Two-Photon Calcium Imaging in the Macaque Monkey

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Introduction

The nonhuman primate has proven to be an invaluable animal model for neuroscience research. Nonhuman primates such as the Rhesus macaque have a complex brain that is organized similarly to the human brain (Sereno and Tootell, 2005). In addition, monkeys can be trained on the complex tasks necessary to study the neural underpinnings of such complicated processes as perception, cognition, and decision making. Because these are the types of behaviors affected by mental disorders, the monkey will remain a crucial animal model for investigating brain function. To date, a wealth of research has established which areas in the monkey brain are likely to be involved in a particular task, as well as the tuning properties of neurons in these areas. Going forward, we will have to build on this knowledge to pursue questions such as: How are the neural circuits within and between these areas organized? What is the function of their individual elements, and how do they interact? Only by solving these puzzles will we be able to address what goes awry in mental disorders.

Investigating these issues is complicated by the fact that neural circuits are formed with a high degree of specificity and on very fine scales. Thus, we will need tools that operate at a resolution appropriate to appreciate the fine-scale organization of neural circuits. Two-photon microscopy (Goeppert-Mayer, 1931; Denk et al., 1990) fills an important technological gap in this respect. At its coarsest resolution, two-photon calcium imaging allows the simultaneous recording of activity of tens to hundreds of neighboring neurons while maintaining single-neuron resolution. At the finest resolution, responses of individual spines on a single dendrite can be investigated. (General reviews of two-photon microscopy can be found in So et al., 2000; Helmchen and Denk, 2005; Svoboda and Yasuda, 2006; and Kerr and Denk, 2008.) No other technique currently is able to operate at these resolutions *in vivo*.

Two-photon calcium imaging is by now routinely used in anesthetized and even awake mice. In contrast, only few studies have applied two-photon imaging to investigate brain functions in larger animals, in particular, monkeys (Nauhaus et al., 2012a; Ikezoe et al., 2013). This chapter will describe the necessary steps to perform two-photon microscopy in monkeys, including some of the commonly encountered complications, and discuss how to interpret the data. Most of the text will focus on an established imaging paradigm using an injectable calcium dye, Oregon Green BAPTA-1AM (OGB), in acute experiments in anesthetized animals. Genetically encoded calcium indicators (GECIs) and experiments in awake animals are discussed in the last section. This text does not include more general details on how to perform acute experiments in anesthetized monkeys, such as the choice of anesthesia, paralysis, or vital-sign monitoring. For more information on these issues, refer, for example, to Nauhaus et al. (2012a).

Setup

Choice of two-photon microscope

A large number of two-photon microscopes are commercially available, and are used to perform calcium imaging in mice. However, the size and curvature of the monkey brain place specific demands on the two-photon microscope. In the monkey, most brain areas are located laterally or posteriorly enough that their surface is at a significant angle relative to the horizontal plane in which imaging is typically performed. In some cases, it may be feasible to rotate the animal's head to bring the brain area of interest into the horizontal plane. Often, this is not possible, either because the angle difference is too large (e.g., posterior V1 is tilted approximately 50° relative to the horizontal) or because rotating the animal's head would interfere with stimulus presentation (e.g., rotating the head downward poses problems for visual stimulus presentation). In these cases, the only solution is to rotate the microscope and to image in a plane tilted relative to the horizontal plane. Increasingly more commercial two-photon microscopes offer this capability. Another important consideration in selecting a two-photon microscope for imaging in larger animals, such as the monkey, is the amount of space available under the objective. This space has to be large enough to fit the animal's head, or (as in our typical setup) the animal and a stereotaxic frame.

