New Angles on Neuronal Dendrites In Vivo

by

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Abstract

Imaging technologies are well suited to study neuronal dendrites, which are key elements for synaptic integration in the CNS. Dendrites are, however, frequently oriented perpendicular to tissue surfaces impeding in vivo imaging approaches. Here we introduce novel laser scanning modes for 2-photon microscopy that enable in vivo imaging of spatiotemporal activity patterns in dendrites. First, we developed a method to image planes arbitrarily oriented in 3D, which proved particularly beneficial for calcium imaging of parallel fibers and Purkinje cell dendrites in rat cerebellar cortex. Second, we applied free line scans – either through multiple dendrites or along a single vertically oriented dendrite – to reveal fast dendritic calcium dynamics in neocortical pyramidal neurons. Finally, we invented a ribbon-type 3D scanning method for imaging user-defined convoluted planes enabling simultaneous measurements of calcium signals along multiple apical dendrites. These novel scanning modes will facilitate optical probing of dendritic function in vivo.

Introduction

Most neurons in the mammalian CNS possess elaborate dendrites forming numerous synaptic contacts with their presynaptic partners. With their diverse morphological and physiological features dendrites are essential for synaptic integration in single neurons and for signal processing in neuronal circuits (Häusser et al. 2000; Yuste and Tank 1996). A key function of dendrites is to spatially confine electrical and biochemical signals. Such compartmentalization occurs on multiple levels ranging from dendritic spines (Yuste et al. 2000) to dendritic arbors that might act as nonlinear thresholding units for local integration of synaptic potentials (Polsky et al., 2004). The dendritic distribution of intracellular calcium ion concentration changes is
particularly relevant because calcium ions are a key regulator of synaptic plasticity (Häusser et al. 2000; Holthoff et al. 2006). Measurements of the spatiotemporal dynamics of dendritic excitation therefore are crucial for further understanding of dendritic integration, synaptic plasticity, and neural network function.

While dendritic function can be directly probed using electrical recordings with micropipettes (Stuart and Spruston, 1995) this approach permits simultaneous recordings from a few locations at most. Alternatively, various imaging techniques have been applied to characterize spatiotemporal activity patterns in dendrites including camera systems and laser scanning microscopes (Häusser et al. 2000; Yuste and Tank 1996). Two-photon microscopy (Denk et al. 1990) has the particular advantage that it enables calcium imaging from dendrites in the mammalian brain in vivo (Svoboda et al. 1997); reviewed in (Helmchen and Waters 2002)). It has been difficult though to simultaneously image large parts of dendritic trees in the intact brain because many dendrites are oriented ‘vertically’, with their main axis perpendicular to the tissue surface and thus to the conventional xy-imaging plane. As a consequence in vivo measurements from dendrites in the neocortex (Helmchen et al. 1999; Svoboda et al. 1997; Svoboda et al. 1999; Waters and Helmchen 2004; Waters et al. 2003), the olfactory bulb (Charpak et al. 2001; Debarbieux et al. 2003), and the cerebellum (Sullivan et al. 2005) have been restricted to selective and sequential sampling from dendritic cross-sections.

Novel approaches for more comprehensive imaging of dendritic activity in vivo are desirable, particularly in view of the recent advances in calcium indicator labelling (Heim et al. 2007; Kerr et al. 2005; Nagayama et al. 2007; Nevian and Helmchen 2007; Stosiek et al. 2003). New scanning technologies and strategies for 2-photon microscopy (Iyer et al. 2006; Kurtz et al. 2006; Nguyen et al. 2001; Salome et al. 2006) so far aimed at fast imaging in a single plane rather than from arbitrarily
oriented structures. Recently, we introduced a simple 3D laser scanning method, which is based on a 3D scan trajectory created by galvanometric scan-mirrors in combination with a piezoelectric focusing device (Göbel et al. 2007). This approach enables optical recordings from large cell populations in 3D space. Here, we present several additional 3D laser scanning modes that are especially advantageous for imaging dendritic excitation in vivo. For the first time we demonstrate imaging of dynamic signaling in dendrites of cerebellar Purkinje cells and neocortical pyramidal neurons with viewing angles as if the observer were sitting inside the intact brain.

Methods

Animal Preparation and Fluorescence Staining

All experiments were carried out according to the guidelines of the Center for Laboratory Animals of the University of Zurich and were approved by the Cantonal Veterinary Office. We anesthetized Wistar rats (16-28 days old) with urethane (1.5 g/kg body weight) and performed a craniotomy above somatosensory cortex or cerebellum as described (Sullivan et al. 2005; Waters et al. 2003). We carefully removed the dura and superfused the exposed brain with normal rat Ringer (NRR) solution (in mM: 135 NaCl, 5.4 KCl, 5 HEPES, 1.8 CaCl2, pH 7.2 with NaOH). To dampen heartbeat- and breathing-induced motion, we filled the cranial window with agarose (type III-A, Sigma; 1% in NRR) and covered it with an immobilized glass coverslip.

