Optogenetics through windows on the brain in the nonhuman primate


* These authors contributed equally to this work.

1 Vision Center Laboratory. Salk Institute for Biological Studies, La Jolla, CA, USA
2 Department of Psychology, Vanderbilt University, Nashville, TN 37203, USA
3 Systems Neurobiology Laboratory. Salk Institute for Biological Studies, La Jolla, CA, USA
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Running Title: Optogenetics through windows on the brain of primates

Corresponding author:
Anna W. Roe, Professor
Department of Psychology
Vanderbilt University
301 Wilson Hall
111 21st Avenue South
Nashville, TN 37240
(615) 343-0901 phone, (615) 343-8449 fax
email: anna.roe@vanderbilt.edu
Abstract

Optogenetics combines optics and genetics to control neuronal activity with cell-type specificity and millisecond temporal precision. Its use in model organisms such as rodents, drosophila, and C. Elegans is now well established. However, application of this technology in non-human primates (NHP) has been slow to develop. One key challenge has been the delivery of viruses and light to the brain through the thick dura mater of NHPs, which can only be penetrated with large-diameter devices that damage the brain. The opacity of the NHP dura prevents visualization of the underlying cortex, limiting the spatial precision of virus injections, electrophysiological recordings and photostimulation. Here we describe a new optogenetics approach in which the native dura is replaced with an optically-transparent artificial dura. This artificial dura can be penetrated with fine glass micropipettes, enabling precisely targeted injections of virus into brain tissue with minimal damage to cortex. The expression of optogenetic agents can be monitored visually over time. Most critically, this optical window permits targeted, non-invasive photostimulation and concomitant measurements of neuronal activity via intrinsic signal imaging and electrophysiological recordings. These improvements over current methods should enable the widespread use of optogenetic tools in NHP research, a key step toward the development of therapies for neuropsychiatric and neurological diseases in humans.

Keywords: primate optogenetics, artificial dura, optical imaging, in-vivo epifluorescence, electrophysiology.
INTRODUCTION

To understand the neural mechanisms that mediate perception and cognition in the human brain, we need to investigate species whose brains are, to the extent possible, homologous to those of humans, and in animals capable of performing tasks that allow us to test cognitive functions that fail in these disorders. These considerations made nonhuman primates the model of choice for studying such neural mechanisms. Moreover, an understanding the neural mechanisms underlying any sensory-motor or cognitive function requires not only recording electrophysiological activity during behavior, but manipulating specific neural circuits and measuring the consequences in awake, behaving animals.

The tools currently available to test causal relationships between neuronal activity and behavior in monkeys have major limitations. Cooling and pharmacological manipulations have poor spatial and temporal resolution; electrical stimulation, has poor spatial resolution and creates electrical artifacts that interfere with neurophysiological recording. Moreover, none of these techniques have the capacity to target differentially specific neuronal subtypes.

Optogenetics uses molecular-recombinant techniques to make specific types of neurons responsive to light (Fenno et al. 2011), and it has excellent spatial and temporal resolution. Opsins expressed by neurons can be used to increase (Boyden et al. 2005) or silence (Chow et al. 2010) neuronal activity with millisecond temporal precision (Boyden et al. 2005; Han and Boyden 2007). For example, channelrhodopsin-
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2 (ChR2) and its variants (e.g. C1V1, Cheta, ReaCh) are light-sensitive cation channels used to depolarize neurons. Halorhodopsin (a light-activated chloride pump) and archaerhodopsin (Arch) (a light-activated proton pump) are used to hyperpolarize neurons (Boyden et al. 2005; Han and Boyden 2007). Different combinations of viral vectors (e.g. adeno-associated virus, lentivirus pseudotyped with vesicular-stomatitis-virus glycoprotein) and gene promoters (e.g. alpha-calcium/calmodulin-dependent protein kinase II) favor opsin expression by either excitatory or inhibitory neurons (Dittgen et al. 2004; Han et al. 2009; Nathanson et al. 2009), and the targeting capacity of this approach will likely improve as new promoters are discovered. Optogenetics has been successfully used in rodents to study Parkinson’s disease (Gradinaru et al. 2009; Kravitz et al. 2010), fear and anxiety (Ciocchi et al. 2010; Tye et al. 2011), and behavior (Huber et al. 2008) (for review see (Bernstein and Boyden 2011)). However, in monkeys, the existing methods of virus and light delivery damage the delicate circuitry under study, limiting the insights that can be gained from primate optogenetics.

