Miniaturization of Two-Photon Microscopy for Imaging in Freely Moving Animals

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Introduzione alla Miniaturizzazione della Microscopia a Due Foton per l’Immagazzinamento in Animali in Movercisi

Fritjof Helmchen, Winfried Denk e Jason N.D. Kerr

Questa articolo descrive lo sviluppo e l’applicazione della miniaturizzazione di microscopi a due foton ("microscopi a due foton""). Microscopi a due foton hanno sviluppati con l’obiettivo di permettere l’imaging ad alta risoluzione di attività neurale in animali in movere. Utilizzano i fotonici per fornire luce laser per due-foton excitation. I loro piccoli pezzi frontali tipicamente contengono un meccanismo di scansione e ottica imaging. Microscopi a due foton possono essere sufficientemente piccoli e leggeri da essere portati da ratti e topi e a consentire un movimento quasi non limitato all’interno di un’arena comportamentale. Tipicamente mondati al cranio dell’animale sopra una finestra cranio, microscopi a due foton permettono l’imaging di cellule fino a 250 µm sottosorante (e.g., nel neocortex di topo). In animali in movere, transitori di calcio potenziale d’azione possono essere immagazzinati in singole somate di neuron visual cortex nuovi etichettati ad alta risoluzione durante comportamenti naturali.

INTRODUZIONE

Il microscopia ad due foton è altamente adatto per l’imaging in tessuto intatto e in animali viventi grazie alla sua ridotta sensibilità a diffusione di luce. In neuroscienze, l’imaging ad due-foton ha aperto una nuova area per misurazioni dinamiche neuronali al livello subcellulare, cellulare e locale (per revisioni, vedi Helmchen e Denk 2005; Kerr e Denk 2008; Grewe e Helmchen 2009; Wallace e Kerr 2010). Due-foton imaging ha, per esempio, applicato lo studio della organizzazione funzionale di popolazioni neurale nelle aree primarie del sistema sensoriale (Kerr et al. 2007; Sato et al. 2007), visivo (Ohki et al. 2005; Mrsic-Flogel et al. 2007; Greenberg et al. 2008), e cortiici auditivi (Rothschild et al. 2010) in modo analogo a cortiici cerebellari (Sullivan et al. 2005; Nimmerjahn et al. 2009).

Sotto anestesia, stato del cervello e dinamiche neurali sono, naturalmente, alterate ed è incerto esattamente quanto questi cambiamenti influenzino la percezione del cervello. La necessità di lavorare su animi anestetizzati è, dunque, una limitazione maggiore e è desiderabile, certamente essenziale, per finalmente condurre esperienze in stato di veglia, animi movere. In recenti anni, una serie di approcci hanno emergere che consentono l’imaging optico con risoluzione cellulare in stato di veglia, animi movere, e due principali vie sono state seguite.

- Nella prima via, animali sono addestrati a ricevere fissazione di testa. Un animo movere può essere posizionato sotto un microscopio ad due-foton standard e imaging è eseguito durante anestesia (Dombeck et al. 2007; Greenberg et al. 2008). L’avvantaggio di questo approccio è che un microscopio regolare può essere usato; i principali svantaggi sono che il repertorio comportamentale è limitato e l’addestramento estensivo è necessario.
The second approach, which is the focus of this article, is to fiber-couple and miniaturize the microscope front piece so that it can be carried by a rat (Helmchen et al. 2001) or even a mouse (Flusberg et al. 2005b). The benefit of this approach is that animal behavior is far less restricted and it is possible to obtain high-resolution optical recordings during behavioral experiments, very much like chronic electrophysiological recordings in rodents. The difficulty with this approach lies in the special optical design. Miniaturization has to be achieved while maintaining sufficient sensitivity and resolution to measure calcium signals in individual neurons and neuronal processes in the brain.

In this article, we first survey the different technologies that have been explored during recent years for miniaturization of two-photon microscopy and introduce the key technological challenges. We then discuss some applications of two-photon fiberscopes, including the recent demonstration of calcium measurements with single-cell resolution in the visual cortex of freely moving rats (Sawinski et al. 2009).