We achieved extracellular staining by bolus injection of the red fluorescent dye Alexa-594 (200 µM; Molecular Probes; diluted in NRR) through a micropipette positioned in cortical layer 2/3 or cerebellar molecular layer, respectively. Population staining with a calcium indicator was performed using the multi-cell bolus loading
technique (Stosiek et al. 2003). Briefly, the acetoxyethyl (AM) ester of Oregon Green-BAPTA-1 (OGB-1 AM; 50 µg; Molecular Probes) was dissolved in DMSO plus 20% Pluronic F-127 (BASF; Florham Park, NJ) and diluted in NRR to a final concentration of 1 mM. The dye was pressure ejected into the molecular layer of cerebellar cortex using a micropipette. For electrical stimulation of parallel fibers, current pulses (100 µs; 0.1-5 µA) were delivered through a glass pipette (about 2 µm tip size) placed in molecular layer 200-400 µm distant from the imaging area. Alexa-594 (20 µM) was included in the pipette for visualization.

For loading subsets of neurons in neocortex or cerebellum with calcium indicator we adopted the recently introduced method of local electroporation (Nagayama et al. 2007). A micropipette (about 2 µm tip size) was filled with a high concentration (30 mM) of Oregon Green BAPTA-1 (OGB-1; Molecular Probes; diluted in NRR). After positioning the pipette in cortical layer 3 or in the molecular layer of cerebellar cortex we applied a series of current pulses at 2 Hz (5 µA; 25 ms pulse width; 1200–2400 pulses in total). Typically 5-15 neurons were stained with calcium indicator dye within 15 min. Washing the brain surface with NRR removed most of the remaining extracellular stain.

**Two-Photon Laser Scanning Microscopy**

We used a custom built 2-photon microscope with about 100-fs long laser pulses at 870-nm wavelength provided by a Ti:sapphire laser system (Tsunami and Millenia-X; Spectra-Physics). We adjusted beam size with a telescope and modulated laser intensity with a Pockel’s cell (Conoptics). We used two galvanometric scan mirrors (model 6210; Cambridge Technologies) for xy-scanning and a piezo-electric focusing element (P-725.4CD PIFOC®, Physik Instrumente) with up to 400 µm travel in
closed-loop mode for rapid z-scanning along the optical axis (Göbel et al. 2007). In all experiments, we used a water immersion objective (40x LUMPlanFl/IR, 0.8 NA; Olympus) and acquired fluorescence images with laser-scanning software custom-written in the LabView environment (National Instruments). We incorporated the novel axial scanning modes in our LabView program.

**Arbitrary plane imaging.** For arbitrary plane imaging the xyz-scan signals were subject to two coordinate transformations: first a rotation around the x-axis by an angle \(\theta\) followed by a rotation around the z-axis (the optical axis) by \(\phi\). Mathematically, we applied two rotation matrices

\[
\begin{pmatrix}
    x_i(\theta, \phi) \\
    y_i(\theta, \phi) \\
    z_i(\theta, \phi)
\end{pmatrix} =
\begin{pmatrix}
    \cos \phi & -\sin \phi & 0 \\
    \sin \phi & \cos \phi & 0 \\
    0 & 0 & 1
\end{pmatrix} \cdot
\begin{pmatrix}
    1 & 0 & 0 \\
    0 & \cos \theta & -\sin \theta \\
    0 & \sin \theta & \cos \theta
\end{pmatrix} \cdot
\begin{pmatrix}
    x_i(0) \\
    y_i(0) \\
    z_i(0)
\end{pmatrix} \quad (i = 1...n) \quad (1)
\]

where \(x_i(0), y_i(0), z_i(0)\) denote the vector components of the initial horizontal xy-scan pattern (\(x_i(0)\) represents the fast line scan signal, \(y_i(0)\) the saw-tooth like slow scan signal, and \(z_i(0)\) equals 0 for all \(n\) initial vectors). To ensure equal image dimensions in all spatial directions the z-scan signal was multiplied with a constant factor \(\sigma\). In addition, a constant offset \(\tau\) was added so that the objective was centered at the middle position of the z-range of the piezoelectric focus (in our case 200 \(\mu m\)):

\[
z_{\text{final, } i}(\theta, \phi) = \sigma \cdot z_i(\theta, \phi) + \tau \quad (2)
\]

These transformations enabled \(\theta\) rotations around the midline of the xy-image. Coordinate transformations could be applied on-the-fly in the focusing mode and a hot key permitted rapid back-and-forth switching between the xy- and xz-views.

**2D free line scan.** We used our previously described point insertion algorithm (Göbel et al. 2007) to generate a smooth user-defined line-scan in a horizontal plane. Briefly, preselected points of interest were appropriately sorted and novel points were iteratively added in between so that a closed, smoothly curved line was obtained.
passing through all selected points. From this set of 2D scan vectors a new set was then calculated using our pixel redistribution algorithm (Göbel et al. 2007) so that the final scan points \( \tilde{S}_i = (x_i, y_i) \) \( (i = 1...n) \) were equidistantly spaced along the free line scan where \( n \) is the number of points per line as defined by the user.