Specific imaging equipment

In addition to the usual equipment necessary for acute experiments in anesthetized animals, we use two specially designed pieces of hardware: an imaging well and a simplified stereotaxic frame. The imaging well serves the following purpose: The objectives typically used for two-photon microscopy are water-immersion objectives, and therefore require water or artificial CSF (ACSF) between imaging region and objective. This can be relatively easily achieved when imaging in the horizontal plane. However, once the microscope has been rotated and imaging is performed at an angle, a specially designed imaging well becomes necessary to hold water between imaging region and objective. Our imaging well consists of a large metal plate with a hole in the center (Fig. 1A). This metal plate is attached to the skull using dental cement. During imaging, a plastic
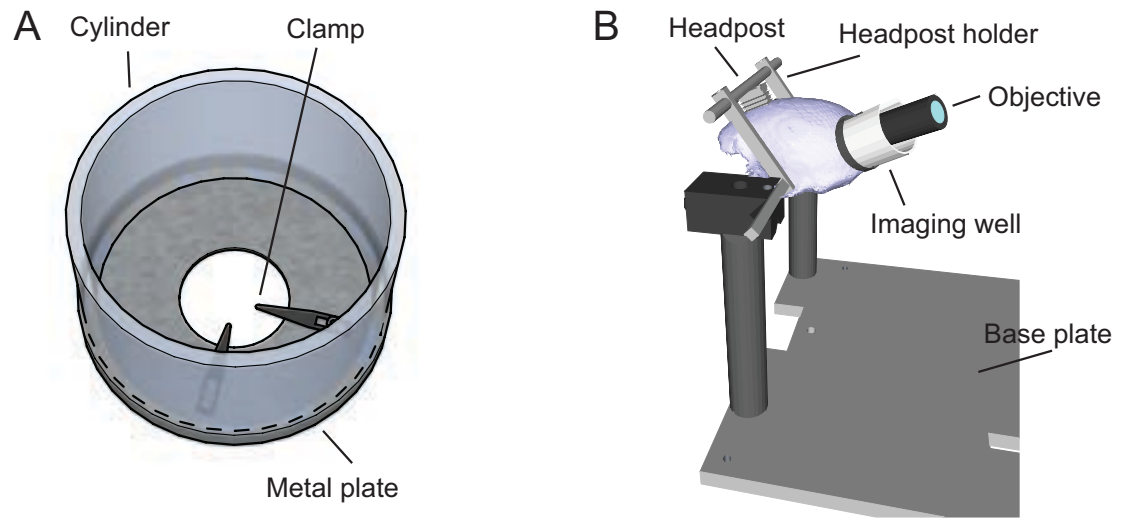
Figure 1. Imaging equipment. *A,* Imaging well, showing the metal plate cemented to the animal's head, clamps used to stabilize the brain, and the cylinder used for water immersion of the objective. *B,* Imaging stereotax. The drawing illustrates how stereotax, head holder, and imaging well are configured during an imaging experiment.

cylinder is fitted into the well, and the interface of cylinder and well is sealed with bone wax to prevent water leakage. The cylinder can then be filled with enough ACSF to cover the imaging region. The cylinder can be removed for easy access to the skull for surgical and other procedures. We also use the imaging well to stabilize the brain during imaging (see Minimizing Movement Artifacts, below).

The second specialized piece of equipment in the imaging setup is a simplified version of a stereotaxic frame (Fig. 1B). The stereotaxic frame is designed to rigidly hold the animal's head while being small enough not to interfere with the two-photon microscope. It consists of a metal base plate, large enough to support the animal's body, and two posts at one end. These posts initially hold the animal's head via attachments for ear bars, eye bars, and a palate bar. This setup provides sufficient stability to perform initial surgeries. To further increase stability during imaging, we cement a headpost to the skull at the beginning of each acute experiment. For the remainder of the experiment, the head is then rigidly held in place by connecting the headpost to the steteotax posts.

Dye Loading
Multiple calcium dyes are available, of which OGB has been most commonly used. OGB has been the calcium dye of choice because of a number of advantages over other dyes: Labeling neurons with OGB is feasible via pressure injection into the brain (Stosiek et al., 2003). Furthermore, because of its acetoxylmethyl (AM) ester group, OGB is not

fluorescent until it is transferred into cells and the AM group is cleaved, which helps to reduce background fluorescence (Tsien, 1981). OGB is stable in the cells for a relatively long period of time (in some cases, up to 12 h) and has a signal-to-noise ratio (SNR) sufficient to detect even single angle potentials (Kerr et al., 2005; Chen et al., 2013). The basic protocol for OGB injections has been described in depth previously (Stosiek et al., 2003; Garaschuk et al., 2006) and widely applies to monkeys. Listed below are the most important steps of OGB injections in the monkey:

(1) *OGB and SR101 solution:* OGB labels both neurons and astrocytes. Sulforhodamine 101 (SR101), a red fluorescent dye that selectively labels astrocytes (Nimmerjahn et al., 2004), is therefore often added to the OGB injection solution. For the monkey, our typical injection solution consists of 2 mM OGB with 10% DMSO, 2% Pluronic F-127, and 25% SR101 (all from Life Technologies, Grand Island, NY).

