Optical Recording of Neuronal Spiking Activity From Unbiased Populations of Neurons With High Spike Detection Efficiency and High Temporal Precision

Gayathri N. Ranganathan and Helmut J. Koester
Center for Learning and Memory, Section of Neurobiology, The Institute for Neuroscience, The University of Texas, Austin, Texas

Submitted 22 February 2010; accepted in final form 30 June 2010

Ranganathan GN, Koester HJ. Optical recording of neuronal spiking activity from unbiased populations of neurons with high spike detection efficiency and high temporal precision. J Neurophysiol 104: 1812–1824, 2010. First published July 7, 2010; doi:10.1152/jn.00197.2010. Activity in populations of neurons is essential for cortical function including signaling of information and signal transport. Previous methods have made advances in recording activity from many neurons but have both technical and analytical limitations. Here we present an optical method, dithered random-access functional calcium imaging, to record somatic calcium signals from up to 100 neurons, in vitro and in vivo. We further developed a maximum-likelihood deconvolution algorithm to detect spikes and precise spike timings from the recorded calcium fluorescence signals. Spike detection efficiency and spike timing detection was determined in acute slices of juvenile mice. The results indicate that the combination of the two methods detected precise spiking activity from unbiased and spatially distributed populations of neurons in high slices with high efficiency of spike detection (>97%), low rate of false positives (0.0023 spikes/s), and high temporal precision. The results further indicate that there is only a small window of excitation intensities where high spike detection can be achieved consistently.

INTRODUCTION

Recording neuronal electrical activity in intact brain tissue using optical methods has been a long-standing quest as it promises probing of activity from populations of neurons in a manner that is much less invasive than mechanical methods, for example multielectrode recordings. Recently functional calcium imaging of somatic calcium transients from neuronal populations has been reported to resolve spiking activity from individual neurons. This technique relies on recording increases in intracellular somatic calcium concentration (calcium transients) to detect neuronal suprathreshold electrical (spiking) activity (Sasaki et al. 2008; Smetters et al. 1999). In combination with the staining of large populations of neurons using membrane-permeant, fluorescent calcium indicators (Stosiek et al. 2003) or genetically encoded calcium indicators (Hendel et al. 2008; Wallace et al. 2008), such a technique would record precise spiking activity from large populations of neurons.

Functional calcium imaging to record neuronal activity is a combination of an imaging technique to record somatic calcium signals with a detection algorithm that extracts spike information from the fluorescence signals. Many different techniques have been used to record somatic calcium fluorescence signals. These techniques include CCD cameras (Sasaki et al. 2008), Nipkow disk confocal scanning (Ikegaya et al. 2005), photodiode arrays (Mao et al. 2001), acousto-optical devices in conjunction with two-photon imaging (Otsu et al. 2008), two-photon imaging using galvanometric scanners (Greenberg et al. 2008; Kerr et al. 2007; Sato et al. 2007), and targeted-path scanners using two-photon excitation (Lillis et al. 2008). Only the methods using two-photon excitation can be used in opaque brain tissue to reject background signal from the neuropil surrounding neuron somata that carries other information (Kerr et al. 2005). Two-photon imaging using galvanometric scanning, however, results in a tradeoff between temporal resolution, duration of signal collection per cycle (duty cycle), and field of view (FOV). Even at low temporal resolution, sample size (number of recorded neurons) when achieving sufficient signal-to-noise ratio (S/N) for single spike detection in galvanometer based implementations is limited. Further, the low temporal resolution can lead to errors in spike detection. For example, two spikes with a very small interspike interval in a neuron with a small spike-evoked calcium signal and one spike in a neuron with a larger spike-evoked calcium signal are indistinguishable in recordings with low temporal resolution.

Functional calcium imaging is an indirect method of recording neuronal spiking activity. Extraction of precise number of spikes and spike timings from fluorescence recordings requires a method of spike detection and rigorous hypothesis testing (Sjulson and Miesenböck 2007). Thus detection of spikes from fluorescence traces is accompanied by errors of type I (false positives) and type II (undetected spikes). A high spike detection efficiency (low errors of both types) is required when using functional calcium imaging for indirect recording of spikes and spike timings. Prior methods of spike inference from calcium fluorescence signals include template-matching algorithms combined with thresholding (Holekamp et al. 2008; Kerr et al. 2005; Sato et al. 2007), inverse filtering (Mukamel et al. 2009; Yaksi and Friedrich 2006), clustering using principal component analysis (Sasaki et al. 2008), using sequential Monte Carlo methods with calcium transients defined by a particle filter (Vogelstein et al. 2009), and others (Greenberg et al. 2008). Many of these methods have been developed for a specific purpose. The majority of these methods rely on kernels to detect the rise in calcium signal associated with a spike. Spike inference methods, however, that rely on detecting templates (fixed kernels) are expected to lead to large errors in spike detection when used in a cell population with heterogeneous spike-evoked calcium signals. Because of the tradeoff
between temporal resolution and number of recorded neurons, and the limitations of spike detection methods, using functional calcium imaging to detect neuronal spiking activity with single-cell, single-spike resolution and with known detection error rates has been limited to specific experimental conditions (Greenberg et al. 2008; Kerr et al. 2005; Sasaki et al. 2008; Sato et al. 2007).

To overcome the current limitations of functional calcium imaging to detect spiking activity in an inhomogeneous population of neurons, we developed a random-access scanning method (“dithered random access scanning”) that collects multiple samples from each recorded neuron somata and records signals with millisecond temporal resolution and a spike detection method that accounts for the heterogeneity of the spike-evoked calcium signals in populations of neurons. Two-photon imaging using random-access scanning has been used to record calcium fluorescence signals from neuronal compartments with high temporal resolution, high duty cycle, and high S/N (Grewe et al. 2010; Iyer et al. 2006; Lechleiter et al. 2002; Otsu et al. 2008). Using random-access scanning with Acousto-optical deflectors (AODs) for detecting spikes from somatic calcium transients can potentially remedy many of the limitations of current implementations of functional somatic calcium imaging for precise spike detection. As we show, however, using single-point random-access scanning to record somatic calcium fluorescence signals resulted in problems in spike detection that arose from the inhomogeneous distribution of fluorescence within a soma. We present a simple solution to this problem by implementing a dithered random-access scanning method.

We further developed a new spike detection method that utilizes the prior information about the single spike-evoked calcium fluorescence signal. The algorithm infers spikes and spike timings by determining the maximum likelihood model. It is useful for detecting spontaneous and/or evoked activity in a population of neurons. We also determined the limitations of excitation intensity in terms of photodamage. Here we show that the combination of dithered random-access imaging, and the maximum-likelihood detection algorithm resulted in a high efficiency of spike detection (>97%) low rates of false positives (0.0023 spike/s), and low errors in spike timing detection when recording from L2/3 neurons in acute brain slices.

