium concentration ([Ca$^{2+}]_i$). Both of these can be imaged optically by the introduction of fluorescent probes into the brain. Voltage-sensitive dyes (VSDs) insert into the plasma membrane and change their fluorescence intensity dependent on the potential across the lipid bilayer. Previous studies recorded VSD signals in invertebrate preparations (Antic and Zecevic 1995; Salzberg et al. 1973), from cultured cells (Bullen and Saggau 1998), brain slices (Antic et al. 1999; Contreras and Llinás 2001; Laaris and Keller 2002; Petersen and Sakmann 2001), and in vivo (Borgdorff et al. 2007; Civillico and Contreras 2006; Derdikman et al. 2003; Ferezou et al. 2006; Grinvald and Hildesheim 2004). Fluorescent calcium-sensitive dyes (CaSDs) such as Fluo-3 and Oregon Green BAPTA-1 (OGB-1) have been developed that respond rapidly and selectively to changes in the cytosolic free calcium ion concentration (Tsien 1980). CaSDs can be applied extracellularly in a membrane-permeable ester form, which is subsequently cleaved intracellularly by esterases releasing the functional fluorescent CaSD (Tsien 1981). Recently, network activity was imaged with CaSD both in brain slices and in the intact brain (Borgdorff et al. 2007; Kerr et al. 2005; Nimmerjahn et al. 2004; Ohki et al. 2005; Peterlin et al. 2000; Stosiek et al. 2003; Wachowiak and Cohen 2001; Yaksi and Friedrich 2006).

In this study, we combined epifluorescence VSD and CaSD imaging together with whole cell (WC) $V_m$ recordings to allow a quantitative comparison of what is measured using these techniques, focusing on their application to the study of the mouse barrel cortex (Petersen 2003; Woolsey and Van der Loos 1970). We recorded VSD and CaSD signals in three different experimental conditions: in single cells, in brain slices, and in vivo. In single cells, we studied the dynamic range of VSDs and CaSDs during controlled membrane potential changes. In the barrel cortex in vitro, we characterized the link of the VSD and CaSD signals to sub- and suprathreshold electrophysiological network activity and thereafter studied the spatial spread of synaptic activity and action potentials. We next transferred these methods to make in vivo optical measurements of activity in layer 2/3 barrel cortex of anesthetized mice, analyzing both the evoked and the spontaneous spatiotemporal dynamics of the VSD and CaSD signals. The data are
consistent with VSD signals reporting predominantly subthreshold activity and CaSD reporting suprathreshold activity.

**METHODS**

All experiments were carried out in accordance with the Swiss Federal Veterinary Office.

**Voltage-sensitive dye biophysics**

Defolliculated *Xenopus* oocytes were incubated for about 30 min in 1.6 mM RHI691 (Optical Imaging, Rehovot, Israel) dissolved in a solution containing (in mM): 135 NaCl, 5 KCl, 5 HEPES, 1.8 CaCl2, and 1 MgCl2. The oocyte was transferred to the recording bath filled with a standard ND96 *Xenopus* oocyte medium containing (in mM): 96 NaCl, 2 KCl, 5 HEPES, 1.8 CaCl2, and 2 MgCl2 (pH 7.4 with NaOH). Two microelectrodes filled with 2 M KC1 were used to voltage-clamp the oocyte using an Axoclamp 2B amplifier (Axon Instruments, Union City, CA). A computer running Igor Pro (WaveMetrics, Lake Oswego, OR), interfacing with an ITC-18 board (Instrutech, Port Washington, NY), not only controlled the command potential but also sampled the achieved membrane potential at 20 kHz. The oocyte membrane was imaged using an Olympus BX51WI microscope equipped with an Olympus 20×0.95 numerical aperture (NA) objective. Excitation light from a 100 W halogen lamp with a shutter (Uniblitz, Vincent Associates, Rochester, NY) was band-pass filtered (630/30 nm) and reflected toward the sample by a 650 nm dichroic mirror. Emitted fluorescence was long-pass filtered (665 nm) and imaged using a MiCam Ultima camera (SciMedia, Irvine, CA). Fluorescence measurements were synchronized to electrophysiology through TTL pulses. In one set of experiments the membrane potential was stepped from a holding potential of −60 mV to test membrane potentials (~100 to +40 mV in 20 mV steps) for 200 ms periods and fluorescence changes were recorded at 10 ms frame rates (Fig. 1A). For each oocyte fluorescence signals from 10 to 20 trials were averaged. Fluorescence signals were integrated across the field of view (FOV) containing 100 × 100 pixels covering roughly 300 × 300 μm. This spatial averaging and averaging across different trials was carried out using the MiCam Ultima analysis software. The fluorescence time-course traces were subsequently exported to Igor Pro (WaveMetrics) for further analysis, where the data from six oocytes were combined and a polynomial fitting was subsequently exported to Igor Pro (WaveMetrics) for further analysis, where the data from six oocytes were combined and a polynomial fit was applied to the fluorescence time-course traces. The fluorescence time-course traces were subsequently exported to Igor Pro (WaveMetrics) for further analysis, where the data from six oocytes were combined and a polynomial fit was applied to the fluorescence time-course traces.

**Brain slice preparation**

Parasagittal or thalamocortical slices (300 or 400 μm thick, respectively) (Agmon and Connors 1991) of the somatosensory cortex were obtained from the somata of neurons in acute slices of the barrel cortex with Multiclamp 700A (Axon Instruments) or BVC-700A (Dagan, Minneapolis, MN) amplifiers. Patch pipettes were made from borosilicate glass with resistances of 3–7 MΩ filled with the following solution (in mM): 135 K-glutamate, 5 KCl, 5 HEPES, 4 Mg-ATP, 0.3 Na2-GTP, and 10 Na3-phosphocreatine (pH 7.3 with KOH). The pipette contained in addition either 200 mM of the CaSD Oregon Green 488 BAPTA-1 potassium salt (Takechi et al. 1998) (OGB-1, Molecular Probes, Eugene, OR), 0.9–5.5 mM of the VSD JPW1114 (di-2-ANEPEQ, Molecular Probes) or 6.8 mM of the VSD RH1691. Stock solutions of these dyes were prepared in pipette solution. JPW1114 and RH1691 were added only in the 4 μl backfill of the pipette, whereas its tip was filled with 1 μl of dye-free solution to prevent staining of the slice with expelled VSD. However, no functional VSD signals could be recorded using RH1691 applied intracellularly. Current injections into the cell under study with exponentially shaped rise and decay ("alpha currents") induced both inhibitory and excitatory postsynaptic potentials (IPSP and EPSP, respectively)—like membrane voltage deflections and action potentials (Fig. 1, D and F). Alternatively, synaptic potentials around action potential threshold were evoked with extracellular stimulation using an ACSF-filled pipette. Corresponding changes in fluorescence were imaged with a Zeiss Axioskop 2FS+ microscope [×40 0.8 NA objective; 75-W xenon short-arc lamp; Deltaram V monochromator (PTI, Lawrenceville, NJ)]. A low-resolution (80 × 80 pixel), fast (1–2 kHz) CCD camera (Redshirt NeuroCCD, RedShirtImaging, Fairfield, CT) running under computer control (Neuroplex software) was used to image changes in fluorescence in the FOV covering the soma and proximal dendrites (Fig. 1, D and F). All experiments were done at 35°C. The following filter sets were used: for OGB-1, excitation filter BP470/20 nm, dichroic mirror 510 nm, emission filter BP540/25 nm; for JPW1114, excitation filter BP520/20 nm, dichroic mirror 580 nm, emission filter LP590 nm; for RH1691, excitation filter BP630/30 nm, dichroic mirror 650 nm, emission filter LP665 nm.