(2) *Craniotomy:* The hole in the imaging well allows access to a large skull region. We usually make small craniotomies (~5 × 5 mm) within its perimeter. Each craniotomy is used once to attempt dye loading. If the dye loading is unsuccessful, or after completion of data collection, the next craniotomy is made. Craniotomies are kept relatively small to help stabilize the brain (see Minimizing Movement Artifacts, below). It is important to thin the skull around the perimeter of each craniotomy to be
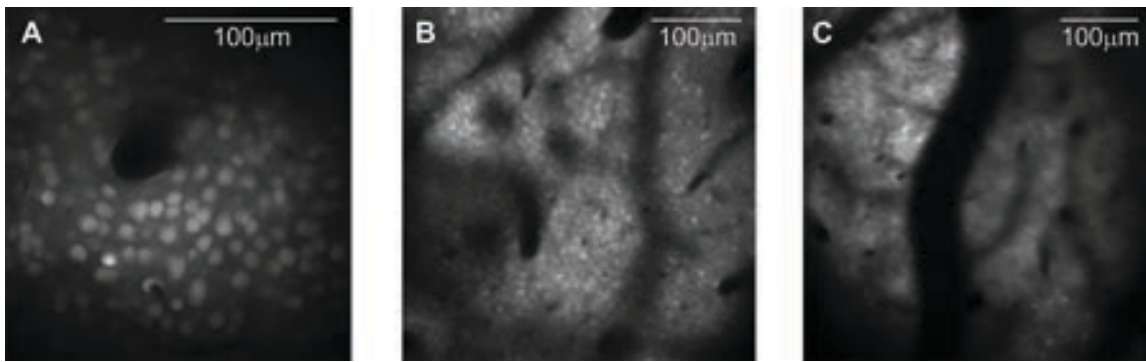
Figure 2. Example OGB loading in monkey V1. *A,* Fine-scale imaging using a 40× objective, showing a large number of labeled neurons. *B,* Large-scale imaging using a 16× objective. *C,* Failure of OGB loading. Notice the uniform label in *C* versus the visible cell bodies in *B*. Data collected as part of Nauhaus et al. (2012a). Scale bars, *A–C,* 100 μm.

able to place the objective close enough (within its working distance [WD]) from the brain.

(3) *Durotomy:* Monkey dura is opaque and needs to be removed before imaging. Usually, one durotomy of approximately 3 × 3 mm is made per craniotomy, trying to avoid major blood vessels in the dura. Care needs to be taken to avoid bleeding while cutting through the last layer of dura: Blood touching the brain seems to interfere with dye loading.

(4) *Dye injection:* Dyes are pressure-injected into the brain using a Picospritzer (Parker Hannifin, Hollis, NH) and glass pipettes with a relatively blunt taper and a tip size of ~5 μm. We have found that the blunt taper helps with preventing backflow of the dye along the track of the pipette and onto the surface of the brain. Tip sizes smaller than ~4 μm tend to clog, whereas larger tip sizes tend to lead to backflow. Pipettes are inserted into the brain at an angle of 35°–45° relative to the surface of the brain, and the dye is injected at a depth of ~300–400 μm. The progress of each injection is monitored by observing the flow of SR101 into the brain. This is a crucial step of the procedure, as it ensures that (a) the pipette is not clogged, and (b) dye is not flowing back onto the surface of the brain. The latter needs to be avoided, as staining the surface of the brain with SR101 drastically degrades imaging quality. Unlike OGB, SR101 is fluorescent before entering a cell. Monitoring the dye spread during injection is thus feasible by observing SR101 fluorescence using the two-photon microscope. Alternatively, most two-photon microscopes have an epifluorescence path that will allow observation of the dye using low power, dry objectives. Multiple injections can be made within the same durotomy, as long as they are done within a short amount of time (~1 h) after each other and after making the durotomy. Reloading OGB into a previously labeled, "old" durotomy appears to not be feasible.