**3D free line scan.** To create a 3D scan trajectory along a single apical dendrite we used our recently introduced user-defined 3D line-scan method (Göbel et al. 2007). In this mode, the microscope objective is set into sinusoidal motion along the optical axis (frequency 20 Hz). The cross-section of a single dendrite was marked at different focal depths based on a previously acquired reference image stack. Constrained by the sinusoidal movement of the objective we then calculated a smooth scan line passing through the preselected set of points along the dendrite. Because of the inertia of the piezoelectric focusing element plus objective load the actual \( z \)-position of the objective in general does not accurately match the imposed drive signals. For a sinusoidal motion, a frequency-dependent amplitude reduction and phase shift result, which need to be corrected. To this end we first determined the phase shift and amplitude reduction by comparing the original drive signals with the resulting position feedback signals. Then, the drive signal was negatively phase shifted by the determined angle and multiplied by the amplitude reduction factor (Göbel et al. 2007). Due to a deviation of the \( z \)-motion from a sinusoid at 20 Hz, however, a complete correction was not possible. While this problem impeded accurate vertical scanning along the entire dendrite, we could always assign the measured signals to dendritic segments based on the position-feedback signals from the piezoelectric focusing element and the reference image stack.

**3D ribbon scanning.** For 3D ribbon scanning we selected the cross-sections of multiple dendrites (using the same order) in two horizontal \( xy \)-planes at focal
depths $z_1$ and $z_2$ and calculated two 2D free line scans $\tilde{S}_i^1$ and $\tilde{S}_i^2$ at $z_1$ and $z_2$, respectively ($i = 1...n$ for both sets of points with $n$ denoting the number of pixels for the scan trajectories). We then calculated a full 3D ribbon scan pattern by linearly interpolating $\tilde{S}_i^1$ and $\tilde{S}_i^2$:

$$\tilde{R}_{i,j} = \tilde{S}_i^1 + \frac{i}{j}(\tilde{S}_i^2 - \tilde{S}_i^1)$$

(3)

with $j = 0...(n-1)$ (whilst $i=\text{constant}$) and $i = 0...(n-1)$. We implemented vertical motion of the objective between focal planes $z_1$ and $z_2$ by using a saw-tooth like z-signal. A sinusoidal z-motion would be equally possible. Because of the low z-scanning frequency (maximally 8 Hz) amplitude reduction was negligible and only small phase shift corrections were necessary.

**Laser intensity adjustment.** Assuming an exponential decrease of 2-photon excitation with focal depth due to light scattering (Kleinfeld et al. 1998) we compensated excitation loss by modulating the Pockel’s cell according to

$$I(\Delta z) = I(0) \cdot e^{\Delta z / \lambda}$$

(4)

where $\lambda$ denotes the scattering length for the excitation light, $I(0)$ the intensity used at the initial $xy$-plane and $\Delta z$ the axial deviation from this plane. For example, in the arbitrary plane imaging mode the image is tilted by rotation around the midline of the initial $xy$-image. Excitation intensity is decreased for those parts of the tilted image that lie above the initial plane ($z<0$) and increased for the parts below ($z>0$). The scattering length was empirically adjusted during imaging until a rather homogeneous illumination of the tilted image was achieved. In our experiments, we used $\lambda$–values between 200 and 250 $\mu$m with no apparent difference for neocortex and cerebellum.
Data Analysis
We analyzed calcium signals in defined regions of interest (ROIs) as relative fluorescence changes ($\Delta F/F$) after background subtraction. Decay time-constant were obtained from exponential fits to calcium transients. For PF beams, we defined the responding region as full-width half maximum of the spatial $\Delta F/F$ profiles through the center of the beam. We obtained $\Delta F/F$ images using ImageJ by merging the average fluorescence image of an entire movie with the time series of fluorescence changes into a single HSB-color coded image. $\Delta F/F$ changes were appropriately mapped onto the hue (H) scale and were also partially used for the saturation (S) channel. The average intensity image was used for the brightness (B) channel. All results are given as mean ± S.D.

Results

Arbitrary Plane Imaging in 3D Space
A major challenge for imaging dendrites in vivo is their variable orientation in 3D space. In a first approach we devised a laser scanning mode that permits imaging of arbitrary planes in three dimensions (Fig. 1A). We used a custom 2-photon microscope with galvanometric scan-mirrors for $xy$-scanning and a piezoelectric focusing element for $z$-scanning (Göbel et al. 2007). By transferring part of the slow, saw-tooth-like $y$-scan signal to the $z$-scan (using appropriate scaling to maintain image dimensions) the image plane could be rotated by an angle $\theta$ around the $x$-axis (Fig. 1B). In the extreme case ($\theta=90^\circ$) the entire $y$-scan signal was transferred to the piezoelectric focus tilting the image plane to the vertical $xz$-plane. By implementing a second rotation around the $z$-axis by an angle $\phi$ we could arbitrarily choose the orientation of the image plane in 3D space (Fig. 1B). For imaging deep in biological
tissue, the reduction of 2-photon excitation with focal depth caused by light scattering has to be taken into account. We compensated changes in excitation intensity during z-scanning by automatic adjustment of the average laser power according to focal depth (see Methods).