Methods

Preparation of acute brain slices and slice experiments

C57Bl6 mice [postnatal days 14–20 (P14–P20)] were anesthetized by intraperitoneal injection of ketamine/xylazine (80 and 20 mg/ml) decapitated, and the brain was quickly removed. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin. Acute thalamocortical brain slices (350–400 µm thick) were prepared in cold slicing solution (containing, in mM: 2.5 KCl, 1.25 NaH2PO4, 25 NaHCO3, 1.7 CaCl2, 7 MgCl2, 7 dextrose, 240 sucrose, 1 ascorbic acid, and 3 sodium pyruvate) as described previously (Agmon and Connors 1991). Slices were transferred to a chamber with extracellular solution containing, in mM: 125 NaCl, 2.5 KCl, 1.25 NaH2PO4, 25 NaHCO3, 1.7 CaCl2, 1 MgCl2, 10 dextrose, 1 ascorbic acid, and 3 sodium pyruvate. Both solutions were bubbled with 95% O2-5% CO2. Slices were incubated at 35°C for 30 min and stored at room temperature prior to experiments. Neurons within 1–2 mm² of cortex were stained by multiple bolus injections of the cell permeant ester form of a Ca2⁺ indicator [50 µg Oregon Green 488 BAPTA-1 (OGB-1) AM dissolved in 5 µl DMSO, diluted with 34 µl extracellular solution containing pluronic (20%); final concentration of OGB-1 was 1 mM]. Neurons were visualized by differential interference contrast microscopy (DIC). Neurons were allowed to take up the indicator for ≥1 h. All experiments were carried out at ~35°C bath temperature.

In vivo recordings

For in vivo recordings, Sprague-Dawley rats P20–P35 were anesthetized using 1–2 g/kg urethan. After anesthesia was established, a 3–5 mm wide craniotomy was opened above the right barrel cortex (5.5 mm from the midline and 2.5 mm posterior to bregma) and the dura removed. Neurons were stained by 4–10 bolus injections 100–150 µm apart. The craniotomy was filled with agarose (1%) in sterile saline and covered with a coverslip, mounted on a metal frame. The metal frame was mounted over the craniotomy using dental acrylic cement to restrain the head for optical recordings. Animals were placed under a microscope, and anesthesia was maintained for the entire duration of the experiment (3–6 h). Level of anesthesia was continuously monitored by monitoring heart rate and foot pinch reflexes. A single whisker was deflected in a short ramp and hold stimulus (200 ms) using a piezoelectric bimorph. Additional urethan was administered as needed. At the end of the experiments, animals were killed by additional injection of urethane and subsequent decapitation.

Electrophysiology

Whole cell or loose-patch recordings were made from the soma of layer 2/3 neurons using a Multiclamp 700-B amplifier. Electrical signals were low-pass filtered at 6 kHz and sampled at 12.5 kHz using a ADC data acquisition board (PCI-6115, National Instruments). Data acquisition and analysis was carried out with custom written software in MATLAB (Mathworks) and Igor Pro (Wavemetrics). Patch pipettes had resistances of 4–7 MΩ (whole cell) 8–10 MΩ (loose-patch) when filled with intracellular recording solution (containing, in mM: 115 KGluc, 20 KCl, 10 HEPES, 4 MgATP, 10 Na-phosphocreatine, and 3.5 GTP, pH 7.3 adjusted with KOH). For whole cell recordings, we added 50 µM of OGB-1 to the intracellular solution.

Dithered functional random-access calcium imaging

An upright microscope (BX50WI, Olympus) was coupled with the scanning module consisting of two AODs (ATD-655CD2/6510CD2, IntraAction) to deflect the laser beam in two dimensions. The Ti:sapphire laser source (Chameleon Ultra II, Coherent) was operated at 840–880 nm and had a mode-locked average power output of 2–2.2 W. The beam exiting the laser cavity was expanded using a telescope and relayed through a prism-pair prechirper to impart a net negative group velocity dispersion (GVD) to compensate for temporal dispersion caused by the AODs (Fork et al. 1984). To compensate for the spatial dispersion introduced by the AODs, a single reflective diffraction grating (53-01R, Newport) was placed at a 45° angle immediately after the AODs (Iyer et al. 2003). The pivot point was relayed to the back focal aperture of the objective lens using an afocal magnification telescope. To record from individual neurons, a ×40 water immersion objective (UMPlanFI, 40 ×, NA 0.8, Olympus) was used. AODs with large apertures (10 mm) were used for imaging large field of views of 300 × 300 µm. We also tested AODs with apertures of 5 mm, resulting in a field of view of 120 × 120 µm. The AOD scanning system and the DIC imaging were aligned for parfocality. The AOD beam deflection angles were controlled by voltage controlled oscillators connected to the output of a DAC board (PCI-6115, National Instruments). The DAC board was operated at a rate of 156.25 kHz when using the large AODs and at 125 kHz when using the small aperture AODs. The sample was initially imaged in raster format to
obtain locations of neuron somata. Coordinates for cell-to-cell scan were selected manually from fluorescence raster images. Temporal resolution (time required for one cycle to record from all neurons once) in cell-to-cell scan was 0.4–4 ms. In the majority of recordings, temporal resolution was 0.998–1.04 ms. Fluorescence was collected by detection modules with photomultiplier tubes (PMT) (H-9305, R-6357, Hamamatsu). Two detection units were used to detect both epifluorescence and transfluorescence (Koester et al. 1999) in acute slice recordings. For in vivo recordings, only epifluorescence was detected.

Random-access scanning is a sequential recording technique that records from a single location at any given time. To record from many neurons, the focal point was moved in a rapid manner from neuron to neuron during one cycle. This cycle was repeated for total recording times of 1–35 s. While the beam was moved from neuron to neuron (access time), no signal was collected. Access time was 12.8 ms for the large aperture AODs and 8 ms for the small aperture AODs. In each neuron, five spots were recorded before moving to the next neuron (dithering). Recordings were discarded from analysis only if the baseline was unstable or if the action potential (AP) timing could not be distinctly ascertained from the electrophysiological recording.

**Galvanometric functional calcium imaging**

A resonant galvanometric scanner (TCS RS, Leica Microsystems) in combination with an ultrafast pulsed laser system (Mai Tai, Newport) was used to record images of (60–80 μm)² with frame rates of ≤150 Hz. From image series, fluorescence signals from for 5–10 neuron somata were extracted using custom-developed software.

**Deconvolution of calcium fluorescence signals for spike and timing detection**

In the first step of the algorithm, for all 1 s time windows we compared the least square error of the following two functions

\[
F_1(t) = \begin{cases} 
F_0, & t < 0.5 s \\
F_0 \times 1.2, & t > 0.5 s
\end{cases}
\]

\[
F_2(t) = \begin{cases} 
F_0, & t < 0.5 s \\
F_0 / 1.2, & t > 0.5 s
\end{cases}
\]

\[F_0\] was calculated as average \(F(t)\) of the first 0.5 s for both functions. For all continuous intervals where the least square error of \(F_2\) was smaller than that of \(F_1\), we determined the location of the minimum least square error \(t_{\text{max}}\) calculating only entries \(F(t) < F_1(t)/F_2(t)\). Each of these instances represented potential spikes and were thus examined in a second step by maximizing the likelihood of a model (see following text). For each instance, baseline fluorescence \(F_0(1...N)\) was calculated as the average fluorescence in the interval \(t_{\text{min}} - 0.5 s; t_{\text{min}} - 0.08 s\).