Here, we describe an approach in which a soft, transparent window replaces a section of the dura mater over the monkey brain. This optical window gives visual access to the underlying tissue for injection of viruses without cortical damage, permits in vivo tracking of opsin expression via epifluorescence, and allows for highly precise targeting of recording electrodes and light delivery to sites of opsin expression. Moreover, the artificial dura allows optogenetics to be combined with intrinsic imaging techniques. The approach thus provides a means of probing functional domains of the cortical map, measuring the effects of this stimulation across the cortical surface, and relating these changes to perception and behavior. Taken together, these capacities
hold considerable promise for enhancing our understanding of cortical computation in the behaving primate.

Glossary
AD: Artificial dura
ArchT: Archaerhodopsin-T inhibitory opsin
ChR2: Channelrhodopsin-2 excitatory opsin
EYFP: Enhanced yellow-fluorescent protein
GFP: Green-fluorescent protein
mCherry: A red-fluorescent protein
OI: Optical imaging
VSVg: Vesicular-stomatitis-virus glycoprotein
Lenti-CaMKIIα-ArchT-GFP, Lenti-ArchT-GFP or Lenti-ArchT: VSVg-coated lentivirus carrying a CaMKIIα promoter and genes for ArchT fused to the fluorophore GFP.
Lenti-CaMKIIα-ChR2-EYFP: VSVg-coated lentivirus carrying a CaMKIIα promoter and genes for ChR2 fused to the fluorophore EYFP
Lenti-CaMKIIα-ChR2-mCherry or Lenti-ChR2: VSVg-coated lentivirus carrying a CaMKIIα promoter and genes for ChR2 fused to the fluorophore mCherry.

MATERIALS AND METHODS

Surgical Procedures
Head posts, chambers and artificial dura (AD) were implanted using strictly aseptic surgical procedures. Monkeys were sedated with ketamine (10 mg/kg), anesthetized with isoflurane and artificially ventilated. They were placed on a heating blanket, secured in a stereotaxic frame, and monitored continuously for end-tidal CO₂, heart rate, temperature, and respiratory function. All surgical procedures and experimental protocols were conducted in accordance with NIH guidelines and approved by the Animal Care and Use Committees of the Salk Institute and Vanderbilt University.

The optical window

Optogenetic recording chamber. Chambers and chamber caps (Fig 1A) were constructed from either FDA approved Nylon (Chen et al. 2002) or titanium. All chambers and caps were MRI-compatible. Chambers were implanted in a craniotomy (Fig 1A) positioned over the cortical region of interest (primary visual cortex, in the three monkeys reported in this study). Figure 1C shows the titanium chamber. The three threaded holes serve to mount a removable stage that holds micro-positioners during recording, to hold the electrode and the optical fiber in place over the cortex. All chambers had inner threading to fasten a screw cap to seal the chamber between recording sessions.

The artificial dura. The key innovation of our approach is a durotomy into which a silicone artificial dura (AD) is inserted. The custom-made AD is molded from silicon, in the shape of a cylinder attached to a disk (Fig. 1B) (Chen et al. 2002; Shtoyerman et al. 2000). The flange – the part of the disk that extends beyond the cylinder like the brim of a hat, is inserted between the native dura and the cortex (Fig. 1A).
The placement of the AD flange beneath the native dura ensures that the durotomy cannot close beneath the artificial dura; the cylindrical wall, inside the durotomy, prevents the native dura from growing into the chamber and over the window. The base of the hat within the cylindrical wall protects the cortex, serves as an optical window (Fig. 1C), and is penetrable by fine glass micropipettes, microelectrodes, and light.