The biggest challenge with OGB loading in monkeys is the observation that, in many cases, OGB is taken up not only by neurons, but also by the neuropil that makes up the extracellular space. This results in uniform OGB fluorescence throughout the entire imaging region (Fig. 2). As a consequence, neurons can no longer be discriminated from the surrounding neuropil. It appears that the likelihood of excessive OGB label in the neuropil strongly increases with animal age, making younger animals better suited for two-photon microscopy. Similar observations have been made in mice (Eichhoff et al., 2008). The reason for this increased label in the neuropil is unclear, but one possible explanation is an increase in acetylesterase activity in the neuropil with age. It remains to be seen whether coinjection of esterase blockers such as eserine, which has been used to improve loading of OGB in the retina (Kurth-Nelson et al., 2009; Srienc et al., 2012), could prevent the neuropil loading of OGB without interfering with brain function.

Minimizing Movement Artifacts

Besides complications with OGB loading, the largest challenge in performing two-photon microscopy in monkeys is posed by artifacts caused by brain movements. In the anesthetized monkey, the largest sources of movement are breathing and pulse. Breathing usually causes vertical displacements of the brain, whereas the pulse tends to be correlated with horizontal displacements. Of these two artifacts, the vertical movements are particularly disruptive for imaging because they cause cells to move in and out of the imaging plane. Because data cannot be collected from a cell outside the imaging plane, vertical artifacts

cannot be corrected offline. It is thus necessary to eliminate at least the vertical brain movements as much as possible before collecting data.

In our experience, vertical brain movements can be minimized using the following approach: First, the brain region to be imaged is covered with a thin layer of 1.5% agarose (Type-III agarose, Sigma-Aldrich) in ACSF, followed by a small coverslip. It is important to avoid using too much agarose, as it interferes with imaging. The coverslip can be placed either on top of the bone, or between bone and dura. Second, the coverslip is held in place by small clamps attached to the imaging well (Fig. 1), which should be set up to provide gentle pressure on the brain. Care needs to be taken that neither coverslip nor clamps block the objective's access to the brain.

The goal of the mechanical stabilization is to eliminate vertical movements. Remaining horizontal movements can then be corrected offline after data collection. A successful strategy for offline motion correction has been to use a two-stage process described in detail in Nauhaus et al. (2012a): First, slow drifts are corrected by computing the average image in every trial and aligning these average images to the average of the first trial using two-dimensional (2D) cross-correlation. Second, fast movements are corrected using an optic flow-based method. Other approaches are also possible and have been described in the literature (Greenberg and Kerr, 2009).

Offline motion correction is feasible only if data are acquired in raster scan format. In this acquisition mode, the laser beam is systematically swept over a 2D region. As a consequence, the collected data consist of a series of frames that show cell bodies and other structures, and horizontal displacements can be corrected by spatial transformations of the collected frames. Other acquisition modes increase acquisition speed by no longer sampling the entire 2D region. In random access scanning, for example, the laser beam "jumps" from one preselected point in the imaging region to the next (Reddy et al., 2008; Grewe et al., 2010). Because no data are recorded outside of the preselected points, data collected in this way cannot be corrected offline. Thus, these acquisition modes place more stringent requirements on brain stability.

Data Analysis

The acquisition system of the two-photon microscope records raw fluorescence time courses $F(t)$ for every pixel. Usually, raw fluorescence time courses are first transformed into $\Delta F(t) = (F(t) - F_0)/F_0$, where F_0 is the average fluorescence during some baseline period, e.g., a time period preceding stimulus onset. In a second step, $\Delta F(t)$ is then averaged across all pixels falling within a neuron. Any further analyses are then based on the $\Delta F(t)$ time courses for every cell. In these analyses, it is important to take into account how the measured changes in OGB fluorescence relate to the underlying changes in neural firing rates. The spike-to-fluorescence transfer function of OGB can be modeled as a linear–nonlinear cascade (Nauhaus et al., 2012b; Fig. 4): Each spike causes a transient influx in calcium, whose time course is characterized by a sharp rise followed by a slow exponential decay with a time constant of ~0.5–1 s (Helmchen et al., 1996; Kerr et al., 2005; Sasaki et al., 2008; Smith and Häusser, 2010; Chen et al., 2013). This signal is then transformed into the observed fluorescence signal via a saturating nonlinearity (Yasuda et al., 2004; Yaksi and Friedrich, 2006; Hendel et al., 2008).