Relative fluorescence increases \(\Delta F/F\) were calculated by

\[
\frac{\Delta F}{F} = \frac{F(t) - F_0}{F_0}
\]

To reduce the impact of slow baseline drifts and to reduce computational costs, we determined a second time \(t_{\text{max}}\). Model likelihood was calculated in the interval \(t_{\text{min}} - 0.5 s; t_{\text{max}}\). To determine \(t_{\text{max}}\) time was increased from \(t_{\text{min}} + 0.5 s\) until a window of 0.5 s was found that had an average fluorescence \(F_0\) that was \(F_0 < 1.001 \times F_0\), indicating that at this time \(t_{\text{max}}\) fluorescence had decayed back to baseline (if there was a spike). To limit computational costs associated with the maximum-likelihood deconvolution step, we limited the time interval \(t_{\text{max}} - t_{\text{min}}\) to maximal 4 s. This was sufficient for the spike trains examined here as they did not contain prolonged periods of spiking activity. The interval can be increased to 10 s with reasonable times to complete the algorithm. For all intervals \(t_{\text{min}} - 0.5 s; t_{\text{max}}\) s, we determined maximum likelihood by using a genetic algorithm.

The only instance of user interaction was when spikes occurred within the first 0.5 s of a recording. In such a case, both the 1 s time window for calculating \(F_1\) and \(F_2\) and the interval for calculating baseline fluorescence \(F_0\) were shortened.

For spike detection, we developed a deconvolution algorithm that modeled fluorescent calcium transients using a high-resolution kernel that we defined as follows

\[
h(t) = A \times \left(1 - \exp \left(-\frac{(t - t_{\text{min}})}{\tau_{\text{rise}}} \right)\right) \times \exp \left(-\frac{(t - t_{\text{min}})}{\tau_{\text{decay}}} \right) \quad \text{for} \quad t > 0
\]

This kernel is described by the following four parameters: the average lag between the peak of AP and onset of fluorescence signal \(t_{\text{rise}}\), the average time constant of the rising phase \(\tau_{\text{rise}}\), variable peak amplitude \(A\), and variable decay time constant \(\tau_{\text{decay}}\). Note that amplitude and decay time constant were variable and were adjusted by the algorithm for each cell individually. Fluorescence signal was modeled by a discrete time model with \(S\) spikes

\[
M(\Delta F/F) = \sum_{n=1}^{S} \left( t_{\text{rise}}(n) + \sigma_{\text{shot}} + \sigma_{\text{dark}} + \sigma_{\text{electrical}} \right)
\]

Thus the parameters of the model \(M(\Delta F/F)\) were \(A, \tau_{\text{decay}}, S,\) and \(t_{\text{rise}(1...S)}\).

A low-pass Butterworth filter with a cut-off frequency of 100 kHz was used to minimize the noise contribution from electrical sources \(\sigma_{\text{electrical}}\) and PMT dark current \(\sigma_{\text{dark}}\), and were hence neglected. As the number of photons per data point \(N_i\) was \(>100\) for most recordings, we did not use a Poisson-distribution for shot-noise. Instead \(\sigma_{\text{shot}}\) was approximated by

\[
\sigma_{\text{shot}}(\Delta F/F) = \sqrt{\frac{N_i}{2 \pi \exp \left(-\frac{(\Delta F/F)^2 N_i}{2} \right)}}
\]

In vivo recordings, additional sources of noise such as tissue movement may contribute. To compare in vitro to in vivo recordings, we approximated all noise by a simple Gaussian distribution

\[
\sigma_{\text{noise}}(\Delta F/F) = \sqrt{\frac{1}{2 \pi \sigma^2 \exp \left(-\frac{(\Delta F/F)^2}{2 \sigma^2} \right)}}
\]

The signal in rate of detected photons is denoted by \(r\) and was calculated as

\[
r = \frac{N_i}{\Delta t}
\]

Even for calcium transients described by kernels with very small amplitudes the number of photons contributing to spike detection was \(>2^5\).

The deconvolution maximized the log-likelihood using a genetic algorithm (Holland 1992). The genetic operators were specifically designed to utilize the topography of the log-likelihood as a function of the kernel, and the monotonic increase in likelihood for decreasing timing error. Each operator was used with a certain probability \(\leq 1\). Genetic operators included the standard operator crossover, displacement of spike timings by a random \(\Delta t = -10, …, +10\) ms with a probability of 0.05–0.2, simultaneous small random change \((\pm 0.05 \pm 0.05)\) in \(A\) and \(\tau_{\text{decay}}\) such that \((A \times \tau_{\text{decay}})\) remained constant (probability 0.02), and standard mutation operators (insertion and deletion of spikes with a probability of 0.005–0.02). The population of models consisted of 200–400 models, grouped into 20–40 families that had similar values of \(A, \tau_{\text{decay}}\). The genetic operator crossover was only applied within a family. In each iteration, the likelihood for all models was calculated and ranked. A new generation of models for the next iteration was generated by crossover using models with a probability according to their likelihood. The algorithm was terminated when no improvement in likelihood for the best model was found for 2,000 iterations. The algorithm was initialized using values drawn at random from the prior

*J Neurophysiol.* • VOL. 104 • SEPTEMBER 2010 • www.jn.org
Amplitude distribution of fluorescence signals within a soma

To determine the spatial distribution of the amplitude of the fluorescence signal, we evoked single APs by loose-patch recordings and simultaneously recorded the optical signals. Optical recordings were obtained using either the galvanometric scanner or the random-access scanner. In the recordings using the galvanometric scanner, sampling from individual locations resulted in very low S/N. We therefore averaged 4–31 recordings in each cell when using the galvanometric scanner. Despite this averaging over multiple trials the low S/N was not sufficient to fit each recorded location with a single exponential with variable amplitude and variable decay time constant. We therefore first analyzed the decay time constant by averaging across all recorded locations in one soma (4–10 locations for the random-access scanner, 184–225 pixels using the galvanometric scanner). The decay of the averaged fluorescence signal was fitted with a single exponential function to obtain the decay time constant. This decay time constant was then used for fitting the fluorescence signals from all locations with a single exponential to obtain the amplitude of the AP-evoked fluorescence signal in each location. When using the AOD scanner, each of nine points placed in a single soma was recorded using the same dwell time as if recorded in dithered mode (96 μs in this set of experiments).

Statistical analysis

For recording spatiotemporal activity from a cortical column, focal extracellular stimulation was used to activate thalamocortical afferents in thalamocortical slices. Experimental conditions were chosen such that cortical responses localized to a single column and restricted to L4, or L4 and L2/3 were observed.