To demonstrate in vivo imaging of vertically oriented dendrites we applied a simple extracellular stain by bolus injection of the red fluorescent dye Alexa-594 into superficial layers of neocortex and cerebellar cortex of anaesthetized rats (Fig. 1, C and D). In both brain regions cell bodies were clearly visible as negatively stained (‘black’) holes in the conventional xy-view. Smaller black spots between cell bodies indicated possible locations of dendritic cross-sections (Fig. 1, C and D, upper images). Rotation of the image plane allowed inspection of these structures under all possible orientations. More specifically, in neocortical tissue $\theta$ -rotation by values between 75° and 105° tilted the image plane so that it was aligned with apical dendrites that either emerged from layer 2/3 pyramidal cell bodies or apparently originated from deeper layers (Fig. 1C, bottom image; for all side views the specific tilt angle $\theta$ is given as xz($\theta$)). Similarly, by appropriately choosing rotation angles the cross-sectional xy-view of the cerebellar cortex turned to a sagittal view so that the different layers were discernible and individual negatively stained Purkinje cells could be resolved including the main arbors of their dendritic tree (Fig. 1D, bottom image; see also supplemental movie 1). Note that the spatial resolution generally depends on the effectively used NA and that the transformation to a sagittal view results in a lower spatial resolution in the z-direction because of the elongation of a diffraction-limited focus along the optical axis.

Because the travel range of the piezoelectric focus is 400 $\mu$m, we easily achieved field of views of several hundred micrometer side-length for all orientations. The temporal resolution of arbitrary plane scanning is similar to standard xy-scanning
(2-10 frames/s depending on the line duration and the number of lines). This novel scanning mode thus is excellently suited for functional imaging of structures that are anatomically arranged perpendicular to the brain surface.

**Calcium Imaging in the Molecular Layer of Cerebellar Cortex**

We first used arbitrary plane imaging for functional measurements in the cerebellum. The molecular layer of cerebellar cortex has a well-defined anatomical organization with parallel fibers (PFs) running along the mediolateral axis, making numerous contacts with flat dendritic trees of Purkinje cells that lie in parasagittal sections (Fig. 2A). Multi-cell bolus loading (Stosiek et al. 2003) of the calcium indicator Oregon Green BAPTA-1 (OGB-1) resulted in unspecific staining of cell bodies and neuropil within a diameter of several hundred micrometers (Sullivan et al. 2005). In the axial view ($\theta \approx 90^\circ$), the different layers of the cerebellar cortex were clearly distinguishable (Fig. 2B). We could hardly resolve individual cells in the granule cell layer though, presumably due to enhanced light scattering and laser beam distortion in the Purkinje cell layer. Because multiple cellular structures are labeled using bolus loading, we next aimed to isolate calcium signals in the various compartments.

Electrical stimulation in the molecular layer excites bundles of parallel fibers ('PF beams') that extend several hundred micrometers laterally (Pichitpornchai et al. 1994). Using arbitrary plane imaging, we could directly visualize PF beam cross-sections in a sagittal view. We stimulated PFs with a micropipette inserted into the molecular layer 200–400 $\mu$m distant from the imaging site. Single stimuli evoked calcium transients confined to circularly shaped regions in the molecular layer (Fig. 2C; supplemental movie 2). Consistent with progressive recruitment of PFs, the peak amplitude and spatial extension of the calcium transients increased with stimulation intensity while the time course did not change (Fig. 2, D-F; average decay time-
constant $578 \pm 40$ ms, $n = 3$ animals). These results are consistent with a previous in vivo study, in which sagittal views of PF beam cross-sections were reconstructed from sequential line-scan measurements at different focal depths (Sullivan et al. 2005).

Dendrites of single Purkinje cells lie within a two-dimensional plane perpendicular to both the PF axis and the brain surface. In several experiments using bolus-loading of calcium indicator we were able to identify the main dendritic arbors of Purkinje cells. Moreover, in sagittal views we observed spontaneous activity that apparently originated from Purkinje cell dendrites (Fig. 3A; supplemental movie 3). In three animals these putative Purkinje cell signals were characterized by brief calcium transients (decay time constant $354 \pm 119$ ms, $n = 7$ cells), in agreement with previous $xy$-measurements (Sullivan et al. 2005). Calcium transients either occurred rather regularly (mean frequency $0.39 \pm 0.18$ Hz, $n = 6$ cells) or were superimposed on slower oscillations on the time scale of 10-30 seconds (8 out of 14 cells; Fig. 3B).

**In Vivo Imaging of Dendritic Activity in Purkinje Cells**

To further examine dendritic activity in Purkinje cells we applied local electroporation as an alternative method to selectively label only few Purkinje cells (Nagayama et al. 2007). Local electroporation of OGB-1 in the molecular layer resulted in dye loading of 3-22 Purkinje cells and also labeled a spatially restricted bundle of crossing PFs. Arbitrary plane scanning enabled calcium imaging in vertically oriented optical sections that contained dendrites of either a single or a few neighboring Purkinje cells (Fig. 4). As in the bolus loading experiments we observed variable spontaneous activity patterns in Purkinje cell dendrites of 4 animals. Nearly all dendrites exhibited fast calcium transients (mean frequency $0.67 \pm 0.29$ Hz, $n = 19$ cells). In 15 cells measured at high frame rates ($\geq 4$ Hz) the mean decay time-constant was $266 \pm 57$
ms (Fig. 4B). Fast calcium transients were prominent in dendritic regions but absent or very small in somata when simultaneous measurements could be made (Fig. 4, B-D). These features suggest that calcium transients originated from dendritic calcium spikes that presumably were associated with complex spikes and triggered by climbing fiber activation (Keating and Thach 1997; Miyakawa et al. 1992; Sullivan et al. 2005). In about 25% of cells we in addition observed slow oscillations in the calcium signal with periods of enhanced activity (duration 2-30 s) alternating with more quiet periods. In the example in Figure 4C large brief calcium transients preferentially occurred at the beginning and the end of such slow oscillations, which is reminiscent of the recently reported bistability of Purkinje cells in vivo and complex spike-induced transitions between ‘up’ and ‘down’ states (Loewenstein et al. 2005).