**In vitro network imaging**

Stock solutions (10 mM) of the acetoxymethyl (AM) ester forms of OGB-1 (OGB-1 AM; Molecular Probes) or RH1691 (Biotium, Hayward, CA) were made in 80% fresh DMSO/20% pluronic acid and diluted 1:19 in a Ringer solution containing (in mM): 135 NaCl, 5 KCl, 5 HEPES, 1.8 CaCl2, and 1 MgCl2. The final solution (500 μM) was vortexed, sonicated, aliquoted, and stored in the freezer. The AM ester solution (10 μl) was sucked into an oil-filled application pipette (tip diameter 15–25 μm). To obtain even staining of the approximately 900 × 900-μm cortical region of interest, the AM ester was vortexed, sonicated, aliquoted, and stored in the freezer. For OGB-1 AM, excitation filter BP470/20 nm, dichroic mirror 510 nm, emission filter BP540/25 nm; for JPW1114, excitation filter BP520/20 nm, dichroic mirror 580 nm, emission filter LP590 nm; for RH1691, excitation filter BP630/30 nm, dichroic mirror 650 nm, emission filter LP665 nm.

**In vitro cellular imaging**

Current-clamp recordings in the whole cell mode of the patch-clamp technique were obtained from the somata of neurons in acute slices of the barrel cortex with Multiclamp 700A (Axon Instruments) or BVC-700A (Dagan, Minneapolis, MN) amplifiers. Patch pipettes were made from borosilicate glass with resistances of 3–7 MΩ filled with the following solution (in mM): 135 K-glutamate, 5 KCl, 5 HEPES, 4 Mg-ATP, 0.3 Na2-GTP, and 10 Na3-phosphocreatine (pH 7.3 with KOH). The pipette contained in addition either 200 mM of the CaSD Oregon Green 488 BAPTA-1 potassium salt (Takechi et al. 1998) (OGB-1, Molecular Probes, Eugene, OR), 0.9–5.5 mM of the VSD JPW1114 (di-2-ANEPEQ, Molecular Probes) or 6.8 mM of the VSD RH1691. Stock solutions of these dyes were prepared in pipette solution. JPW1114 and RH1691 were added only in the 4 μl backfill of the pipette, whereas its tip was filled with 1 μl of dye-free solution to prevent staining of the slice with expelled VSD. However, no functional VSD signals could be recorded using RH1691 applied intracellularly. Current injections into the cell under study with exponentially shaped rise and decay ("alpha currents") induced both inhibitory and excitatory postsynaptic potentials (IPSP and EPSP, respectively)—like membrane voltage deflections and action potentials (Fig. 1, D and F). Alternatively, synaptic potentials around action potential threshold were evoked with extracellular stimulation using an ACSF-filled pipette. Corresponding changes in fluorescence were imaged with a Zeiss Axioskop 2FS+ microscope [×40 0.8 NA objective; 75-W xenon short-arc lamp; Deltaram V monochromator (PTI, Lawrenceville, NJ)]. A low-resolution (80 × 80 pixel), fast (1–2 kHz) CCD camera (Redshirt NeuroCCD, RedShirtImaging, Fairfield, CT) running under computer control (Neuroplex software) was used to image changes in fluorescence in the FOV covering the soma and proximal dendrites (Fig. 1, D and F). All experiments were done at 35°C. The following filter sets were used: for OGB-1, excitation filter BP470/20 nm, dichroic mirror 510 nm, emission filter BP540/25 nm; for JPW1114, excitation filter BP520/20 nm, dichroic mirror 580 nm, emission filter LP590 nm; for RH1691, excitation filter BP630/30 nm, dichroic mirror 650 nm, emission filter LP665 nm.
Alternating sweeps were imaged with or without stimuli delivered to the C2 whisker representation. The cortical surface was visualized through the intact skull covered with a glass coverslip. The surface blood vessels were visualized using light at 530 nm to enhance contrast. The illumination was switched to 630 nm for functional imaging. The reflected light was imaged using a Quicam CCD camera (Q-imaging; 10-Hz frame rate, 800 × 800 pixels covering approximately 2.5 × 2.5-mm area). Image acquisition by a Firewire and stimulus control of the piezo by an ITC18 board were governed by custom routines running in Igor Pro. To avoid interference with VSD signals in the intrinsic imaging experiments described in Supplementary Fig. 1, we illuminated at 700 nm and to obtain the same FOV as the VSD imaging, we also imaged the intrinsic signals using a MiCam Ultima camera (20-Hz frame rate, 100 × 100 pixels covering a 2 × 2-mm area). Alternating sweeps were imaged with or without stimuli delivered to the C2 whisker. Stimuli were applied at 10 Hz for 4 s and repeated 7 to 15 times with a 120 s interval. The intrinsic signal was quantified as the difference in the reflected light on stimulus compared with a 4 s time interval immediately before. The local signal was mapped onto the blood vessel pattern to guide surgery for the monkey. The cortex was covered with 1% agarose and a coverslip placed on top to stabilize the cortex. The excitation light was focused onto the cortical surface with a 25 mm Navitar video lens (or 50 mm Nikon lens). Fluorescence was collected by the same optical pathway but without reflection of the dichroic mirror, long-pass or band-pass filtered, and focused onto the camera by a glass pipette connected to a hydraulic micromanipulator, avoiding blood vessels (about 50 nl of a 500 μM or 1 mM solution per injection site). Fluorescence from the esterase-cleaved OGB-1 AM increased gradually over time and stabilized 1 h after injection as previously reported (Stosiek et al. 2003). Fluorescence at an injection site covered a circular area with half-maximal values at 330 ± 70 μm diameter. The cortex was covered with 1% agarose and a coverslip placed on top to stabilize the cortex. The excitation light was focused onto the cortical surface with a 25 mm Navitar video lens (or 50 mm Nikon lens). Fluorescence was collected by the same optical pathway but without reflection of the dichroic mirror, long-pass or band-pass filtered, and focused onto the camera by a glass pipette connected to a hydraulic micromanipulator, avoiding blood vessels (about 50 nl of a 500 μM or 1 mM solution per injection site). Fluorescence from the esterase-cleaved OGB-1 AM increased gradually over time and stabilized 1 h after injection as previously reported (Stosiek et al. 2003). Fluorescence at an injection site covered a circular area with half-maximal values at 330 ± 70 μm diameter. The cortex was covered with 1% agarose and a coverslip placed on top to stabilize the cortex. The excitation light was focused onto the cortical surface with a 25 mm Navitar video lens (or 50 mm Nikon lens). Fluorescence was collected by the same optical pathway but without reflection of the dichroic mirror, long-pass or band-pass filtered, and focused onto the camera by a glass pipette connected to a hydraulic micromanipulator, avoiding blood vessels (about 50 nl of a 500 μM or 1 mM solution per injection site). Fluorescence from the esterase-cleaved OGB-1 AM increased gradually over time and stabilized 1 h after injection as previously reported (Stosiek et al. 2003). Fluorescence at an injection site covered a circular area with half-maximal values at 330 ± 70 μm diameter.