**IMAGING SETUP**

**Equipment**

To build a two-photon fiberscope, several key components are the same as those required for a standard two-photon microscope. Hence, a fiberscope can be built as an addition to an existing setup. In the excitation pathway, an ultrafast laser source providing ~100-fsec laser pulses in the 700- to 1050-nm wavelength range is required. Typically, a tunable commercial Ti:sapphire laser system is used, although a custom-built laser may provide certain advantages (Sawinski and Denk 2007). For efficient two-photon excitation, a laser with large maximal output power is preferable because light guidance through optical fibers involves power losses, and fluorescence excitation is further reduced by pulse distortions caused by dispersion and nonlinearities (see below). Further standard components required include an intensity controller and a telescope for laser beam size adjustment. Specific to the fiberscope is a fiber coupler to stably and efficiently couple the excitation light into the single-mode optical fiber. If fluorescence is collected through a large-core fiber and detected at the fiber end remote from the front piece of the two-photon fiberscope, a standard detector system can be used. Besides these standard components, special technology is required for building a miniaturized front piece (see below). A list of useful components is given in Table 1.

**Considerations for Two-Photon Fiberscope Design**

First, mobilization of the microscope requires delivery of the excitation light via an optical fiber, through which it is difficult to maintain short pulse widths at high powers as is needed for efficient two-photon excitation. Second, a miniaturized scanning device is needed to scan the output beam from the optical fiber. Third, to focus the scanned beam, a small water-immersion objective suitable for imaging in the living brain is required. Fourth, fluorescence needs to be efficiently collected and detected, either directly at the fiberscope front piece using a small photodetector or with a remote detector following guidance through a large-core collection fiber (Fig. 1A–C). Finally, a simple and flexible mounting mechanism should permit easy fixation and removal of the fiberscope onto the animal’s head. In the following sections, we provide some details about these various technological aspects. Further information can be found in several reviews (Helmchen 2002; Flusberg et al. 2005a) and in the original papers cited below.

**Two-Photon Excitation through Optical Fibers**

The standard optical fibers for delivering laser light are step-index single-mode fibers, that is, solid glass fibers with a small (~5-μm diameter) core of slightly higher refractive index. In these optical
TABLE 1. Components useful for designing a two-photon fiberscope

<table>
<thead>
<tr>
<th>Component</th>
<th>Specifications</th>
<th>Model (vendors)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single-mode fiber</td>
<td>Core size 5–6 μm; NA 0.12–0.15; cladding diameter 125 μm</td>
<td>F-SF, F-SPF (Newport); FS-SN-4224 (3M)</td>
</tr>
<tr>
<td>Hollow-core photonic crystal fiber</td>
<td>Air core 9 μm; zero-dispersion wavelength 812 nm; NA 0.12–0.15; cladding diameter 125 μm</td>
<td>HC-800-01 (NKT Photonics, Denmark)</td>
</tr>
<tr>
<td>Piezoelectric elements</td>
<td>Benders (~2 mm × 8 mm in size); tubes, etc.</td>
<td>(EDO; Polytrec PI; Morgan Matroc; Piezo Systems; Argillon)</td>
</tr>
<tr>
<td>Tube lens</td>
<td>Aspheric (NA 0.15)</td>
<td>C280TM, C260TM (Geltech lenses; Thorlabs)</td>
</tr>
<tr>
<td>Long-pass dichroic</td>
<td>Cold mirror or dichroic beam splitter</td>
<td>M43-960 (Edmund Optics)</td>
</tr>
<tr>
<td>Short-pass dichroic</td>
<td>Hot mirror, near-infrared blocking filter, or dichroic beam splitter</td>
<td>Calflex X (Linos Photonics)</td>
</tr>
<tr>
<td>Objective</td>
<td>Water immersion; NA &gt;0.8; large fluorescence collection field of view</td>
<td>Custom design (Throl Optical Systems) (modified from design by Syncoctec; now defunct)</td>
</tr>
<tr>
<td>Collection lens</td>
<td>Small lens; NA matched to collection fiber</td>
<td>For example, C230TM (Geltech lenses; Thorlabs)</td>
</tr>
<tr>
<td>Fluorescence collection fiber</td>
<td>Large core 0.6–1 mm; high transmission at visible wavelengths; high NA</td>
<td>FT-600-URT (Thorlabs); MO2-534 (Edmund Optics); plastic fiber PJUB1000 (Toray Deutschland)</td>
</tr>
<tr>
<td>Small photomultiplier tube</td>
<td>High quantum efficiency at visible wavelengths; weight 4 g</td>
<td>R7400U (Hamamatsu)</td>
</tr>
<tr>
<td>(front piece detection)</td>
<td></td>
<td>Smoovy motor (Micro Precision Systems)</td>
</tr>
<tr>
<td>Small DC motor</td>
<td>Weight 300 mg; gear ratio 1:125</td>
<td>2L10SP (ON-TRAK Photonics); SS991 (Hamamatsu)</td>
</tr>
<tr>
<td>Position-sensitive detector (PSD)</td>
<td>Two-dimensional silicone-based detector with several milliliters side length</td>
<td></td>
</tr>
</tbody>
</table>