In one experiment, two different dendritic arbors, which could clearly be assigned to the same Purkinje cell, were differentially activated (Fig. 4D; supplemental movie 4).

From these examples we conclude that arbitrary plane imaging is well suited to study calcium signaling in various components of the cerebellar cortex, particularly in Purkinje cell dendrites.

**Horizontal and Vertical Free Line Scans Resolve Fast Dendritic Calcium Signals**

We further aimed at functional imaging of dendrites in the neocortex in vivo. Using local electroporation in layer 2/3 of somatosensory cortex we were able to load 5-10 neocortical pyramidal neurons with OGB-1. Although arbitrary plane imaging permitted sideways imaging of segments of the labeled apical dendrites (data not shown), the improvement provided by this mode was limited because vertically oriented planes contained 2 or 3 apical dendrites at most. We therefore devised
additional scanning modes more suitable for measurements from pyramidal cell dendrites.

First we applied user-defined free line scans in the horizontal plane to simultaneously measure fast calcium signals from multiple neighboring dendrites (Fig. 5). In the xy-view individual dendrites appeared as small, bright spots. Dendritic cross-sections were pre-selected and a smooth 2D scan trajectory was calculated passing through these points (Fig. 5, A and B; see Methods). User-defined line-scans provide excellent temporal resolution (up to 1 kHz). In 5 experiments we measured spontaneously occurring fast calcium transients from multiple dendrites (Fig. 5C). This scanning mode thus is suitable for studying the distribution and synchrony of dendritic activation in subnetworks of pyramidal neurons.

Several important questions (such as where action potentials are initiated, how far they spread, and where synaptic inputs arrive) require measurements of the spatial profile of dendritic calcium transients. In a second approach, we therefore devised a method to scan along the vertically oriented dendrites of pyramidal neurons in vivo (Fig. 6). The method employs our recently introduced user-defined 3D linescan technique, which is based on the pre-selection of points of interest in a 3D volume (Göbel et al. 2007). Here, we applied this method to image the spatial profile of calcium signals in individual apical dendrites filled with OGB-1. Several points were selected from a reference image stack to define a vertically oriented free line scan trajectory along the dendrite (Fig. 6, A and B). To achieve fast z-scanning the objective was vibrated sinusoidally at 20 Hz, which requires compensation of amplitude reduction and phase shift (see Methods). Because of remaining deviations from the desired path the dendrites typically were not followed accurately along the entire imaged segment. Nevertheless, fluorescence signals from dendritic sections at multiple focal depths could be analyzed.
In four experiments on distal apical dendrites (typically 100 µm downwards from the main bifurcation and in one case 300 µm downwards almost to the soma) we observed spontaneous calcium transients that closely resembled action potential-evoked calcium transients observed in previous in vivo studies (Waters and Helmchen 2004; Waters et al. 2003). Interestingly, when we analyzed the spatial profile of calcium transient amplitudes (normalized to the most proximal recording site), we found a variable degree of attenuation, even for events in the same recordings (Fig. 6C). While some calcium transients were almost completely attenuated in the most distal part of the dendrite others obviously spread much farther distally (Fig. 6, D and E). This variability was not due to movement artefacts. Rather, this finding is consistent with the idea that action-potential evoked calcium transients are small in the distal dendrite unless they are boosted by coincident distal synaptic input or during ongoing spontaneous activity (Waters and Helmchen 2004; Waters et al. 2003).

3D Ribbon Scanning from Multiple Pyramidal Cell Dendrites

Finally we aimed at simultaneous imaging of spatiotemporal activity in multiple apical dendrites of neocortical neurons. To this end we devised a ribbon-like, user-defined scan pattern that creates a fluorescence image of a convoluted plane in 3D space. The principal idea is to define two horizontal free line scans at two different focal depths and to interpolate linearly between these two lines assuming a saw-tooth like z-scan signal (Fig. 7, A and B; see Methods). By appropriately selecting dendritic cross-sections the 3D ribbon could be adjusted to include several (typically 5-10) dendrites with variable orientations. Figure 7B shows an example of a 3D ribbon scan through 10 apical dendrites of neocortical pyramidal neurons, filled with OGB-1 via local electroporation. In the unfolded ribbon, individual dendrites appeared as bright,
vertical lines (Fig. 7B). Spontaneous calcium transients measured simultaneously in the various dendrites were directly visualized (Fig. 7C; supplemental movie 5) and analyzed separately for the more proximal and distal segments, respectively (Fig. 7D). Similar results were obtained with 3D ribbon scanning in 3 animals with frame rates up to 8 Hz and axial ranges up to 100 µm.

Discussion

We have presented novel laser scanning modes for 2-photon microscopy that provide new viewing angles on neural dynamics in vivo. The new scanning modes are especially well suited for imaging dendritic signaling because they enable spatially resolved functional measurements from vertically oriented dendrites. Specifically, we have demonstrated sideways imaging of dendritic calcium signals in cerebellar Purkinje cells, vertical scanning along neocortical pyramidal cell dendrites, and simultaneous calcium imaging along multiple apical dendrites of neocortical pyramidal cells using 3D ribbon scanning. The ability to optically record spatiotemporal patterns of dendritic excitation in vivo will facilitate further studies of dendritic integration in the intact brain.