The first important consequence of the spike-to-fluorescence transfer function of OGB is the temporal summation of action potentials caused by the slow exponential decay of the linear stage. As long as firing rates are low enough, individual spikes lead to clearly visible calcium transients, and spike times can be identified. However, from firing rates >5 Hz on, the calcium transients of individual spikes can no longer be resolved because of the slow decay kinetics of the OGB signal (Sasaki et al., 2008). Thus, for firing rates >5 Hz, spike times cannot be computed from the OGB data. In most areas of the monkey brain, firing rates will be well beyond this rate even under anesthesia.

The second important consequence of the spike-to-fluorescence transfer function of OGB is the distortion in signal caused by the saturating nonlinearity. We have demonstrated that this saturation can have a significant impact on measurements of tuning functions by clipping the fluorescence signal at higher firing rates (Nauhaus et al., 2012b). Distortions caused by the saturating nonlinearity were observed not only in ferret visual cortex, which has neurons with high firing rates, but also in mouse visual cortex, where firing rates are significantly lower. It is critical to keep these distortions in mind when interpreting OGB data. While some measurements (e.g., the tuning curve maximum) may not be affected by a monotonic nonlinearity, other measurements (e.g., tuning curve width) can be strongly distorted. As a consequence, differences among neuron groups or conditions with different firing rates could be artificially enhanced or diminished.

Future Developments

So far, we have focused on how to perform two-photon calcium imaging in anesthetized animals using an injectable calcium dye. Although extremely valuable data can be collected under these conditions, it will be important for future research to be able to perform two-photon experiments in awake monkeys performing a task. A number of technical hurdles need to be overcome to make these experiments feasible. First, injectable calcium dyes will likely no longer be useful in an awake animal, in which repeated measurements will need to be carried out. As described earlier, injectable dyes can be used only in young animals, once per durotomy, and only in "fresh" durotomies. Instead, GECIs appear to be a better choice because they can be stably expressed in cells. The most recent generation of these indicators (GCaMP6s, 6m, and 6f) show great promise for future experiments because of their relatively fast response kinetics and good SNR (Chen et al., 2013).

GECIs require the injection of a virus into the brain to be expressed. Thus, going forward, we will need to identify which virus (e.g., different serotypes of AAV, lentivirus) will lead to sufficient expression in terms of spatial spread, expression density, as well as expression level within each cell. In addition, an appropriate promoter will need to be chosen. Another issue with respect to the use of GECIs is their impact on cell health. All GECIs are calcium buffers. Because calcium is important for many "housekeeping" functions in a cell (Berridge et al., 2000, 2003), excessive calcium buffering leads to cell death. How long cells remain viable after virus infection will depend on the particular indicator used and the rate at which it is expressed, which will in part depend on the virus chosen for expression. In the mouse, stable expression of GCaMP6 over several months has been demonstrated (Chen et al., 2013). Whether similar durations are possible in the monkey will need to be determined.

The switch from OGB to GECIs is one important issue that needs to be solved to be able to perform two-photon microscopy in awake monkeys. Other hurdles are the development of an imaging well, and the development of strategies to deal with brain movement. The largest engineering challenge with respect to the imaging well is the fact that most two-photon objectives are very large (diameters ≤3 cm) and need to be placed quite close to the brain (typical WD ~3 mm). Thus, large implants are required. In addition, because monkey dura is opaque, it will need to be removed and replaced with an artificial dura. So far, one two-photon implant has been developed (Stettler et al., 2006); implant designs similar to the ones used for optical imaging may be another possibility (Roe, 2007). Because of the necessary size of the well, and the removal of the dura, motion artifacts will be further exacerbated beyond the inevitable increase caused by using an awake animal. Thus, imaging wells likely will have to include provisions to stabilize the brain. Additionally, it may be necessary to develop ways to track brain movements online and to move the microscope in synchrony with the brain.

To summarize, two-photon microscopy is a promising addition to the toolkit available for research in the macaque monkey. Because of its unique capabilities—high resolution and high sampling density—it will open important novel avenues to study local circuit function. Two-photon calcium imaging in the anesthetized monkey is feasible today, but experiments in the awake monkey will require further technical developments.

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