### Statistical tests

Data are expressed as means ± SE and were tested for statistical significance using Student’s t-test or ANOVA.

### RESULTS

**VSD fluorescence correlates linearly with V_m**

Our first goal was to characterize the relationship between membrane potential changes and fluorescence changes under controlled conditions. We applied the VSD RH1691 to voltage-clamped *Xenopus* oocytes and found linear millisecond changes in fluorescence with respect to V_m (n = 6 oocytes; Fig. 1, A–C). However, we were unsuccessful in our attempts to obtain functional fluorescence signals from single neurons labeled with RH1691. As a positive control for our experimental procedures, we introduced the VSD JPW1114 into individual layer 2/3 neurons in brain slices of the mouse barrel cortex by the patch pipette during WC recordings. VSD fluorescence (Fig. 1, D and E) linearly followed both sub- and suprathreshold changes in neuronal V_m induced by alpha currents (n = 9 cells). In agreement with previous studies (Antic and Zecevic 1995; Antic et al. 1999), these data indicate that VSD fluorescence (from both RH1691 and JPW1114) correlates linearly with V_m at sub-millisecond precision.

**CaSD signals reflect action potential firing**

Having found a linear relationship of V_m and VSD fluorescence in individual cells, we next investigated the behavior of...
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Fig. 1. Voltage-sensitive dye (VSD) fluorescence correlates linearly with membrane potential ($V_m$), whereas calcium-sensitive dye (CaSD) signals are dominated by action potentials (APs) with little contribution of subthreshold events. A: VSD RH1691 was applied at 1.6 mM extracellularly to Xenopus oocytes and fluorescence was imaged during 2-electrode voltage clamp. Voltage steps (black trace, $V_m$) evoked fluorescence changes (red trace, $\Delta F/F_0$). B: fluorescence changes recorded at high temporal resolution followed $V_m$ with submillisecond precision ($\Delta F/F_0$ red data points; solid red line shows a sigmoidal fit to the fluorescence data yielding a time constant 0.15 ms; $V_m$, black line). C: RH1691 fluorescence changes were linearly correlated with $V_m$ with a slope of 0.7% $\Delta F/F_0$ per 100 mV. D: whole cell recordings of neurons were performed in acute brain slices with 1.8 mM of the VSD JPW1114 included in the backfill of the patch pipette. Dye diffused into the cell during recording (inset shows fluorescence image). Hyperpolarizing and depolarizing alpha current injections mimicked both inhibitory and excitatory postsynaptic potentials (IPSPs and EPSPs, respectively) and evoked spikes recorded in the whole cell $V_m$ trace (black trace). Corresponding changes in the JPW1114 fluorescence were measured over the proximal dendritic arbor of the cell. JPW1114 fluorescence changes ($\Delta F/F_0$ green trace) closely follows $V_m$. E: plot of JPW1114 fluorescence changes vs. $\Delta V_m$. Subthreshold $\Delta F/F_0$ values were obtained at the peak of the IPSP or EPSP from low-pass (100-Hz) filtered traces, whereas suprathreshold values were obtained from unfiltered traces. A linear fit of the subthreshold values (3.1% $\Delta F/F_0$ per 100 mV) was extrapolated and crossed also the data points for APs, thereby reflecting a linear relationship over the whole activation range. F: 200 $\mu$M of the CaSD OGB-1 was included in the pipette solution and $V_m$ (black trace) was changed by injecting hyperpolarizing or depolarizing alpha currents. Large calcium signals (blue trace) were recorded only after APs and not in response to subthreshold $V_m$ changes. G: plot of changes in OGB-1 fluorescence as a function of $\Delta V_m$. $\Delta F/F_0$ values of somatic CaSD signals (blue trace) were measured from low-pass filtered traces at the peak of the calcium response, which was delayed by 15 ms relative to the $V_m$ peak. A linear fit of the subthreshold values was extrapolated toward higher $\Delta V_m$ values and did not cross the data points for APs, thereby reflecting a steep-like relationship between $\Delta F/F_0$ and $\Delta V_m$. This plot points contain attributes which were determined by alpha current injection (black, data from 11 cells) and extracellular synaptic stimulation (magenta, data from 8 cells).