Arbitrary Modes of Laser Scanning

Our study demonstrates how beneficial novel non-rasterlike imaging schemes can prove for experimental studies of neural dynamics. Traditionally, laser scanning microscopes mostly employ raster-like scanning in the xy-plane - normal to the optical axis – combined with stepwise focal changes for the acquisition of image stacks. Typically, a compromise has to be made between scanning of a single or few structures of interest at high speed (e.g., using line scans) and a more extensive sampling of many structures albeit at reduced speed. For fast imaging researchers
still adhere to two-dimensional imaging, which provides only a limited view on the
three-dimensionally organized cellular circuits.

Recently, we have introduced a simple 3D laser scanning approach that
collects fluorescence signals along a predefined 3D scan trajectory and thereby
enables 10-Hz measurements from populations of several hundred cells in volumes
of up to 250 µm side length (Göbel et al. 2007). The key element of our 2-photon
microscope setup is the piezoelectric focusing element, which is used equivalently to
the two galvanometric scan mirrors. Axial scanning of a piezoelectrically-driven
objective has been described previously for xz-scanning in confocal and 2-photon
microscopy (Callamaras and Parker 1999; Nguyen et al. 2001). However, only one
study reported elementary calcium release events in Oocytes using this method
(Callamaras and Parker 1999). Here we extended this approach to arbitrary plane
imaging in 3D space, which permits optimal adjustment of the viewing angle for
imaging particular structures of interest. We exemplified the advantage of this method
by performing in vivo calcium imaging in the cerebellar cortex. The same approach
should be useful for functional imaging in many other areas of the brain as well as in
other research fields such as immunology, dermatology, or cancer research
(Helmchen and Denk 2005; Zipfel et al. 2003). Here we applied rather slow frame
rates of 2-10 Hz in xz-imaging mode. In principle frame rates of 20 Hz or even video
rate should be possible (Göbel et al. 2007; Nguyen et al. 2001).

In our further approach we departed from straight line scans or planar imaging
by employing user-defined curved line scans or convoluted image planes. Horizontal
free line scans permitted rapid measurements from multiple apical dendrites. Such
measurements are flexible and fast, with line durations of only a few milliseconds.
They promise to enable detailed investigation of the variability and the synchrony of
dendritic excitation in neighboring neurons, particularly in response to sensory input.
As a complementary technique we demonstrated user-defined vertical line scans along individual pyramidal cell dendrites. We could achieve acquisition rates of 20 Hz, which should be sufficient to study spatiotemporal profiles of dendritic calcium signaling. Finally, we combined these two approaches and devised the 3D ribbon scan technique, which can spatially resolve calcium signals in multiple, arbitrarily oriented dendrites. In summary, these ‘free’ modes of laser scanning, though generally applicable, are especially suited for in vivo studies of dendritic signaling.

Although alternative laser scanning technologies are currently being explored for fast and comprehensive 3D imaging, for example axial scanning using acousto-optical deflectors (AODs) (Reddy and Saggau 2005) or fast addressing of optical fibers (Rozsa et al. 2007), these methods have not yet been applied to the study of neural dynamics. The main advantages of our approach are that it is simple and that it lacks any additional optical aberrations.

**Functional Imaging of Dendrites In Vivo**

Fluorescence imaging of ion concentrations or biochemical activity has been extremely useful for characterizing intrinsic excitability and synaptic integration in dendrites of various cell types. Following the first demonstration of functional dendritic imaging in the mammalian brain in vivo (Svoboda et al. 1997), several obstacles prevented widespread use of this technique. Besides the high cost of a two-photon microscope, calcium indicator labeling in vivo ten years ago relied on intracellular filling of individual neurons via an electrode, which still is a demanding task. Consequently only few studies succeeded in characterizing action potential-evoked calcium transients in dendrites of neocortical pyramidal neurons (Helmchen et al. 1999; Svoboda et al. 1997; Svoboda et al. 1999; Waters and Helmchen 2004; Waters et al. 2003) and of mitral cells in the olfactory bulb (Charpak et al. 2001;
Debarbieux et al. 2003). Meanwhile, several new in vivo labeling methods have become available, including bulk loading of membrane-permeable calcium dyes (Kerr et al. 2005; Stosiek et al. 2003; Sullivan et al. 2005), electroporation (Nagayama et al. 2007; Nevian and Helmchen 2007), and the expression of genetically-encoded calcium indicators (Diez-Garcia et al. 2007; Hasan et al. 2004; Heim et al. 2007; Miyawaki 2005). These advances, together with the flexible 3D scanning modes presented here, should enable far more detailed studies of dendritic activation patterns in the intact brain.