Surrogate data for analysis of spatiotemporal activity patterns was constructed by two methods, shuffling spikes between neurons within a vector and shuffling spikes between neurons across vectors. The former method of spike shuffling preserved the number of responding neurons in a given trial as well as the average response probability across the population. The latter preserved average response probability across trials and the number of spikes from all neurons within a vector. Errors are expressed as ±SD unless stated otherwise.

\[
P(y) = \left( \frac{N}{2\pi} \right)^{N} \exp \left\{ -\frac{1}{2} \sum \left[ y - \left( \frac{\Delta F/F}{N} \right) \right]^{2} \right\}
\]
nometric scanner as well as the random-access scanner \((n = 7)\) cells galvanometric scanner, \(n = 5\) cells random-access scanner.\) We determined the spatial distribution of the amplitude of the fluorescence signal from single-exponential fits to the fluorescence transients. In all neurons recorded using galvanometric scanning, amplitudes \((\Delta F/F)\) of single spike-evoked calcium transients displayed large variations between different locations across the soma. In some neurons, the range of \(\Delta F/F\) amplitudes was as large as \([-0.22 \text{ to } 0.43]\), (average \(0.11 \pm 0.07, n = 7\)) across different locations. In recordings using random-access scanning, we sampled nine different spots distributed over the soma. Similarly amplitudes had a wide range (Fig. 1C). With such a large range of response amplitudes, not surprisingly, single-point random-access scanning resulted in many recordings that lead to failures in spike detection (see following text).

As sampling from multiple locations strongly reduced the probability of recording only from locations with low spike-evoked calcium transients, we developed a dithered scanning technique of sampling five points within each neuron soma (Fig. 1B). Somata locations and outlines were determined by obtaining a full \(x-y\) raster image. For dithering, five points were positioned in a star pattern in each neuron soma with a point centered in the middle of the soma and four points at a distance of \(1.8-2.5 \mu m\) from the central point (Fig. 1B). The five samples from one neuron were averaged off-line to yield one data point \(F(t_i)\). When using a low dwell time of \(25.6 \mu s\), we reduced the number of dithered points to three. As described in the following text, dithering significantly increased the fraction of spikes detected compared with single-point imaging. Note that in this comparison the total time spent on each neuron was the same. Dithered scanning presumably also decreased the cumulative photodamage in each sampled neuron by distributing excitation over multiple locations. In comparison to galvanometric scanners that are currently used for functional calcium imaging of neuronal populations, dithered random-access scanning achieved a high temporal resolution, high duty cycle, and hence a high S/N (Fig. 2, A–C). As a result, dithered random-access scanning can also be used to record activity from neurons that are spatially distributed while maintaining a high duty cycle.

**Spike-evoked calcium signals in cortical neurons**

As our method of extracting the spike information from fluorescence signals required prior knowledge about the spike-evoked calcium signals, we first characterized the spike-evoked calcium signals in L2/3 neurons with high temporal resolution. We used four parameters (rise time constant \(\tau_{\text{rise}}\), time between peak of action potential and start of fluorescence response \(t_{\text{start}}\), amplitude of the calcium signal \(A\), and decay time constant \(\tau_{\text{decay}}\)) to describe the single-spike evoked calcium signal \(h(t)\) (see Eq. 1). This model is based on a single compartment model for the intracellular calcium concentration (Augustine and Neher 1992; Helmchen et al. 1996).

![FIG. 2. Functional dithered random-access calcium imaging. A: examples of fluorescence calcium transients recorded in L2/3 neurons in acute brain slices. Left: traces show recordings using a galvanometric scanner (temporal resolution: 27 ms); middle: traces show recordings using dithered random-access scanning (temporal resolution: 1.28 ms). Right: traces show dithered random-access data filtered with a 30 ms Hamming window. \(N\) denotes the average number of detected photons from the neuron per data point. B: illustration comparing the duty cycles of dithered random-access scanning and galvanometric scanning. Each bar represents the time during which signal was collected from 1 neuron (dwell time), 25.6–96 \(\mu\)s every 1 ms (dithered random-access scanning) and 150 \(\mu\)s every 60–100 ms (galvanometric scanning), respectively. C: illustration of high signal-to-noise ratio (S/N) achieved with random-access scanning. Left: trace shows a recording using dithered random-access scanning in a L2/3 neuron. Bottom: trace shows cell-attached recording, indicating 1 spike at 0.313 s. Right graph shows integral of the relative fluorescence change \((\Delta F/F)\). As the algorithm presented in this study uses all the information contained in the long decay of the spike-evoked calcium signal, the signal of spike detection \((S)\) is approximately given by the integral compared with the noise \((N)\) of the baseline.](http://jn.physiology.org/)

To determine lag and rise time, pyramidal neurons in layer 2/3 (L2/3) in acute brain slices were patched in whole cell or loose-patch configuration. Single APs were evoked with brief current injections. Neurons were also recorded optically at a high temporal resolution (0.4 ms). Onset of the calcium fluorescence signal was determined by the point of intersection between baseline and a linear fit to the first 2 ms of the fluorescence signal. Average lag between peak of action potential and start of rise of fluorescence was \(\bar{t}_{\text{start}} = 485 \pm 214 \mu s (n = 17\) cells, 9 slices, Fig. 3, A and B). To obtain the time constant of the rise in fluorescence following an AP \(\tau_{\text{rise}}\), the first 7–10 ms of the calcium fluorescence signal were fit with a kernel with variable rise time constant and amplitude while lag and decay constant were held constant. Average value for rise time constant was \(\tau_{\text{rise}} = 2.25 \pm 1.09 ms (n = 17\) cells, 9 slices, Fig. 3).

To obtain amplitude and decay time constant, the fluorescence signals from recordings with a temporal resolution of 1.04 ms were fit with the model with amplitude and variable decay time constant as free parameters while rise time constant and lag were held constant at their average values \(\bar{t}_{\text{rise}}\) and \(\bar{t}_{\text{start}}\) (Fig. 3, C and D). Precise spike timing was determined from the electrophysiological data and hence was not a free parameter. Although the average values of amplitude and decay time constant were not used by our deconvolution algorithm, we nevertheless report these values here. Average amplitude was \(A = 0.057 \pm 0.0207 (\Delta F/F)\) and average decay time constant...
was $\tau_{\text{decay}} = 0.657 \pm 0.405$ s ($n = 218$ spikes, 47 cells, 20 slices from 17 animals, Fig. 3F).

The joint distribution of $(A, \tau_{\text{decay}})$ was used as a prior for the detection algorithm. To reduce the impact of the fluorescence noise (photon shot noise) on this distribution, we determined the joint distribution $(A, \tau_{\text{decay}})$ by averaging three to five recordings in each tested neuron (Fig. 3G, $n = 47$ cells). Before averaging, fluorescence traces were aligned by spike timing (Fig. 3E). In this distribution, amplitudes ranged from 0.0284 to 0.1546 and decay time constants from 0.128 to 1.403 s and $\tau_{\text{decay}} = 0.6365 \pm 0.297$ ms.