CaSD fluorescence with respect to membrane potential changes. CaSD OGB-1 was loaded intracellularly into single layer 2/3 neurons in brain slices of the mouse barrel cortex during WC recordings. $V_m$ changes were evoked either with alpha current injection (Fig. 1F and black data points in Fig. 1G) or through synaptic potentials evoked by an extracellular electrical stimulus (magenta data points in Fig. 1G). Subthreshold $V_m$ changes evoked only small fluorescence signals, but an action potential (AP) was followed immediately by a prominent CaSD signal with a prolonged time course (half-width of 231 ± 19 ms; $n = 11$ cells) largely determined by calcium buffers (Helmchen et al. 1996). Threshold synaptic stimulation evoked some trials with subthreshold $V_m$ responses and small CaSD responses ($\Delta F/F_0 = 0.13 \pm 0.10\%$; $n = 8$ cells) and other trials in which an AP was triggered together with a large CaSD response ($\Delta F/F_0 = 7.3 \pm 0.2\%$; $n = 8$ cells). In good agreement with previous studies (Kerr et al. 2005; Peterlin et al. 2000; Stosiek et al. 2003; Svoboda et al. 1997; Yaksi and Friedrich 2006), our data therefore indicate that CaSD signals predominantly reflect APs, with only a minor contribution from subthreshold potentials (2 ± 1%; $n = 8$ cells; calculated as the ratio of the subthreshold calcium signal $\Delta F/F_0 = 0.13$ relative to the supraphathreshold calcium signal, $\Delta F/F_0 = 7.3$). Cortical spatiotemporal dynamics of VSD and CaSD signals in vitro

After delineating the behavior of VSD and CaSD at a cellular level, we next investigated how their fluorescence signals compared when applied to the study of neuronal networks in vitro. Brain slices of the mouse somatosensory barrel cortex were labeled with VSD RH1691 by topically applying the dye to the slice surface. CaSD OGB-1 was injected into multiple locations across the cortical area of interest in its AM ester form and nearly 1 h later cells were strongly labeled (Stosiek et al. 2003). In some experiments, the imaging was combined with WC recordings of $V_m$ from neurons in layer 2/3. The imaged area covered three to four barrel columns and spanned from cortical layer 2 to layer 5. Neuronal network activity was evoked with a stimulation electrode in a layer 4 barrel and the time course quantified in layer 2/3 (Fig. 2, A–C). Compared with the VSD signal, the CaSD signal was roughly 20 times larger (peak amplitude $\Delta F/F_0$ VSD 0.34 ± 0.07%, $n = 9$; CaSD 8.3 ± 2.2%, $n = 13$; in four experiments only CaSD and not VSD signals were imaged) and lasted about five times longer than the VSD signal (half-width duration VSD 35.5 ± 3.1 ms, $n = 9$; CaSD 181 ± 14 ms, $n = 13$).

Pharmacological manipulations were carried out to explore the underlying nature of these signals. Bath application of the ionotropic glutamate receptor antagonists N-methyl-D-aspartate (NMDA), D-2-amino-5-phosphonovaleric acid (APV, 50 $\mu$M) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 $\mu$M) blocked WC responses and VSD signals (1.3 ± 0.8% of control; $n = 9$ slices) (Fig. 2, C and D). This suggests that the VSD response almost exclusively reflects postsynaptic potentials (PSPs). In contrast to the profound block of the VSD response, adding APV/CNQX reduced the peak of the CaSD response to only 56.3 ± 6.9% of the control value ($n = 6$ slices; Fig. 2, C and D). This reveals that the CaSD signal evoked by local electrical stimulation is not driven primarily by synaptic potentials. Additional application of 1 $\mu$M tetrodotoxin (TTX) abolished all responses (WC recording, VSD and CaSD) (Fig. 2, C and D). Evoked $V_m$ and CaSD signals were not changed by blockade of metabotropic glutamate receptors [500 $\mu$M (S)-a-methyl-4-carboxyphenylglycine (MCPG); peak CaSD response was 92.6 ± 3.2% of control; $n = 7$] or the endoplasmic reticulum calcium ATPase (5 $\mu$M thapsigargin; peak CaSD response was 101.7 ± 6.0% of control; $n = 5$). These results indicate that the entire CaSD signal is thus mediated by action potential firing. The fraction of the CaSD signal that is sensitive to ionotropic glutamate receptor antagonists likely reflects APs in postsynaptic cells.
driven by synaptic potentials. The majority of the CaSD signal (which is not blocked by D-APV/CNQX) is likely evoked by the direct stimulation of APs in neurons with axons near the stimulation electrode.

To separate the stimulation site from the measuring location and, in addition, to excite the cortical network in a more physiological manner, we prepared thalamocortical slices (Agmon and Connors 1991), with CaSD injected into the barrel cortex (Fig. 2E). Stimulation of the VPM nucleus of the thalamus evoked calcium signals in the connected layer 4 barrels (Beierlein et al. 2002; MacLean et al. 2005). These thalamically evoked responses were almost completely abolished by blockade of postsynaptic ionotropic glutamate receptors. We found that bath application of 50 μM D-APV and 10 μM CNQX reduced the peak of the thalamic evoked cortical CaSD response to 7.6 ± 1.6% of the control response (n = 10 slices; Fig. 2E). These calcium transients are therefore not mediated by signals in the thalamic axons, but instead are likely to relate primarily to cortical action potentials evoked after the release of glutamate from the thalamocortical synapses.

Consistent with our cellular measurements (Fig. 1), these data from neuronal networks suggest that VSD signals measure predominantly PSPs and that CaSD signals measure local AP activity. We therefore applied these two optical techniques to image the spatiotemporal dynamics of subthreshold and suprathreshold activity evoked by electrical stimulation of a layer 4 barrel in the mouse somatosensory cortex (Fig. 3; Supplemental Movie 1). Excitation began in the stimulated layer 4 barrel, then spread in a columnar fashion into layer 2/3, and finally into neighboring columns. In layer 2/3 of the neighboring barrel column, the VSD response was reduced to 71.7 ± 9.3% (n = 9 slices) and the CaSD response was reduced to 25.5 ± 4.0% (n = 13 slices) of the peak response in the stimulated layer 4 barrel (P = 0.0001; Fig. 3C). These data likely reflect a signaling sequence that begins with the stimulation of APs in layer 4 evoking PSPs in the columnar layer 2/3 through the strong axonal projection from layer 4 to layer 2/3 (Larais and Keller 2002; Petersen and Sakmann 2001; Wirth and Löscher 2004). If in turn the layer 2/3 neurons fire APs, then these will evoke PSPs across a large area of cortex through the extensive horizontal axonal arborizations of the layer 2/3 pyramidal neurons (Petersen et al. 2003a). The VSD signal therefore spreads further than the CaSD signal, which remains more tightly localized to the stimulus location. These results are consistent with the hypothesis that VSD images PSPs, which originate from neurons firing APs localized close to the stimulating electrode and are imaged with CaSD.