NA, numerical aperture.

fibers, ultrafast laser pulses broaden because of material dispersion and also, at the high laser powers required for deep imaging, because of nonlinear effects (Agrawal 1995). Such broadening limits the efficiency of two-photon excitation and therefore has to be reduced to a minimum. It is possible to compensate for dispersion-induced broadening by giving the short-wavelength components a sufficient head start (a negative “prechirp”) so that blue and red components arrive at the fiber end at the same time. Prechirping the laser pulses can be accomplished before coupling to the fiber with a pair of diffraction gratings (Treacy 1969) or with prism sequences (Walsmsley et al. 2001; Sawinski and Denk 2007). For example, two reflection gratings (400 grooves/mm and 9.7° blaze angle; Richardson Grating Laboratory) in double-pass configuration provide around −60,000 fsec² at 17-cm spacing, compensating for ~1.5 m of optical fiber (Helmchen et al. 2001). The optimal spacing between gratings (or prisms) can be found by measuring the pulse width after the fiber with an autocorrelator and adjusting the spacing for minimal pulse width. Even with prechirping, however, laser pulses progressively broaden at increasing power levels because of nonlinear optical effects in the fiber core (mainly self-phase modulation; Agrawal 1995). Nonlinear pulse broadening becomes substantial starting at surprisingly low average powers (~10 mW) and is dominant at the high average power levels needed for imaging deep into tissue (Helmchen et al. 2002). As a result, relatively long laser pulses (~1 psec or longer) are used in effect at high excitation powers when standard step-index fibers are used, resulting in suboptimal two-photon excitation.

Several approaches have been devised to alleviate the problem of nonlinear pulse broadening. For example, large-core specialty fibers (Helmchen et al. 2002; Ouzounov et al. 2002) as well as spectral and temporal shaping of the input pulse (Clark et al. 2001) have been tested but are not widely used. The problem of pulse broadening can be nearly completely circumvented by using hollow-core photonic crystal fibers (Göbel et al. 2004b). In these fibers, most of the laser light travels through an air-filled core and thus encounters little dispersion or nonlinearity, minimizing pulse broadening even at high average power (Göbel et al. 2004b). No prechirping is required and the laser beam can be coupled straight into the fiber. Photonic crystal fibers have been successfully used in a number of two-photon fiberscope designs (Plusberg et al. 2005b; Engelbrecht et al. 2008; Piyawattanametha et al. 2009). A disadvantage is, however, their susceptibility to contamination of the fiber tip, probably attributable to the intricate photonic crystal structure, which poses a particular problem when the fiber end is integrated into a fiber scanner device and thus cannot be easily recleaved.
\[ V_{\text{piezo}} = A \sin(2\pi f_{\text{rest}}) \]
\[ V_{\text{piezo}} = A_X \sin(2\pi f_X t) + A_Y \sin(2\pi f_Y t) \]

**A** Two-photon fiberscope setup. Excitation light is delivered through fiber optics to a miniaturized microscope front piece that is attached to the head of a freely behaving animal. Two principle options for fluorescence detection are indicated. **(B)** Example for Option 1: Original design used by Helmchen et al. (2001). Excitation light is guided through a single-mode fiber. Scanning is achieved using resonant mechanical vibration of the fiber tip. Near-infrared excitation light (red) is passed through a long-pass dichroic beam splitter and fluorescent light (green) is detected by a small photomultiplier tube (PMT) at the front piece. **(C)** Example for Option 2: Recent design used by Sawinski et al. (2009). A nonresonant fiber scanner is used and near-infrared excitation light is collimated and deflected by a short-pass dichroic beam splitter. Fluorescence is collected through a large-core multimode fiber and detected remotely. **(D)** Resonant fiber scanning. **(Left)** Line scan. **(Middle)** Lissajous scan. **(Right)** Spiral scanning.