Detection of spikes in a heterogeneous cell population

The task of detecting spikes from fluorescence signals from an inhomogeneous cell population is difficult as neurons can have a wide range of spike-evoked calcium signal characteristics as seen in the distribution $(A, \tau_{\text{decay}})$. We therefore developed a maximum-likelihood approach using a genetic algorithm to maximize likelihood (see METHODS). The prior distribution $(A, \tau_{\text{decay}})$ was used by the detection algorithm to update model probability using Bayes’ theorem.

The algorithm tested models $M(\Delta F/F)_k$ with the parameters $A$, $\tau_{\text{decay}}$, number of spikes $S$, and spike timings $t_{\text{start}}$, $t_{\text{rise}}$, $t_{\text{decay}}$. Note that as part of the procedure the algorithm adjusted $A$ and $\tau_{\text{decay}}$ for each neuron. This is highly useful when detecting spikes from responses of a heterogeneous population of neurons, where spike-evoked calcium signals vary between neurons. Lag and rise time, on the other hand, were not free parameters of the model. Instead we used the average values $t_{\text{start}}$ and $t_{\text{rise}}$. Nevertheless, as shown in Fig. 4, the algorithm reliably detected spikes not only in recordings where only a single spike was observed (Fig. 4A) but also in recordings where multiple spikes occurred at low inter-spike intervals (Fig. 4B).

Maximizing signal rate

The largest limiting factor in dithered random-access scanning as well as two-photon calcium imaging in general is given by the photodamage effects that alter neurons structurally as well as impact functional dynamics (Koester et al. 1999). To characterize the impact of photodamage on spike detection and to establish an indicator of onset of photodamage for our imaging method, we recorded for prolonged periods of time from individual neurons. At a given laser intensity 20–75 trials (9.5 s each, 20–60 s between trials) were recorded. A single spike was elicited by brief current injection. From these recordings we calculated average baseline fluorescence, baseline slope across trials, amplitude and decay time constant of the spike evoked calcium signal, and spike detection rate. Average baseline fluorescence of each trace was calculated from all data points before and 3 s after the spike. The average baseline fluorescence values from consecutive runs were fit with a straight line to obtain baseline slope. This slope was normalized. As shown in Fig. 5, at high laser intensities, changes in baseline fluorescence were observed along with a decrease in the amplitude of spike-evoked calcium transient. This decrease in ampli-
Low errors of spike and spike timing detection

Using AODs with an aperture of 10 mm, a large FOV of 300 × 300 μm was achieved, resulting in recordings of high temporal resolution (0.4–4 ms) from ≤103 neurons that were distributed within the entire width of a cortical column (Fig. 6). The detection of spikes from noisy fluorescence recordings involves hypothesis-testing (Sjulson and Miesenböck 2007). As our approach of recording calcium fluorescence signals and extracting spike and spike timings using our detection algorithm did not allow us to determine the reliability of spike detection analytically, we determined spike detection errors empirically. Error type II was defined as the fraction of undetected spikes, error type I as the rate of false positives. These errors and the error in spike timing detection were determined from data with simultaneous optical and electrophysiological recordings, using a temporal resolution of 1.04 ms for all optical recordings. As the S/N had a large impact on these

FIG. 4. Deconvolution of spike information from fluorescence signals. A: example of a cell with a single spike at \( t = 5.0466 \) s. Spike timing was determined from electrophysiological data. Top: graph shows a random sample of 40 models at the initialization of the deconvolution algorithm. Middle: graph shows these 40 models after just 200 iterations of the algorithm. Bottom: graph shows the fluorescence recording (gray trace) and the final maximum likelihood model (black line). In this example the algorithm found 1 spike with a spike timing of \( t = 5.04712 \) s. Note that the algorithm not only extracted the correct number of spikes (1) and the spike timing with an error of only 0.6 ms but also adjusted amplitude and decay time constant for this particular neuron. Note that the appearance of the fluorescence trace is dominated by the large outliers (±3 SD because of the large amount of data points). B: example of the algorithm extracting spike information from a recording with multiple spikes at short interspike intervals. Bold line indicates best model determined by algorithm. The algorithm detected 5 spikes at \( t_1 \ldots t_5 = [0.9995, 1.0053, 1.0176, 1.9945, 2.0045] \). Analysis of the simultaneously collected electrophysiological data revealed 5 spikes with spike timings (peak of action potential) \( t_1 \ldots t_5 = [0.9996, 1.004, 1.0151, 2.0001, 2.004] \). The vertical bars below the trace illustrate actual spike timings (top row of bars) and deconvolved spike timings (bottom row of bars).

We therefore used the decrease in normalized baseline fluorescence as an indicator for onset of photodamage. Furthermore, as shown in Fig. 5B at higher negative baseline slopes, spike detection decreased rapidly. We used a conservative criterion of −0.0002/s for the normalized baseline slope as an indicator of photodamage (see Fig. 5B). Spike detection efficiency was high only in recordings where normalized baseline decline was below our criterion. At higher laser intensities baseline slope exceeded our criterion and spike detection rapidly decreased (Fig. 5B). At very high laser intensities, dramatic structural changes were observed (data not shown) such that neurons were no longer visible after brief (<10 s) exposure.

When limiting laser power according to our criterion, the signal we obtained from neurons ranged from \( 0.16 \sim 5.2 \times 10^6 \) photon/s. Not surprisingly, dwell time did not affect the photon rate obtained before onset of photodamage. Average signal rate across all neurons recorded at below photodamage threshold was \( 1.54 \times 10^6 \) photon/s (\( n = 43 \) neurons, dwell time = 96 μs, temporal resolution = 1.04 ms) and \( 1.35 \times 10^6 \) (\( n = 88 \) neurons, dwell time = 25.6 μs, temporal resolution = 0.998 ms).

FIG. 5. Photodamage and signal. A: traces and graphs show 8 s recordings from a L2/3 neuron in a slice at increasing excitation intensities as given by \( P \) in each row. Laser intensity was measured at the sample. Left: traces show overlay of all recordings for a certain excitation intensity (traces were filtered with a 30 ms Hamming window for illustration purposes. Left: graphs show the baseline fluorescence \( F_0 \), middle graphs show the deconvolved amplitude \( A \), right graphs show the decay time constant \( \tau_{\text{decay}} \). At higher excitation intensities, \( P = 31.8 \) and \( P = 46 \) mW) fluorescence baseline had a negative slope and spike detection failed consistently. Failure in spike detection is illustrated by an \( A = 0 \). Right: plot shows the relationship between detection efficiency and normalized slope of baseline fluorescence. The vertical line indicates the criterion of change in baseline fluorescence for photodamage (0.0002/s) and the horizontal line indicates 90% spike detection efficiency.
errors, we calculated signal as the rate of detected photons from the baseline fluorescence distribution for each recorded cell using Eq. 3.

Our combined data set consisted of data collected at signal rates of $0.16 \times 10^6$ to $5.4 \times 10^6$ photons/s. On testing spike detection in this data set, the error type II was low (2.5%, 9 of 362 spikes undetected in $n = 43$ cells). The error type I was also low (0.0023 spikes/s, 5 false positives, $n = 43$ cells, 575 recordings, 2,192 s total time). Similarly, the error in spike timing, calculated from the successfully detected spikes, was small, (average absolute error = 3.47 ms, Gaussian fit $\sigma = 2.07$ ms, SD = 8.3 ms, SE = 0.44 ms, $n = 353$ recordings in 43 cells). The theoretical limits of spike detection using our maximum-likelihood method were determined by simulating fluorescence responses. As shown in Fig. 6 (E–H), our method resulted in detection rates and errors that were close to these theoretical limits.