So far most in vivo studies have focused on dendritic calcium signals evoked by action potentials. Far less is known about calcium signaling in the subthreshold regime. Axial scanning modes may facilitate the search for localized dendritic calcium signals as they might be induced by specific synaptic activation patterns. In the neocortex, individual dendritic branches might generate local dendritic spikes under certain conditions acting as isolated functional units (Gulledge et al. 2005; Holthoff et al. 2006; Polsky et al. 2004). Moreover, it is unclear when dendritic calcium waves induced by release of calcium from intracellular stores (Ross et al. 2005) might occur in the intact brain. In the cerebellar cortex, it might be possible to resolve natural activation patterns in both parallel fibers and Purkinje cell dendrites, e.g., upon sensory stimulation. Local dendritic calcium signals in Purkinje cells have been implicated in various forms of synaptic integration and plasticity (Hartmann and Konnerth 2005) but so far have resisted investigations in vivo. Moreover, Purkinje cells display complex dynamics comprising bistability (Loewenstein et al. 2005) that could be further analyzed using the imaging techniques presented here. Similar questions could also be addressed in mitral cells in the olfactory bulb (Charpak et al. 2001; Nagayama et al. 2007).
Finally, novel laser scanning technologies may not only provide means to read out neuronal signals. Patterned photostimulation could in addition be used to purposely impose activation patterns on single cells (Shoham et al. 2005) or cellular circuits (Arenkiel et al. 2007) in particular using genetically-encoded optical switches (Zhang et al. 2007). We therefore expect that the presented 3D scanning approaches will be powerful tools to dissect fundamental mechanisms of neural circuit function.

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References


Figure Captions

FIG. 1. Principle of arbitrary plane imaging in 3D. A: 2-photon microscope setup. Two galvanometric mirrors are used for xy-scanning and a piezoelectric focusing element for z-scanning. Laser intensity is adjusted with a Pockel’s cell. Fluorescence is detected in 2 color channels using photomultipliers (PMTs). B: Schematic of arbitrary plane scanning. The imaged plane can be tilted by an angle \( \theta \) and freely rotated around the optical axis by \( \phi \). C: Examples of vertical in vivo imaging in neocortical layer 2/3. Cellular structures were negatively stained by applying an extracellular stain. The standard xy-view is compared to the side view with the angle \( \theta \) optimized to image dendritic structures. Arrow heads indicate a corresponding dendrite (solid marker) and soma (open marker) in top and side view. Scale bar 40 \( \mu m \) D: Comparison of top and side view in the molecular layer of cerebellar cortex. A negatively stained Purkinje cell dendrite and a blood vessel are marked by a solid and open arrow head, respectively. Scale bar 40 \( \mu m \).

FIG. 2. Live imaging of parallel fiber beams in the cerebellar cortex. A: Schematic of xz-imaging in cerebellar cortex. Electrical stimulation with a distant electrode excites bundles of parallel fibers (PF; red), that make synaptic contact onto perpendicularly oriented Purkinje cell dendrites (grey). Purkinje cells receive additional synaptic input from climbing fibers (CF). B: Sagittal fluorescence image following bolus loading with the calcium indicator OGB-1 AM. The image orientation was aligned with the major branch of a Purkinje cell dendrite. Molecular layer (ML), Purkinje cell (PC) and granule cell (GC) layer are discernible. Scale bar 50 \( \mu m \). C: Three examples of snapshots of cross-sectional activation of PF beams at different stimulation intensities. Images were taken at the peak of the calcium transients. Scale bar 50
µm. 

D: Time course of the calcium transients in the center of the PF beam for the three examples shown in C. 

E: Spatial profile of the PF beam through the center of the PF beam at three different stimulus intensities. Same responses as in D. 

F: Increase of the diameter (full-width half maximum) of the responding region with stimulus intensity in three different experiments.

FIG. 3. Dendritic calcium signals in Purkinje cells in bulk-loaded tissue. 

A: Side view of cerebellar cortex (left) and single ΔF/F image (right) from a time series of spontaneous activity (4 Hz frame rate). A region of interest (ROI) was defined around the activated region, which corresponds to the outline of one Purkinje cell dendrite. The soma of this Purkinje cell presumably was located outside the field of view. 

Lower trace: time course of the relative fluorescence change in the ROI. Dashed vertical line indicates the time point of the ΔF/F image shown in the upper right. Scale bar 50 µm. 

B: Similar experiment as in A. In this example four responsive regions were analyzed, which presumably correspond to dendrites of different Purkinje cells. 

Lower traces: time course of the relative fluorescence change in the four ROIs. Dashed vertical lines indicate the time points of the snapshot ΔF/F images shown in the upper right. Scale bar 50 µm.

FIG. 4. In vivo calcium Imaging of Purkinje cell dendrites. 

A: Left: Sagittal average fluorescence image of several labeled Purkinje cells following local electroporation (top). Two ΔF/F snapshot images from two different time points are shown below. A sketch of the region of interests (ROIs) is shown in the upper right panel. ROIs correspond to dendritic arbors from two different cells (D1 and D2). 

Right: time course of the relative fluorescence changes for D1 and D2. Vertical dashed lines
indicate time points of the $\Delta F/F$ snapshots. Acquisition rate 4 Hz. B: Sideview, $\Delta F/F$ snapshot, and ROI sketch for another cell. The time course of spontaneously occurring fluorescence changes is plotted to the right for a dendritic and a somatic ROI. Three calcium transients (*brackets*) are shown on an expanded time scale to show the decay time-course. Acquisition rate 10 Hz. C: Side view, $\Delta F/F$ snapshot, and ROI sketch for a third cell. In this cell slow modulations in the calcium signals were apparent in both the dendritic and the somatic ROI. Sharp calcium transients occurred in the dendritic ROI preferentially near the beginnings and the ends of plateaus. Acquisition rate 2 Hz. D: Side view of a fourth example cell. $\Delta F/F$ snapshot images are shown for two time points, for which the two dendritic arbor regions D1 and D2 displayed differential activation. Acquisition rate 4 Hz. For all cells the tilt angle is given in brackets. Scale bar is 50 $\mu$m for all images.