**F**

- **Resonance frequency (kHz)**
- **Tip length (mm)**
- **Amp. |A|^2 (a.u.)**

**FIGURE 1.** (A) Two-photon fiberscope setup. Excitation light is delivered through fiber optics to a miniaturized microscope front piece that is attached to the head of a freely behaving animal. Two principle options for fluorescence detection are indicated. (B) Example for Option 1: Original design used by Helmchen et al. (2001). Excitation light is guided through a single-mode fiber. Scanning is achieved using resonant mechanical vibration of the fiber tip. Near-infrared excitation light (red) is passed through a long-pass dichroic beam splitter and fluorescent light (green) is detected by a small photomultiplier tube (PMT) at the front piece. (C) Example for Option 2: Recent design used by Sawinski et al. (2009). A nonresonant fiber scanner is used and near-infrared excitation light is collimated and deflected by a short-pass dichroic beam splitter. Fluorescence is collected through a large-core multimode fiber and detected remotely. (D) Resonant fiber scanning. **(Left)** Line scan. **(Middle)** Lissajous scan. **(Right)** Spiral scanning. A piezo tube with quadrant electrodes induces spiral scanning by driving the fiber coordinates at the same resonance frequency but with a 90° phase shift. (E) Nonresonant fiber scanner. The “piezolever fiber scanner” achieves fiber tip deflections by leveraging the movements of two crossed pairs of piezo benders. The fiber is glued to the tips of the cross-connected piezo pairs at the cross point close to the clamped fiber base. Driving the piezo benders with sawtooth-like waveforms (fast and slow) at frequencies below the resonance frequency creates raster scanning similar to conventional two-photon microscopes. Offset voltages to the x and y piezo benders provide the unique option to shift the scanned field laterally. Moreover, free-line scanning is possible. (F) Fiber resonance characteristics.
Miniaturized Fiber-Scanning Devices

A compact scanning device is essential for miniaturization. One possibility is to move the tip of the illuminating fiber, which is positioned in an optical plane conjugate to the focal plane (Giniunas et al. 1991). In this approach, the output light beam emerging from the fiber core is collimated and then refocused to a focal spot using small optics (see below), so that any movement of the fiber tip translates into a lateral movement of the focal spot. Multiple options exist for how to move the fiber tip. In particular, we need to distinguish resonant and nonresonant scanning (Fig. 1D,E). We first discuss several ways to exploit resonant vibration before describing a nonresonant fiber-scanning method.

Resonant mechanical vibration of a free-standing fiber end can be easily induced with a piezoelectric bending element (“piezo bender”). The bare fiber (with the coating stripped off) is glued to the piezo bender so that 10–20 mm of the fiber end remains free to vibrate (Fig. 1D). For a 125-μm (standard) diameter fiber, this results in a resonance frequency $f_{\text{res}}$ ranging from 200 to 1000 Hz (Fig. 1F). In general, for transverse vibration of a cylindrical rod, $f_{\text{res}}$ is given by the equation

$$f_{\text{res}} = \frac{s_0^2 R}{4\pi L^2} \sqrt{\frac{E}{\rho}}$$

where $R$ is the radius, $L$ is the length of the rod, $\rho$ is the density ($\sim 2.3$ g/cm$^3$), $E$ is the elasticity module of the fiber ($\sim 75$ GPa), and $s_0 = 1.875$ (Bishop and Johnson 1960). Thus, $f_{\text{res}}$ depends inversely on the square of the length $L$ (Fig. 1F).