When limiting the data set to those recordings where photon rates were maximal before the onset of photodamage (optimized condition, mean $2.3 \pm 1.01 \times 10^6$ photons/s, $n = 192$ recordings from 36 cells, 2nd data point in Fig. 6E) the error type II was 3.1% (6 in 192 spikes undetected), error type I was 0.0025/s (3 false positives in 1195.6 s) and timing error was (average absolute error = 2.81 ms, Gaussian fit $\sigma = 2.1$ ms, SD = 6.1 ms, SE = 0.45 ms, $n = 186$ spikes in 36 neurons).

We also tested spike detection on data obtained with single-point random-access scanning (no dithering). In these experiments, we recorded data from single locations on neuron soma. Dwell time and duty cycle were the same as in the dithered data. The results demonstrated that recording from multiple locations on a neuron is required for high spike detection efficiency.
As the data set used to determine the prior kernel distribution and the test data partially overlapped, we tested the performance of the algorithm after separating the training data set (used to determine the prior distribution of the kernel) and the test data set (used to determine errors of our method) for dithered scanning. There were five errors of type 1 (error type I of 0.0029 spikes/s, \( n = 415 \) traces in 35 neurons, total time = 1,679.6 s) when testing our data in this way. There were also only four failures of spike detection (error rate type 2 = 1.8%, \( n = 218 \) spikes in 35 neurons). The timing error tested in the successfully detected spikes was also small (average absolute error = 3.95 ms, Gaussian fit \( \sigma = 1.71 \) ms, SD = 10.07 ms, SE = 0.24 ms, \( n = 214 \) spikes in 35 neurons).

As illustrated in Fig. 6, C and D, dithered random-access scanning can be used to record somatic calcium signals in vivo. Figure 6, C and D, shows fluorescence traces of calcium transients evoked by both spontaneous activity and by whisker deflection in L2/3 neurons in mouse barrel cortex in vivo. As expected, signals recorded in vivo had a larger baseline noise compared with in vitro recordings (Fig. 6G). The largest contribution to this difference was presumably the higher photon shot noise because of a lower rate of detected photons. Furthermore, tissue movements due to heartbeat and background signal from the surrounding neuropil are also likely to contribute to the recorded baseline noise in vivo. We did not quantify spike detection in vivo by simultaneous electrophysiological and fluorescence recordings.

**Spatial and temporal patterns of spiking activity in mouse barrel cortex**

We used our methods to examine the responses of cortical populations of neurons for spatial patterns of activity. The cortical responses to thalamic inputs were recorded in L4 of mouse barrel cortex. In acute thalamocortical slices, we used focal extracellular stimulation to activate thalamocortical afferents. Twenty-two neurons were recorded using dithered random-access scanning. From spike raster plots we extracted a population vector, with entries of 1 denoting neurons that responded with \( \geq 1 \) spike (Fig. 7A). A set of population vectors from 225 recordings was examined for repeating spatial patterns as a function of number of neurons (pattern rank) that make up a pattern. By calculating the number of times a pattern repeats in the set of population vectors for all patterns of a given rank, we obtained the probability distributions shown in Fig. 7, B and C. When surrogate data were created by simply distributing spikes across neurons (thus keeping firing rate per trial constant), the number of repeating spatial patterns in experimental data exceeded that in the surrogate data (Fig. 7B).

When exchanging spikes between trials such that average firing rate across trials as well as number of spikes in all neurons within a trial was maintained, no difference between experimental and surrogate data were found (Fig. 7C).

**FIG. 7.** Detection of spatial patterns in spatially distributed populations of neurons. A, top left: graph shows spike raster plot of responses of 22 neurons recorded in L4 to focal thalamic stimulation in a thalamocortical slice. The responses of 225 trials (dots indicate \( \geq 1 \) spike) are shown (right graph, arrow shows vector corresponding to spike raster plot shown on the left). B: graphs show the probability of finding patterns of rank \( r = 5 \) (and \( r = 10 \), respectively) that repeat \( N \) times in the 225 recorded trials (top row) and in surrogate data (bottom row) where spikes were shuffled within trials between neurons, equalizing firing rates. Arrows indicate the pattern with the highest number of repetitions (experiment: 151 and 81 for \( r = 5 \) and 10; compared with surrogate data: 50, and 27 for \( r = 5 \) and 10). C: graphs show the probability of finding patterns of rank \( r = 5 \) neurons (and \( r = 10 \), respectively) that repeat \( N \) times in the 225 recorded trials and in surrogate data where spikes were shuffled such that total number of spikes in a trial as well as average firing rates were preserved. Arrows indicate the pattern with the highest number of repetitions (experiment: 151, and 70 for \( r = 5 \), and 10; compared with surrogate data: 145, and 72 for \( r = 5 \) and 10).
**Discussion**

The sum of the methods presented in this study represent the significant achievement of recording spiking activity from unbiased, spatially distributed populations of neurons with low errors from a large sample of neurons. The combination of dithered random-access scanning with the deconvolution algorithm detected spikes with high efficiency and spike timings with millisecond precision. The low errors in spike detection were a result of the combination of the high duty cycle of random-access scanning, high temporal resolution (typically 1 ms), dithering, determining the prior kernel distribution, maximizing excitation intensity by determining photodamage threshold, and optimal use of information from fluorescence signals. Although we do not test spike detection efficiency in vivo, the imaging method can be used to record somatic calcium signals in vivo.

**Comparison to nonoptical techniques**

Dithered random-access functional calcium imaging is an optical technique that can record spiking activity from large populations of neurons. In contrast to techniques using microelectrodes or tetrodes, it is less invasive, can record from a larger local sample including neurons with very low spiking probabilities, and the spatial positions of neurons are known. Thus dithered random-access scanning can be combined with other techniques that record from or manipulate individual neurons. The larger local sample is particularly important to unravel the local computations carried out by highly recurrently connected neurons, for example within a cortical column.

**Functional calcium imaging for detection of supra-threshold neuronal activity**

Probing of the somatic calcium signal can be used to extract quantities related to neuronal activity other than precise spike timings, for example firing rate (Yaksi and Friedrich 2006) or simply discern between responding and nonresponding neurons (Ohki et al. 2006). To use functional calcium imaging for detection of precise number of spikes and spike timings in individual neurons requires rigorous empirical determination of spike detection efficiency, given by errors type I and type II (Sjulson and Miesenböck 2007). Only few studies have characterized this error systematically (Sato et al. 2007). Other studies have reported error rates from smaller test samples (≤14 cells) (Greenberg et al. 2008; Grewe et al. 2010; Kerr et al. 2005, 2007; Sasaki et al. 2008). As functional calcium imaging in vivo faces additional obstacles, we distinguish here between functional calcium imaging in acute brain slices (in vitro) and in vivo. The only study that characterized detection efficiency in vitro used Nipkow disk confocal scanning to record from slice cultures stained with the calcium indicator Oregon Green 488 Bapta-1 AM with a temporal resolution of 20–100 ms (Sasaki et al. 2008). Spike detection was high, but because of a low temporal resolution of the imaging method, this study could not determine spike timings.