In vivo VSD and CaSD imaging of neocortical sensory processing

To investigate the spatiotemporal dynamics of sensory processing evoked by whisker deflection, we recorded VSD RH1691 and CaSD OGB-1 signals in vivo from layer 2/3 of the barrel cortex of anesthetized mice. The staining and imaging

![Image](http://jn.physiology.org/)

**Fig. 2.** Kinetic and pharmacological profile of electrically evoked VSD and CaSD signals in the mouse barrel cortex and thalamocortical neuronal network in vitro. A: schematic drawing of the recording arrangement. An extracellular electrical stimulus was delivered to a layer 4 barrel in a parasagittal slice labeled with VSD RH1691 and CaSD OGB-1. A whole cell (WC) recording from a layer 2 neuron was performed simultaneously with imaging. B: example membrane potential recordings in C relate to the biocytin-filled layer 2 neuron and the 100 × 100-μm purple square is the area from which the fluorescence changes in C were quantified. C: stimulation in the layer 4 barrel evoked changes in fluorescence relating to network depolarization and changes in V_m of the layer 2 cell monitored by WC recording. Stimulus was repeated 10–25 times under different pharmacological conditions: control, α2-aminophosphonovaleric acid (D-APV, 50 μM)/β-cyano-7-nitroquinolinoxaline-2,3-dione (CNQX, 10 μM), and tetrodotoxin (TTX, 1 μM). D: normalized amplitudes of the evoked VSD, CaSD, and WC signals across all experiments are shown for the different conditions. VSD and WC responses were completely blocked by D-APV/CNQX, whereas the majority of the CaSD response remained intact. Under TTX, all responses were completely blocked. E: CaSD was injected into layer 4 of the barrel cortex of thalamocortical slices (brightfield image, top left). Thalamic stimulation evoked cortical CaSD signals, which were quantified in layer 4 (bottom left image with color-coded response amplitude; field of view (FOV) indicated by black outline in the brightfield image). Cortical CaSD signals could be blocked by bath application of D-APV/CNQX (bottom right image and top right traces quantified from area outlined by the magenta square).
procedures did not alter cortical sensory processing as assessed by intrinsic optical imaging (Supplementary Fig. 1; Slovin et al. 2002). The in vivo VSD and CaSD fluorescence measurements were combined with WC recordings targeted to layer 2/3 neurons in the C2 barrel column (n = 7 cells, each in a different mouse). The optical signals were quantified over a barrel-sized area, immediately surrounding the recorded neuron. The C2 whisker was deflected in a highly controlled manner and each whisker deflection measured with an optical displacement sensor. Layer 2/3 neurons typically responded with large subthreshold $V_m$ depolarizations accompanied by APs on some trials (Fig. 4, A–E; peak whisker deflection averaged across the seven experiments was 33 ± 1 µm). The time course of the VSD signal closely followed $V_m$ changes both in individual experiments (Fig. 4, A–C) and in the traces computed by averaging across the seven experiments (Fig. 4, D and E). Our in vivo VSD measurements thus reflect the mean $V_m$ changes of layer 2/3 neurons. These $V_m$ changes in response to whisker stimulation were almost entirely dominated by subthreshold activity. Comparing averages of raw $V_m$ with averages of median-filtered $V_m$ (the median filter used was adjusted in a way that APs were specifically removed) showed that APs made very little impact on the averaged evoked $V_m$ response (Fig. 4D).

The CaSD signal evoked by whisker deflection also showed a rapid onset, but it was somewhat delayed relative to the VSD signal (difference 2.9 ± 1.2 ms; n = 11 animals, P = 0.019). The CaSD signal lasted longer than the VSD signal (222 ± 26 vs. 97 ± 11 ms half-width). The delayed calcium response can relate to the fact that the neurons first need to depolarize through summation of PSPs before reaching AP threshold. In our WC recordings the delay between PSP onset and the first occurring AP was 7.7 ± 2.6 ms (n = 7 cells, range 0.9–21.7 ms). The correlation of single-cell $V_m$ and the VSD signal suggests that, on the scale of a single barrel, many neurons behave in a similar subthreshold manner.

Similar to our observations in vitro (Fig. 2, C–E), we found that topical application of 500 µM d-APV and 125 µM CNQX to the cortical surface blocked the sensory response measured with both CaSD and VSD after a 10 min wash-in period (Fig. 4, F and G). Both VSD and CaSD therefore predominantly measure the local cortical processing of sensory information rather than axonal signals from long-range (e.g., thalamocortical or corticocortical) inputs.

**CaSD and VSD spatiotemporal dynamics**

Different stimulation strengths evoked different temporal and spatial patterns of activity and an example experiment is shown in Fig. 5. A–C. At low stimulation strength (peak whisker displacement amplitude 14 µm) localized VSD responses could be observed without any CaSD signal (Fig. 5A). This likely reflects subthreshold potentials in layer 2/3 neurons without evoking APs. In contrast, at an intermediate stimulation strength (peak whisker displacement amplitude 18 µm), the VSD signal spreads and a localized CaSD signal was recorded (Fig. 5B). The spreading VSD signal likely results from AP firing of layer 2/3 neurons in the C2 barrel column evoking PSPs on target cells distributed across the extensive lateral axonal arborizations of the L2/3 pyramidal neurons. A stronger stimulus (peak whisker displacement amplitude 26 µm) evoked spreading VSD and CaSD signals (Fig. 5C). The horizontal spread of VSD and CaSD signals evoked by C2 whisker stimulation was quantified in seven experiments by comparing the peak amplitude of evoked responses in the C2 barrel column relative to a location about 500 µm away. The VSD signal evoked by an intermediate whisker stimulus (19 ± 3 µm) was reduced to 34 ± 3% and the CaSD response was reduced to 14 ± 2% (n = 7, P < 0.01). Increasing stimulation strength increased both the amplitude and spread of the evoked responses. Doubling whisker deflection amplitude (38 ± 4 µm) increased peak responses in the C2 barrel column to 161 ± 8% for VSD and to 259 ± 52% for CaSD. In a region about 500 µm away from the C2 barrel column, the VSD response amplitude was reduced to 59 ± 4% and the CaSD signal was reduced to 28 ± 5% (n = 7, P < 0.001). In vivo CaSD signals evoked by sensory stimuli thus remain significantly more localized compared with the VSD signals, which can spread over large cortical areas. These results are consistent with small suprathreshold receptive fields reflected in localized CaSD signals and larger subthreshold receptive fields (Brecht et al. 2003; Moore and Nelson 1998; Petersen and...
VSD and CaSD recordings revealed strong variability in both optical signals (Fig. 6A, n = 7 mice). The sensory-evoked CaSD and VSD response amplitudes covaried trial by trial, with small CaSD signals accompanying small VSD signals and large CaSD signals accompanying large VSD signals. These results are consistent with both evoked APs (reflected in the CaSD signal) and evoked PSPs (reflected in the VSD signal) competing with the spontaneous activity (Petersen et al. 2003b; Sachdev et al. 2004). The spontaneous activity itself, occurs as propagating waves, which are nearly simultaneous in CaSD and VSD signals (Fig. 6B; Supplemental Movie 2). Topical application of 500 μM d-APV and 125 μM CNQX to the cortical surface after 10 min blocks this spontaneous activity as measured by both VSD and CaSD (Fig. 6, C and D). These spontaneous fluorescence changes similar to the evoked sensory responses are thus also dominated by local cortical activity rather than long-range axonal signals.