Two-dimensional Lissajous scanning can be achieved by stiffening the fiber end in one direction with a short (2–4 mm) piece of bare fiber glued to the lower edge of the piezo and to the fiber at ~2- to 3-mm distance from the piezo (Fig. 1D). This approach results in two distinct resonance frequencies of the fiber tip, as becomes apparent by driving the piezo with a sinusoidal waveform that is swept in frequency (Fig. 1F). Because the resonance widths are typically only 5–10 Hz, one has to sweep the frequency slowly and finely enough not to miss the resonance. For accurate measurement of the resonance curve, the fiber tip can be imaged onto a two-dimensional position-sensitive detector. When the piezo bender is driven with a superposition of two sine waves at the respective resonance frequencies for the different directions, a Lissajous scan pattern is produced, which has been used in several two-photon fiberscope implementations (Helmchen et al. 2001; Flusberg et al. 2005b). With up to 100-V (peak-to-peak) drive amplitude, resonant vibrations of >1-mm peak-to-peak amplitude can be reached and the Lissajous scan pattern can be tuned so that a rectangular area is covered rather homogenously (for details on scan parameters and data acquisition, see Helmchen et al. 2001).

An alternative method of resonant fiber scanning is spiral scanning (Fig. 1D). Here, the fiber is glued inside a piezoelectric tube with quadrant electrodes that enable independent excitation of vibration in $x$ and $y$ directions (Seibel and Smithwick 2002; for review, see Lee et al. 2010). The advantage of spiral scanning is that two-dimensional scanning is easily achieved without stiffening of the fiber end by driving vibrations in the two orthogonal directions with a 90° phase shift (Fig. 1D). Because small piezo tubes are sufficient to drive fiber vibration, spiral scanners can be made very compact, probably smaller than is possible for Lissajous scanners. For example, in combination with a small gradient-index (GRIN) lens system, a miniature fiberscope front piece (<1 g) can be realized (Engelbrecht et al. 2008). Moreover, using a short fiber end with a correspondingly high resonance frequency of a few kHz, frame scanning at video rate is possible.

Resonant scanning lacks flexibility and, in particular, does not support lateral offsets or random access scanning. To overcome these limitations, a nonresonant, leverage-based fiber scanner has been developed (Fig. 1E) (Sawinski and Denk 2007). Here, the fiber end is deflected near its clamped base with the help of piezo benders. Because of the leverage, small deflections of the piezo benders are transduced into relatively large fiber tip deflections. Operating below the resonance frequency of the fiber tip, this design permits raster-like scanning of an area, similar to standard galvano metric scan mirrors. It also allows one to randomly access scan points within a field of view, which might be useful.
in future optogenetic applications. This type of scanner was used recently to measure calcium signals in individual neurons of neocortex at $\approx 15$ Hz frame rate in freely behaving rats (Sawinski et al. 2009).

Instead of deflecting the fiber tip, the beam can be scanned using a microelectromechanical system (MEMS), whereby a two-dimensional scanning mirror (Piyawattanametha et al. 2006) is inserted between the fiber end and miniature objective. The use of a coherent fiber bundle avoids scanning in the front piece at all (Göbel et al. 2004a). None of these methods, however, has become widely adopted and currently fiber tip scanning appears to provide the best results for two-photon excitation.

**Small Microscope Objectives**

For high-resolution in vivo imaging, a water-immersion objective with a numerical aperture (NA) of $\geq 0.8$ is desirable. Standard high-quality microscope objectives are too large and too heavy to be useful for microscope miniaturization. Thus, the first two-photon fiberscope took advantage of a water-immersion objective, in which the core part could be removed and reassembled in a minimal holder (Helmchen et al. 2001). This type of objective is not available anymore. In the more recent fiberscope design shown in Figure 1C, a specially designed water-immersion objective with NA 0.9 (Throl Optical Systems) was used (Sawinski et al. 2009). As an alternative, GRIN lens systems have been used in two-photon fiberscopes, typically with limited NA (0.48–0.58) of the front GRIN lens (Flusberg et al. 2005b; Engelbrecht et al. 2008; Piyawattanametha et al. 2009). The recent development of thin compound lens systems, made of a GRIN lens and a planoconvex lens, now enables NA values up to 0.85 (Barretto et al. 2009). These new lens systems should be highly suitable for two-photon fiberscope designs.

**Fluorescence Detection**

In principle, there are two options for fluorescence collection: (1) to detect fluorescence light directly at the fiberscope head piece and (2) to collect and guide it through an additional optical fiber and then project it onto a remote detector (Fig. 1A–C). The advantage of the first approach is its simplicity and the high detection efficiency. In the first fiberscope design (Helmchen et al. 2001), a small photomultiplier was used, but in the future a small avalanche photodiode array might provide superior quantum efficiency. The disadvantage here is that more weight is added to the animal’s head. Moreover, depending on the design of the photomultiplier tube, high voltages can be present close to the animal. Collecting fluorescence through a large-core fiber suffers from some transmission losses but has the great advantage of being very flexible at the remote end of the collection fiber (Fig. 1C). Any highly sensitive detector can be used irrespective of its size and weight, and the fluorescence light can be separated into multiple spectral windows for multichannel imaging of several fluorescent dyes. As only a small coupling lens is required in the front piece, this detection scheme also helps to further reduce the size and weight of the fiberscope.