FIG. 5. Horizontal free line scan for fast measurements from multiple neocortical dendrites. A: Schematic drawing of a subset of labeled neocortical pyramidal cells. Dendritic cross-sections are selected in a particular focal plane and a curved, smooth scan trajectory (*red*) is defined. B: *Left*: Sideview of labelled L2/3 neocortical cell population, stained with the calcium indicator OGB-1 using local electroporation 250-300 $\mu$m below the pia (not labeled cell bodies). A user-defined linescan is indicated in red. *Upper right*: Two-photon fluorescence image with selected dendritic cross sections marked by vertical lines (*yellow*) and the resulting 2D line scan (*red; slightly elevated*). Lower right: Numbering and color code of the sampled dendrites. C: Spontaneous calcium signals from the 9 selected dendrites. The color code corresponds to panel B. Note that synchronous activity occurred in some of the dendrites.
FIG. 6. Vertical line scan along a single dendrite. A: Principle of user-defined vertical line scan. The scan trajectory (red) is calculated based on the selection of the dendritic cross-section at different focal depths from a previously acquired reference image stack. B: Example of a scan trajectory (red) along the distal portion of an apical dendrite of a pyramidal neuron filled with OGB-1 (100 µm z-range). Four cross-sectional planes are shown in addition to the side view of the dendrite. The dendritic segment covered by the scan line was divided into two proximal parts (black and green) and two distal parts (blue and red). C: Spontaneous calcium transients in the four regions of the dendrite shown in B. Same color code as in B. Note the highly variable amplitude of calcium transients in the distal part of the dendrite. Decay time constants of exponential fits were $228 \pm 101$, $276 \pm 122$, $308 \pm 70$, and $317 \pm 66$ ms (mean ± SD) from proximal to distal. D: Top: example of a calcium transient that spread far into the distal dendrite. Bottom: example of a highly attenuated calcium transient in the same recording. Same color code as in B. E: Summary plot of the spatial profile of the calcium transient amplitude (normalized to the most proximal measurement). Different symbols correspond to different dendrites in 4 experiments.

FIG. 7. 3D ribbon scanning of neocortical dendrites. A: Scheme of scan ribbon pattern enclosing the apical trunks of several dendrites (left). Partially unfolded (right top) and completely unfolded (right bottom) scan ribbon. Dendrites appear as bright, slightly tilted vertical lines. B: Sideways reconstruction of labeled layer 2/3 neurons stained with the calcium indicator OGB-1 via local electroporation in about 450 µm depth (left). The location of a ribbon scan is indicated in red. The actual ribbon-type scan pattern (top) and its unfolded version (bottom) are both shown on the right. Individual dendrites are clearly discernible. C: Four example $\Delta F/F$ images showing activity in different dendrites. D: Time-course of spontaneous calcium signals from
identified dendrites (ordering indicated in B). Signals from proximal (black) and distal (red) regions are shown separately.

Supplemental Movies

Movie 1. Arbitrary Plane Imaging. Live rotation of the image plane by an angle $\theta$. First, rotation is shown for neocortex, second for cerebellar cortex until the image plane is aligned with the extension of individual dendrites. The angle of rotation $\theta$ is indicated in the upper right corner. In both cases a simple extracellular stain was applied, so that cell bodies and dendrites appear as unstained (‘black’) regions. Dimensions: 190 x 190 $\mu$m.

Movie 2. Sagittal Imaging of Parallel Fiber Beams. $\Delta F/F$ movie of three parallel fiber beams at different stimulation intensities (5, 6 and 10 $\mu$A stimulation intensity, respectively; Average of 4 stimulation trials). The extracellular stimulation pipette was positioned about 250 $\mu$m distant. With increased stimulation intensity more parallel fibers are recruited but the decay time-constant of the evoked calcium signals does not significantly change. Speed: 2.5x real time; Dimensions: 190 x 190 $\mu$m.

Movie 3. Sagittal view of Purkinje Cell Activity. The molecular layer of the cerebellum was bolus loaded with the calcium indicator OGB-1 AM. In the sagittal view, the outline of a Purkinje cell dendrite is clearly visible. Note the brief flashes of spontaneous activity in the dendrite. Speed: 5x real time; Dimensions: 175 x 175 $\mu$m.

Movie 4. Sagittal Calcium Imaging of Purkinje Cell Dendrites. A local network of Purkinje cells was stained with electroporation of a calcium indicator. The image
plane was then aligned with an individual dendrite. In this $\Delta F/F$ movie the differential activation of two sub-branches of the dendrite is evident. Speed: 5x real time; Dimensions: 240 x 240 $\mu$m.

**Movie 5.** 3D Ribbon Scanning. $\Delta F/F$ ribbon scan movie from neocortical dendrites. The scan ribbon is shown unfolded, so that individual dendrites appear as bright, vertical lines. Different activation patterns across multiple dendrites can be directly observed. Speed: 5x real time; Dimensions: 250 x 100 $\mu$m.
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Figure 2  Göbel & Helmchen
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