**Comparison of dithered random-access scanning to other calcium imaging methods**

As outlined in the preceding text, functional calcium imaging for spike detection is a combination of an imaging method with a detection algorithm to extract precise spike timings from the noisy fluorescence signals. The aim of this study was to refine existing imaging methods to obtain fluorescence signals from many neuron somata with low noise, high temporal resolution, and multiple samples from each neuron soma to avoid spike detection errors. In contrast to other calcium imaging methods that do not use two-photon excitation, dithered random-access scanning as well as galvanometric scanning can be used for imaging deep within opaque brain tissue and reject background signal from neuropil surrounding the recorded somata that carries other information (Kerr et al. 2005). In contrast to galvanometric scanners that do use two-photon excitation, dithered random-access scanning has a much higher duty cycle. It thus results in a higher temporal resolution and/or much larger sample size. A high temporal resolution can be achieved when using galvanometric scanners in line-scan mode instead of recording full frames. In line-scan mode, however, only very few neurons can be recorded from.

Two-photon imaging using galvanometric scanner as well as AODs are scanning techniques that record from a single spot at any given time. Thus there is a tradeoff among the number of recorded locations, the time spent to record from a location, and temporal resolution. The advantage of dithered random-access scanning over galvanometric scanning is the much smaller fraction of the duty cycle that is not used to record useful signal from neuron somata. In comparison to targeted path scanners (Lillis et al. 2008), the temporal resolution achieved using dithered random-access scanning is ≥10 times higher. Because spike detection efficiency has not been tested for data obtained with targeted-path scanning, it is unclear if targeted-path scanners have an advantage over other techniques when used for functional calcium imaging to detect neuronal spiking activity.

As we show, implementing AODs to collect fluorescence signals from a single-point scanning resulted in large problems of spike detection because of inhomogeneous distributions of fluorescence and calcium transient amplitudes within a soma. As a solution to this problem, we have implemented star-patterned dithering that samples from several points within the soma. This approach reduced the probability of sampling from a location with low spike-evoked calcium transient. Dithering presumably also reduced photodamage by distributing excitation over multiple locations. Averaging over multiple samples potentially may reduce the sensitivity to small movements of the specimen as encountered for example during in vivo recordings. Independently from this study, another group has developed a very similar approach of using AODs in conjunction with two-photon excitation to record multiple samples from tens to hundreds of neuron somata in vivo with high temporal resolution (Grewe et al. 2010). The optical design was similar to our approach except that only one prism was used for spatial and temporal dispersion compensation, resulting in higher throughput of laser intensity.

**Acousto-optical deflectors**

For ultra-short laser pulses AODs introduce spatial dispersion because of the wavelength dependence of beam deflection. Despite dispersion compensation, the spatial resolution of AOD scanning is usually lower compared with galvanometric
scanning. This spatial dispersion increases with angle of deflection. Because dispersion compensation achieved using a diffraction grating is best at the center of the field of view, the spatial resolution deteriorates slightly toward the corners of the field of view (Iyer et al. 2003). AODs also result in a temporal dispersion of short laser pulses. Temporal dispersion was compensated using a two-prism prechirper as otherwise laser power would have limited excitation and thus limited signal. Two-photon imaging in vivo is limited to superficial layers; thus the technique presented here is also limited to superficial layers.

Comparison of spike detection to other methods

There are a number of methods that have been suggested for extracting spikes from fluorescence signals (Holekamp et al. 2008; Mukamel et al. 2009; Vogelstein et al. 2010). However, as these studies have not determined spike detection efficiency, we could not compare their performance to our algorithm. Our algorithm does not rely on a fixed kernel to extract spike information (Grewe et al. 2010; Kerr et al. 2005; Sato et al. 2007; Yaksi and Friedrich 2006) but instead uses a prior distribution. The spike-evoked calcium response (kernel) is adapted to each neuron during the deconvolution process. This is paramount for spike detection efficiency in a heterogeneous population of neurons as shown by high errors of types I and II, when using a fixed kernel on our dataset (error type I = 0.019/s and error type 2 = 19.8%, $P = 1.17 \times 10^{-14}$ Fisher's exact probability test). Our combined imaging/detection method is thus useful to detect precise spike timings in a population of neurons with inhomogeneous spike-evoked calcium signals. The algorithm also does not require knowledge of stimulus timings to increase robustness of spike detection in contrast to (Sato et al. 2007). Once the prior distribution used has been characterized from simultaneous electrophysiological and optical recordings, it can be used for any data set obtained under similar conditions. It is thus similar to other algorithms like (Greenberg et al. 2008; Sasaki et al. 2008). Our algorithm achieves high spike detection efficiency even when there is only a single spike in the observed neuron, and it also decodes the correct number of spikes in short bursts.

For detecting individual spikes in data containing high-frequency spike trains, a high temporal resolution is advantageous. Correct spike decoding from data containing many spikes at short interspike intervals also requires incorporation of indicator saturation into the model. Indicator can also easily be incorporated into our model (Eq. 2). Detecting the precise number of spikes and spike timings in data containing persistent high-frequency spike trains, however, presumably requires higher S/N than is possible to achieve using our technique without eliciting photodamage. This problem, however, can be alleviated to a large degree by using information across many spikes by recording over long time periods. We note that our algorithm can easily be modified to utilize information from many spikes. Genetic algorithms are not guaranteed to converge to global maxima. The genetic operators of the algorithm were designed to minimize convergence errors. As indicated by the high detection rate and also indicated by the analysis of simulated fluorescence data, convergence errors do not play a role in the analysis of single-spike and short burst data recorded in this study. In summary, the combination of the large number of simultaneously recorded neurons, the high S/N, the high temporal resolution, and the flexible deconvolution algorithm is highly useful to detect spiking activity and spike timings in heterogeneous populations of neurons.

Functional calcium imaging and spike detection in vivo

Calcium imaging under in vivo conditions faces obstacles that are usually not encountered in in vitro recordings. These obstacles include tissue movement, for example because of heartbeat or muscle movements and image degradation when recording from neurons deep within tissue. Some of these problems are often more pronounced in recordings in awake animals (Dombeck et al. 2007; Greenberg et al. 2008). To determine spike detection efficiency for a particular combination of imaging/detection method requires simultaneous optical probing and recording of action potentials with another technique. Testing spike detection efficiency for in vivo recordings requires simultaneous electrophysiological and optical recording, which we did not achieve here. We note that baseline noise for in vivo recordings was similar to the first data point (at lower photon rates) in Fig. 6E. As spike detection efficiency is determined by signal and kernel distribution, we estimate that our technique can record from many neurons in vivo with low errors in detection. This prediction is supported by a study that was published during revision of this manuscript (Grewe et al. 2010). In populations of neurons with large spike-evoked calcium signals, for example as has been reported for in vivo recordings in rats (amplitude $\Delta F/F > 0.1$) (Greenberg et al. 2008), spike detection using our approach will be even more robust. When recording from such populations, one could reduce the dwell time per neuron further to increase the number of simultaneously recorded neurons.