**Mechanical Attachment to the Animal**

Three issues arise when designing a suitable head-mounting system for the fiberscope: (1) The microscope must be easily detachable; (2) when attached, the microscope must be mechanically stable, even during high-acceleration movements (jerks) of the head; and (3) attachment must not affect the animal’s behavior or viability. The second point is especially important when using a raster scan, spiral scan, or free-line scan approach, in which the presumed positions of the beam are used to reconstruct images. Although offline movement correction has been successfully applied (Dombeck et al. 2007; Greenberg et al. 2008), there is a limit as to how much movement can be corrected (Greenberg and Kerr 2009). The attachment system that has been successfully applied to the freely moving animal is based on a two-part system in which the headplate remains permanently bonded to the animal’s skull and the fiberscope can be attached (Sawinski and Denk 2007; Sawinski et al. 2009). When the fiberscope is not attached, it should be replaced with a protective cover that protects the cranial window from mechanical damage, such as from the animal’s paws during grooming. For the
stable attachment of the headplate to the skull, we prefer using ultraviolet-cured bonding agents rather than bone screws, especially in younger animals in which the skull is thin. Another important consideration is whether it is easily possible to attach and detach the microscope to and from an awake animal while keeping handling of the animal at a minimum. This last point is important for trained animals in which excessive handling can interfere with their behavioral performance (Stüttgen and Schwarz 2008).

FLUORESCENT DYE LABELING IN VIVO

Imaging with a two-photon fiberscope depends on in vivo fluorescence labeling. Tail-vein injection of a fluorescent dye was used for the first demonstration of two-photon fiberscope imaging in freely moving rats (Helmchen et al. 2001). For imaging neural activity, various methods of labeling neurons can be applied. Individual neurons can be filled with organic indicator dyes via intracellular recording electrodes (high-resistance sharp electrodes; Svoboda et al. 1997) or whole-cell patch pipettes (Helmchen and Waters 2002). This approach has been used for fiberscope measurements of dendritic calcium signals during anesthesia (Helmchen et al. 2001) (Fig. 2A,B). Single-cell labeling is, however, laborious and restricted to small numbers of neurons. When the activity in a larger number of neurons is to be sampled in parallel, population labeling techniques are needed. What has proven popular for fiberscope and fixed-scope experiments is the in vivo loading of populations of neocortical cells with calcium indicators by the local application of membrane-permeant dye (Stosiek et al. 2003). Alternatively, local electroporation of calcium indicator dyes has been used (Nagayama et al. 2007). The use of genetically encoded calcium indicators is also highly promising (Wallace et al. 2008; Tian et al. 2009; Lütcke et al. 2010). The latter approach allows permanent labeling and should, therefore, permit repeated measurements of the same neuronal population in multiple imaging sessions over days, weeks, and perhaps even throughout an animal’s life.

EXAMPLES OF APPLICATION

Using the first Lissajous-type scanning two-photon fiberscope (Helmchen et al. 2001), pyramidal neurons in neocortical layer 2/3 (loaded with calcium indicator via an intracellular electrode) could be resolved in head-restrained, anesthetized rats (Fig. 2A). Using a line-scan mode, with fiber-end vibration induced along one direction, dendritic calcium transients were resolved following intracellular current injection, providing proof-of-concept for the sensitivity of fiberscope calcium measurements (Fig. 2B). As the first awake demonstration, fluorescently labeled blood capillaries were imaged in freely moving rats (Fig. 2C), showing that stable images can be acquired in unrestrained animals, except during periods of strong head movements (Helmchen et al. 2001).