Deep isoflurane anesthesia suppresses CaSD signals more than VSD signals

We were interested to see whether further experimental conditions could be found that would affect the CaSD and the VSD responses differently, in addition to the effect of varying stimulus strength described earlier. Striking differences were found between the two optical signals measured under different levels of anesthesia. We imaged both CaSD and VSD responses under different concentrations of isoflurane and found that VSD responses were relatively little affected by depth of anesthesia (Ferezou et al. 2006), whereas CaSD responses were strongly suppressed by anesthesia (Fig. 7A). Deep (1.5–2%) isoflurane anesthesia reduced the whisker-evoked response amplitude to 85.7 ± 13.5% for VSD and to 33.4 ± 6.8% for

FIG. 4. Combined VSD and CaSD imaging in vivo in the mouse somatosensory barrel cortex. A: WC recording from a layer 2/3 neuron located in the C2 barrel column. A C2 whisker stimulus was briefly delivered at the time indicated by the dotted line with a peak stimulus amplitude of 33 μm. Ten superimposed sweeps are shown. A depolarizing sensory response is consistently observed that is dominated by subthreshold depolarization. In 3 sweeps APs were evoked. B: VSD RH1691 and the CaSD OGB-1 responses were measured in the C2 column during the WC recording. Superimposed time courses of the averaged VSD response (red trace, above) and the averaged Vm (black trace). Averaged CaSD OGB-1 response (blue trace, below) has slower kinetics; for comparison the Vm trace (black trace) is superimposed. C: averaged VSD signal plotted as a function of change in membrane potential (ΔV_m) for this cell indicated a close to linear relationship. D: averaged Vm (black trace) changes recorded in 7 cells located in layer 2/3 of the C2 barrel column in 7 different mice. Responses were evoked by C2 whisker stimulus (mean peak stimulus amplitude 33 ± 1 μm) and recorded simultaneously with the imaging. Superimposed green trace is the result of median filtering to remove APs from the Vm traces. APs make very little impact on the averaged Vm trace. Differences between the averaged original data and the averaged median filtered data are shown in the solid green plot below. Peristimulus time histogram (PSTH, below) averaged across all 7 cells shows when APs were evoked in the WC recordings on trials with (green bars) or without (red bars) whisker stimulus. Firing rate is plotted as the probability of an AP being evoked per stimulus per 2.5-ms time-bin. E: averaged across all experiments, the time course of the VSD response (red trace, above) with the Vm (black trace) superimposed. Averaged across all experiments, the time course of the CaSD response (blue trace, below) with the Vm (black trace) superimposed. F: an example experiment showing that the topical application of 500 μM d-APV and 125 μM CNQX to the cortical surface blocked the sensory-evoked and CaSD signals measured with both VSD and CaSD. G: summary data for experiments involving pharmacological blockade of ionotropic glutamate receptors show that peak response amplitudes of both CaSD and VSD sensory-evoked signals were strongly reduced (n = 4).
**Innovative Methodology**

BERGER ET AL.

**DISCUSSION**

We have demonstrated that VSD and CaSD epifluorescence measurements can be combined to image the spatiotemporal dynamics of cortical activity both in vitro and in vivo. The VSD signal was found to correlate with subthreshold membrane potential changes and the CaSD signal reflected predominantly action potential firing. We therefore imaged in vivo VSD and CaSD to define the sub- and suprathreshold spatiotemporal dynamics in layer 2/3 of spontaneous activity and sensory processing evoked with different stimulus strengths and under different conditions of isoflurane anesthesia.

**Imaging membrane potential with VSD and action potentials with CaSD**

In vivo, the time course of the VSD RH1691 fluorescence followed $V_m$ changes recorded in individual layer 2/3 neurons of mouse somatosensory barrel cortex (Fig. 4, A–C), similar to CaSD relative to the responses recorded at low (0.5–1%) concentrations of isoflurane (Fig. 7B). Because our results suggest that the VSD signals reflect subthreshold $V_m$ changes and CaSD reflects suprathreshold signaling (Figs. 1–5), these data then suggest that increasing isoflurane concentration strongly reduces AP firing with a much smaller effect on subthreshold PSPs. To test whether these observations hold true at the level of individual neurons, we made whole cell $V_m$ recordings to study evoked responses at different concentrations of isoflurane. Some cells responded with only subthreshold PSPs (Fig. 7C) and others also fired APs (Fig. 7D). Deep isoflurane anesthesia reduced PSP amplitude to 73.8 ± 9.5% ($n = 8$) and reduced evoked AP activity to 2.0 ± 2.2% ($n = 5$) of the evoked response recorded under light isoflurane anesthesia (Fig. 7E). Increased isoflurane anesthesia therefore slightly reduces evoked synaptic depolarization of cortical neurons and strongly reduces AP firing. The nonlinear threshold for AP initiation likely allows a small reduction in VSD signal and PSPs to be translated into a large reduction in CaSD signal and suprathreshold activity.

![Figure 5](http://jn.physiology.org/)

**Figure 5.** Spatial spread of sensory responses evoked by different stimulation strengths imaged in vivo with VSD and CaSD. A: both the temporal and spatial dynamics of the signals depend on stimulus strength. Top: time course of the C2 whisker stimulus measured by an optical displacement sensor about 1 mm from the base of the whisker. Color-coded images show temporally averaged VSD responses evoked by the whisker stimulus. Barrel map derived from cytochrome oxidase staining and aligned using the blood vessel pattern is superimposed in white on the VSD images. Three locations where CaSD was injected in this experiment are indicated in thick circles (dysgranular zone). Time course of VSD signals (red traces) and CaSD signals (blue traces) were calculated within a radius of about 150 µm centered on these locations and the thickness of the traces codes the location. At low stimulus strengths there is no CaSD signal and only a localized VSD response is recorded. B: at intermediate stimulus strengths the VSD response spreads and a localized CaSD response is recorded. C: at high stimulus strengths both VSD and CaSD signals spread. These results demonstrate qualitatively different processing regimes in the barrel cortex.