Many combinations of calcium imaging techniques and detection methods have been developed for specific experimental circumstances. For example, Sato et al. (2007) developed a method to detect stimulus-evoked activity in vivo from neurons in mice in somatosensory cortex using two-photon galvanometric scanning. This recording method in conjunction with a thresholding/clustering spike detection algorithm resulted in reliable (>95%) spike detection in 60% of neurons. By limiting the field of view, the galvanometric scanner (Kerr et al. 2007) detected spiking activity in vivo from 10 to 14 neurons at temporal resolutions of 62.5 ms, using a template-matching algorithm. Greenberg et al. developed a method based on two-photon galvanometric imaging and a detection algorithm (Greenberg et al. 2008). The detection algorithm was designed to detect both spontaneous and stimulus-evoked activity in vivo and also to decode the correct number of action potentials. Finally, as discussed in the preceding text, Grewe et al. (2010) developed a random-access scanning technique with a peeling algorithm and reported high spike detection efficiency.

Signal and limitations of dithered random-access scanning

The surprising finding of this study is that very high fluorescence signal rates can be achieved in recordings from neurons before the onset of detectable impact of photodamage. There is, however, only a very small window of excitation intensity where high detection efficiency can be achieved.
consistently. This “high-efficiency window” is limited by the impact of photodamage on calcium signals for high intensities and the low S/N for low intensities. This high-efficiency window is presumably even smaller for in vivo recordings. As this window is rather small, we regard it as important to precisely determine baseline noise and detection efficiency in experimental studies using functional calcium imaging to detect precise spiking activity. In particular, the decline in detection efficiency with increasing exposure time can introduce systematic errors in studies that rely on quantifying changes in neuronal activity. A high S/N is required to detect spikes and precise spike timings in unbiased populations of cortical neurons. Such populations include neurons that have very low-amplitude spike-evoked calcium transients, such as interneurons (Fierro and Llano 1996). The combination of dithered random-access scanning with our deconvolution algorithm was able to detect spike-evoked calcium fluorescence signals that have amplitudes as low as 0.025 (ΔF/F) and thus has a much higher sensitivity and precision than previous implementations of functional calcium imaging and spike detection methods. Dithered random-access scanning has a duty cycle that is ~9–10 times higher compared with a galvanometric scanning. A 10-fold higher duty cycle is expected to amount to gain in signal by the same factor. However, we find that the signal using dithered random-access scanner was ~10 times higher compared with a galvanometer-based implementation. Excitation intensity is not limited by available laser power but by rate of photodamage for both methods. Photodamage in turn depends on peak intensity, which can be quite high for femtosecond laser pulses. In contrast to two-photon calcium imaging in dendrites and axons of neurons, photodamage in dithered random-access imaging for somatic calcium signals manifests as reduction in amplitude and baseline decline not increases in baseline (Koester et al. 1999). The reason for this difference is not known. A possible contribution to this difference may be that bleached fluorescence molecules in a dendrite can be replaced from a large pool (the soma). We speculate that the higher than expected advantage of dithered random-access scanning arises from the highly nonlinear dependence of photodamage on excitation intensity in two-photon imaging (exponent n > 2, indicating contribution of three-photon effects to photodamage) (Hopt and Neher 2001; Koester et al. 1999), the high energy deposit of radiation-less transitions of excited molecules throughout the neuropil, ground state depletion, or a combination of these factors. A simple calculation based on the signal collection efficiency, detection rate and number of excited dye molecules (Koester et al. 1999) shows that ground-state depletion may occur when using a low duty cycle and high excitation intensity. Another limitation to excitation intensity may be given by dielectric breakdown. In fact, as a simple calculation shows, the peak intensity required (~10^{12} W/cm^2) to match the signal of a galvanometric scanner to our implementation would approach the dielectric breakdown of water (~10^{13} W/cm^2) (Xu et al. 1996).

We conclude that the combination of dithered random-access scanning and the maximum-likelihood detection algorithm result in a high spike and spike timing detection efficiency for recording from populations of neurons when operating within the “high-efficiency window” of excitation intensity. Detection of spikes from neuronal populations using functional calcium imaging is not useful for brain areas where somatic calcium transients and spiking activity are not highly correlated (Lin et al. 2007).

**Functional significance**

The result of our technique refinement and tool development is a method to record from up to 100 neurons that are spatially distributed with known and low errors of spike and spike timing detection. It further allows sampling from an unbiased population of neurons not only those with large spike-evoked calcium transients. Our method thus presents the tool to address central questions in neuroscience that require recordings from large populations of neurons. For example, theoretical studies predict that the detection of synfire chains or the detection of “neuronal groups” requires data from ≥50 neurons (Izhikevich et al. 2004; Schrader et al. 2008).

**Further improvements**

As a scanning technique, dithered random-access scanning requires a certain access time to move the focal point between spatial positions. Access time is given by the aperture of the AODs and the speed of the acoustic wave propagating through the crystals. The aperture determines field of view and spatial resolution (as it determines the beam diameter at the back aperture of the objective). For larger AOD apertures, the access time constitutes a significant fraction of the dwell time required to collect sufficient signal for single spike detection. In our recordings, the access time was 8–12.8 μs compared with the dwell time of 25.6–96 μs. Thus access time contributed significantly to the cycle time, reducing duty cycle. A reduction in access time thus would lead to further increase in the number of neurons that can be recorded. Another increase in sample size could be gained by adapting dwell time for each neuron depending on signal (photon rate) and spike-evoked calcium signal. Furthermore, one could also increase spike detection efficiency by assigning different weights to the five samples recorded in a neuron. This, however, would require incorporation of the weights parameters into the model and increase the complexity of the deconvolution algorithm. Further gain in signal, without increase in photodamage, could be obtained from increasing pulse rate (Ji et al. 2008), reducing peak intensities and thus reducing the contribution of higher order excitation processes to photodamage. The deconvolution method can be improved by using information across many spikes, reducing uncertainty about the kernel. The method presented in this paper can also be combined with light-sensitive ion-channels to selectively induce or prevent spiking activity in individual neurons. The combination of recording of neuronal spiking activity with the ability to change spiking activity will be highly useful to test models of neuronal processing and plasticity on the mesoscale in the CNS.

**Acknowledgments**

We thank B. Johnson for assistance in programming the data acquisition software and Dr. T. Middendorf for critically reading the manuscript.

**Grants**

This work was supported by the Albrecht P. Sloan Foundation, the Albert and Ellen Grass Foundation, the Whitehall Foundation and startup funds from The University of Texas at Austin.
No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES

Grewe BF, Langer D, Kasper H, Kampa BM, Helmchen F. Agmon A, Connors BW.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).