Despite the development of various two-photon fiberscope designs during the past decade (e.g., Flusberg et al. 2005b; Engelbrecht et al. 2008; Piyawattanametha et al. 2009), the original goal of enabling functional measurements of neural activity in freely moving animals remained elusive until recently when a light, compact fiberscope (≏5 g) using a leverage-based fiber scanner was combined with bulk-loading of the visual cortex with calcium-sensitive indicator (Sawinski et al. 2009). This study succeeded in recording spike-evoked calcium transients from animals freely exploring a novel environment that contained static visual stimuli (Fig. 2D–H). Intrinsic optical imaging through the thinned skull can be used to coarsely map the cortical responses. Using the pial blood vessel pattern as a reference, this approach allows placement of the cranietomy without the need to map responses again. To decrease the stress to the animal while attaching the microscope, animals were trained over many days to tolerate repeated handling and head-fixing while awake. Once animals were trained, they were head-fixed, the calcium-sensitive indicator was injected in the area of the cortex previously mapped with intrinsic optical imaging, and the fiberscope was attached. After a calcium
Once an indicator-loaded neuronal population (~10–20 neurons) had been located, the animal was released onto the running track (a semicircular track raised ~1.5 m above the ground), which restricted the area the animal could explore while being always fully visible for overhead and side-on cameras that were used to track the animal’s position (Fig. 2E). In addition, this setup allowed the unrestricted movement of the fibers and wires attached to the fiberscope.
Full-frame (64 × 64-pixel) images were then acquired at ~10 Hz while the animal was free to explore a novel environment. This environment included three cathode ray tube monitors, each with a static grating orientated at −45°, 0°, or 45° (Fig. 2E). Using an overhead camera and four infrared light-emitting diodes attached to the fibroscope, the head of the animal could be accurately tracked in three dimensions. Movement within the two-photon frames was corrected offline and calcium transients were detected (Fig. 2G; Greenberg and Kerr 2009). Calcium transients could then be related to the head position of the animal. The relative orientation and distance to the animal’s head were calculated for each screen by using video frames from the video camera, and related to the receptive field of the imaged cortical region (Fig. 2H). A subpopulation of neurons was found to become significantly more active when one specific monitor was in the receptive field, compared with other monitors (Fig. 2H). This fibroscope application shows that neuronal activity can be recorded in a freely behaving animal that is free to interact with a stimulus in a self-determined way.

**DISCUSSION**

During the past few years, optical fibers have been increasingly used to measure neural activity in mobile animals, mainly in connection with fluorescent calcium indicators. In the simplest case, an optical fiber provides a bulk readout of the mean calcium signal in a local population of neurons (Adelsberger et al. 2005; Murayama et al. 2007; Lütke et al. 2010). Achieving cellular resolution with small, portable fiberscopes is much more difficult, not only because of the technical challenges associated with miniaturization but also because of strong light scattering in neural tissue. Even though calcium transients in Purkinje cell dendrites of the cerebellum could be imaged in mobile mice using single-photon imaging through a fiber bundle (Flusberg et al. 2008), single-photon approaches are highly susceptible to light scattering and are thus applicable only when the cells of interest are located near the tissue surface. In contrast, two-photon fiberscopes exploit the well-known two-photon advantages, in particular enhanced depth penetration and intrinsic optical sectioning. In principle, it should be possible to reach imaging depths of ≥400 µm with two-photon fiberscopes, similar to standard two-photon microscopes.

With the demonstration of cellular calcium imaging in freely behaving rats (Sawinski et al. 2009), the application of two-photon fiberscopes has now entered a new phase. Many potential application and further developments can be envisaged. The expression of genetically encoded sensors in transgenic animals or following viral gene delivery will enable long-term measurements from specific, well-identified ensembles of neurons. This method has the potential to directly relate changes in neural population dynamics to behavioral changes, such as an improved performance in a behavioral task. The fiberscope also will allow the study of social interactions as well as tasks in which the animal needs to be free to move within its environment. Although the two-photon fiberscopes discussed here first of all enable imaging studies in accessible regions such as the neocortex and the cerebellum, the use of GRIN lenses as front objectives or relay lenses (Flusberg et al. 2005b, 2008) should permit functional imaging in deeper brain regions, such as the hippocampus or the basal ganglia. Finally, another fascinating possibility is the combination of two-photon fiberscopes with optogenetic tools to eventually achieve optical control of small cell groups or even of individual neurons in freely behaving animals.

**REFERENCES**

Two-Photon Microscopy in Freely Moving Animals