![Figure 6](http://jn.physiology.org/)

**Figure 6.** Single-trial simultaneous VSD and CaSD imaging of evoked responses and spontaneous activity. A: example of the time course of a simultaneously recorded single trial VSD RH1691 and CaSD OGB-1 whisker-evoked response (far left). Average of 100 evoked responses (middle left). On an expanded temporal scale, the VSD response is observed to precede the CaSD response (middle right). Quantifying the peak evoked response amplitude on each individual trial indicates that the VSD and CaSD responses covary (far right). B: spontaneous activity was also closely correlated between VSD and CaSD signals. Spontaneous activity occurred as propagating waves (below, gaussian spatially filtered VSD images show the time period shaded in gray). C: example traces showing that the application of t-APV and CNQX to the cortical surface blocked spontaneous activity measured with both VSD and CaSD. D: spontaaneous activity before and after application of 500 µM t-APV and 125 µM CNQX was quantified by calculating the SD of the fluorescence traces ($n = 4$).
FIG. 7. Deep isoflurane anesthesia strongly suppresses evoked CaSD signals and APs with a smaller reduction in VSD signals and postsynaptic potentials (PSPs). A: increasing the isoflurane concentration in this example experiment did not change the peak VSD RH1691 signals evoked by whisker stimulation, but strongly reduced CaSD OGB-1 signals (each trace is an average of 10 trials). B: plot of the ratio of the peak response amplitude evoked during deep anesthesia (1.5–2% isoflurane) relative to light anesthesia (0.5–1% isoflurane) for VSD and CaSD signals (gray circles represent individual experiments with a line linking VSD and CaSD measurements; black circles show the average ± SE, n = 7). VSD signal was weakly reduced during deep isoflurane anesthesia, whereas the CaSD response was strongly suppressed. C, top: 10 superimposed individual sweeps of evoked PSPs measured with a WC recording under 0.75% isoflurane (left) and 1.5% isoflurane (right). Increasing the concentration of isoflurane did not affect the amplitude of PSPs evoked by whisker stimulation (averaged sweeps below). D: in a different cell, which responded with suprathreshold activity to whisker deflection during light isoflurane anesthesia, the AP firing was almost abolished at deeper isoflurane anesthesia. Ten superimposed trials are shown (above) at 0.75% isoflurane (left) and 1.5% isoflurane (right). Psth for this cell is plotted below, quantified as the probability of evoking an AP in a given 2 ms time bin. E: plot of the ratio of the evoked PSP amplitude and APs during deep isoflurane anesthesia (1.5–2%) relative to light isoflurane anesthesia (0.5–1%) (gray circles represent individual experiments). APs were not evoked in all neurons and a line links experiments where both PSPs and APs were recorded. Black circles show the average ± SE, n = 8 PSPs; n = 5 APs. PSP amplitude is much less affected than AP firing by increasing isoflurane anesthesia, mirroring the strong reduction in CaSD signals with smaller effects on the VSD signals described earlier.

results described previously in rat barrel cortex (Petersen et al. 2003a,b) and mouse barrel cortex (Ferezou et al. 2006). Complete truncation of action potentials through median filtering makes only a small difference to the mean \( V_m \) changes observed in the averaged traces of the WC recording and the accompanying VSD signals are therefore dominated by subthreshold \( V_m \) changes. This situation applies to networks where neurons are on average far from threshold and require substantial depolarization to evoke an action potential. This is the case for both the in vitro and the in vivo measurements. The correlation of single-cell \( V_m \) and the VSD signal suggests that, on the scale of a single barrel, many neurons behave in a similar subthreshold manner and it is likely that specificity in encoding information arises from the subset of neurons that reach threshold to fire action potentials.

Because the VSD stains the brain tissue nonspecifically, changes in \( V_m \) of glial cells will also contribute to the fluorescence signals. However, this is likely to be a small effect because glial responses to direct neuronal transmitter release have small amplitudes as a result of the relatively high glial membrane conductance (Lin and Bergles 2004) and because glial VSD responses resulting from increased extracellular potassium concentrations have much slower kinetics (Konnerth et al. 1987).

CaSD signals require AP firing and reflect suprathreshold neuronal activity. In addition to the AP-evoked calcium signals, we found only a minor subthreshold contribution (estimated to be roughly 2% of the AP-evoked calcium signal in the cellular measurements; Fig. 1, F and G). The nonspecific loading with AM esters labels both neurons and glia, both of which might contribute to our calcium measurements. However, it was recently demonstrated that glial and neuronal calcium signals do not temporally correlate (Nimmerjahn et al. 2004) and that sensory-evoked glial calcium signals are nearly two orders of magnitude slower in onset and duration compared with neuronal calcium signals (Wang et al. 2006). The calcium signals evoked by sensory or electrical stimulation that we recorded are fast, linked to neuronal activity, and are thus unlikely to substantially involve glial calcium signals. We did not observe slow CaSD signals unrelated to VSD signals, although very slow signals are more difficult to detect and so may have gone unnoticed. We conclude that the CaSD signals we observed are likely to result predominantly from AP firing in neurons (Smetters et al. 1999). In particular, local action potential activity seems to play a dominant role for the calcium signal we measured, whereas long-range axonal signals from the thalamus or other cortical areas appear to make only a minor contribution (Figs. 2E, 4, F and G, and 6, C and D). In contrast, the VSD signal is driven by PSPs and dominated by changes in neuronal subthreshold \( V_m \) which it follows with millisecond precision. Although VSD and CaSD signals are clearly related, the highly nonlinear aspect of AP generation allows the two signals to be differentially manipulated.

**Imaging sensory processing with VSD and CaSD**

Whisker deflections evoke responses in the primary somatosensory barrel cortex, through a signaling pathway from the trigminal nerve to the brain stem, and then to the thalamus, which provides input to the neocortex. Three qualitatively different processing modes of layer 2/3 barrel cortex were observed depending on the strength of the whisker stimulus. The weakest stimuli evoked localized...
VSD signals without any CaSD signal, implying a localized subthreshold excitation without supragranular AP activity. Such a local subthreshold signal could modulate other inputs, for example, by summatating with other sensory inputs from neighboring whiskers, other sensory modalities, or with top-down influences. Intermediate stimuli evoked propagating PSPs with localized suprathreshold signals in layer 2/3. In response to this intermediate stimulation strength, the sensory processing of the single whisker stimulus in terms of action potential activity is therefore restricted to the principal whisker barrel column, although the spreading subthreshold VSD signal would allow multiwhisker integration to occur. The strongest stimuli evoked propagating VSD and CaSD signals. Therefore even a single whisker, if deflected sufficiently strongly, can evoke a propagating wave of sensory responses involving regenerative AP firing. These data are in good agreement with receptive field analysis of individual neurons in layer 2/3 of barrel cortex, which are very broad at the subthreshold level, but show more tightly tuned suprathreshold classical receptive fields (Brecht et al. 2003; Moore and Nelson 1998; Petersen and Diamond 2000; Simons 1978; Wilent and Contreras 2005; Zhu and Connors 1999).

Deep isoflurane anesthesia strongly reduces evoked action potentials and CaSD signals

In a further test to probe the relationship of the VSD and CaSD signals, we found that the level of isoflurane anesthesia led to appreciable differences in the behavior of the two fluorescence signals. The VSD signals and PSPs were only weakly affected by increasing isoflurane concentration, whereas the CaSD signals and APs were strongly suppressed (Hentschke et al. 2005). Even small changes in subthreshold activity may strongly affect suprathreshold activity at low firing rate conditions (Brecht et al. 2003; Crochet and Petersen 2006). Thus the action of isoflurane is likely to reflect a small change in subthreshold activity that nonlinearly translates into a strong effect in action potential discharge. Equally, one can construct a membrane potential trajectory by summing either many or few excitatory and inhibitory synaptic inputs. It might therefore be possible to record a similar PSP resulting from sparse AP firing compared with higher levels of AP activity, simply by appropriately balancing excitation and inhibition. Finally, the large CaSD signal at low anesthesia might suggest that AP firing rates are considerably higher in awake mice than in mice under deep anesthesia. Independent of the underlying mechanisms, these results stress the importance of measuring with both dyes because together they give information, which imaging either dye alone could not provide. The nonlinear function linking $V_m$ to firing rate makes it difficult to relate PSPs to APs at a network level. The combined imaging approach presented here is thus likely to be a useful experimental tool for many investigators probing cortical network properties and seeking to differentiate between sub- and suprathreshold effects of a specific manipulation.

Outlook and limitations

The current technique is attractive in that it is relatively simple, requiring only epifluorescence optics and a fast camera. The data collected by this method demonstrate that CaSD and VSD signals can be recorded in the same preparation with millisecond time resolution and that they image different aspects of cortical function, with the VSD reflecting subthreshold PSPs and the CaSD being dominated by APs. Currently, these methods applied in vivo are well suited only for the study of supragranular layers because VSD RH1691 does not penetrate deeply into the cortex (Supplementary Fig. 1; Ferezou et al. 2006). Also the fluorescence excitation light is unlikely to penetrate substantially below the supragranular layers and indeed the blue light needed to excite OGB-1 will penetrate less deeply into the brain than the red excitation light for VSD RH1691. Equally, the clear correlation between the VSD signal and subthreshold activity that we observe in the neocortex may not hold true for other brain areas. In hippocampal Schaffer collaterals, a high degree of synchronized action potential activity results in the detection of the suprathreshold activity with VSDs (Grinvald et al. 1982). Comparably, highly synchronized spiking activity of thalamocortical axons traversing the striatum can be observed with RH1691 in thalamocortical slices after stimulation in the thalamus (Neubauer and Berger, unpublished observations). In the cortical gray matter, however, the part of the VSD signal related to spiking activity (compare Fig. 1D) is extremely small because only a small fraction of membranes experience an action potential, but all neuronal membranes experience subthreshold depolarization. Thus either dye alone (VSD or CaSD) does not seem to be well suited to reflect the complexity of electrical signals in the cortical network. The CaSD responses measured under our conditions with epifluorescence do not have a dynamic range that allows the detection of subthreshold activity and VSDs on the gray matter network level do not reflect AP activity. However, what initially seems to be a limitation turns into an advantage if both dyes are used together, as done in this study for the first time. A combined use of VSDs and CaSDs enables the simultaneous but separate study of synaptic and action potential activity in the barrel cortex.

Using two-photon microscopy combined with OGB-1 AM labeling, Kerr et al. (2005) previously described the detection and separation of local input and output activity in neocortex by imaging the fluorescence of a single dye. Fluorescence changes in the neuropil were suggested to relate to axonal calcium transients, which would reflect local synaptic input. Somatic fluorescence changes resulted directly from action potential discharge of the imaged neuron, which thus provided a measure of output. With epifluorescence optics, the calcium signals of incoming long-range axons do not seem to have a major impact on the overall calcium signal (Figs. 2E and 4, F and G). Thus the calcium signal in our study appears to reflect only the local action potential activity of the local cortical area imaged.

A disadvantage for the use of CaSDs in vivo (in contrast to VSDs) is the need to inject the AM ester dye. AM esters can be applied with pipettes into the brain but they stain only neurons in a small volume of about 300-μm diameter (Stosiek et al. 2003). In a spatially restricted field of three cortical columns in a parasagittal slice, a complete staining can be achieved (Fig. 3), but even with many injections we were unable to obtain uniform staining of a complete cra-
niotomy measuring several millimeters in diameter. Our analyses of the spatial extent of CaSD signals in vivo (Fig. 5) was therefore limited to three well-defined separate injection spots across the cortical map. This spatially restricted labeling is of course true for both epifluorescence and two-photon imaging. However, two-photon imaging is generally applied at high magnification and correspondingly small fields of view, which can be stained easily with one AM ester injection (Kerr et al. 2005; Stosiek et al. 2003). The recent development of transgenic mice expressing genetically encoded calcium-sensitive fluorescent proteins may provide an approach to wide-field epifluorescence measurements of calcium signaling in vivo (Díez-García et al. 2005; Hasan et al. 2004; Nagai et al. 2004). However, current data suggest that the genetically encoded calcium indicators are not sensitive to single action potentials (Polo-Gruto et al. 2004). In the rodent somatosensory cortex, where low-frequency action potential firing was previously observed (both for spontaneous and evoked activity) (Brecht et al. 2003; Crochet and Peterson 2006), the genetically encoded calcium indicators might then miss the majority of activity.

Our approach of wide-field epifluorescence imaging of CaSDs and VSDs therefore appears to be a promising combination to study different aspects of cortical computation. Such simultaneous imaging of subthreshold and suprathreshold neuronal activity will help advance our understanding of sensory processing.

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