Chamber and dura implantation. The chamber and artificial dura are implanted using approaches developed for intrinsic imaging studies. Briefly, a trephine is used to create a circular craniotomy over the cortical area under study (diameter ~ 2 cm). The exposed dura is cut and resected. The AD is then placed in the durotomy so that the flange lies under the edge of the native dura. The chamber is then secured to the bone around the AD (Figure 1A). Two or more screws are placed in the skull, around the chamber, to serve as anchors for the cement. A thin layer of bone cement is applied between the bone and the rim of the chamber, and more cement is added, extending over the screws, to secure the chamber in position (Fig. 1A,C). Chambers were made as low profile as possible to facilitate illumination during optical-imaging experiments and AD replacement. Chamber diameters were chosen based on the extent of cortex to be studied. The chambers used in this study had inner diameters between 17 and 21 mm; they were implanted near the occipital pole, over primary visual cortex (V1), and covered different proportions of V2 and V4 in each monkey. We have successfully implanted long-term optical-chambers in rodents, squirrel monkeys, and macaque monkeys for use in optical-imaging experiments (e.g. (Chen et al. 2005; Lu et al. 2010; Tanigawa et al. 2010)). The present work reports the use of our AD in macaque monkeys for optogenetic experiments.
Chamber maintenance. Proper maintenance of the AD is essential to avoid infection and to prevent excessive tissue growth between the wall of the AD and the chamber. The chamber should be cleaned at least twice each week. The chamber is only opened under aseptic conditions. First, the exterior of the chamber is cleaned and carefully wiped with aseptic solutions such as Nolvasan and Betadyne. The cap is then removed and any fluids within the chamber absorbed with sterile gauze. The top edge of the chamber is wiped with hydrogen peroxide and dried. The interior of the chamber is rinsed with sterile saline, and saline is immediately absorbed with gauze, so as not to permit fluid continuity between the interior and the exterior of the chamber. There is typically some tissue growth, from the edge of the craniotomy, which invades the space between the chamber wall and the silicone cylinder. This gelatinous tissue is removed by wiping it with cotton-tipped swabs. Removal of this tissue must be done regularly (at least once per week) to prevent further maturation into membranous tissue. Prior to closing the chamber, a small piece of gauze with a drop of antibiotic (e.g. amikacin or gentamycin) is placed within the chamber, over the AD window, and a silicon plug placed over the gauze, to fill the empty space between the AD and the cap, and prevent the AD from popping out from the durotomy. Cephazolin should not be used, as it can induce seizures. A sterile chamber cap is then put in place and the space between the chamber and the cap is sealed with melted bone wax to prevent entry of bacteria.

Viral preparation and injections

In the present study two different types of viruses were used (VSVg-pseudotyped lentivirus, and AAV5), carrying constructs that encoded one of three opsins (ChR2,
ArchT, or C1V1), fused to one of three fluorescent reporter proteins (EYFP, GFP, or mCherry). In all cases, expression was driven by the promoter CaMKIIα (Dittgen et al. 2004). The titers of all lentiviruses used were between $0.4 \times 10^{10}$ and $3 \times 10^{10}$ TU/ml. The titer of the AAV5 virus was $2.0 \times 10^{12}$ TU/ml. Table 1 lists the viral constructs used, their source, and the tests performed with each virus. CaMKIIα-ChR2-mCherry, CaMKIIα-ArchT-GFP, and CaMKIIα-ChR2-EYFP lentiviruses were produced by the Salk Institute viral vector core using constructs provided by Dr. Karl Deisseroth (Chr2-mCherry and ChR2-EYFP) or Dr. Ed Boyden (ArchT-GFP). The AAV5-CaMKIIα-C1V1-EYFP virus was a gift from Dr. Deisseroth. The CaMKIIα-C1V1-EYFP lentivirus was produced by Ali Cetin using a construct provided by Dr. Deisseroth. All lentiviruses were pseudotyped with VSV glycoprotein.

Viral injections were made under isoflurane anesthesia using glass pipettes (WPI 1B120F-4) with tips pulled to a diameter of ~30-40 μm. A stereotactic micromanipulator was used to insert pipettes into the cortex, either through the exposed pia mater during the initial implant surgery or, subsequently, through the AD. Care was taken to avoid surface vessels by viewing through a microscope during pipette insertion. We used slightly different parameters for the different injections and monkeys. For monkeys M and C, clusters of 2-5 injections were made. In each pipette penetration, we injected about 0.5 μl of virus, slowly, over ~10-20 minutes at each of three depths (1.2, 0.8 and 0.4 mm) using a picospritzer (WPI, PV820) (8-20ms pulses of ~20-25 psi delivered at ~0.2 – 0.6 Hz). An entire site was completed in 45 minutes. In monkey A, we injected every 200 μm in each penetration, spanning the entire thickness of cortex (~2mm), spending 1-2 minutes per depth. For the AAV5-C1V1 site we made two penetrations,
for the Lenti-C1V1 site, one penetration. In all cases and monkeys, we injected
approximately 0.5 µl per depth.

Histology

Monkey M was given an overdose of anesthetic (100mg/kg pentobarbital) and
perfused transcardially with fixative (4% paraformaldehyde). Prior to sectioning, the
ability to detect fluorescence from opsin-and-fluorophore expressing neurons through
the brain surface was assessed. We used commercial goggles that incorporate
wavelength-specific illumination with the appropriate emission filters for the fluorescent
reporter proteins used in this study (BLS Ltd., Budapest, Hungary). For microscopy,
parasagittal sections were cut at 50-µm thickness, using a freezing microtome. A series
of every 8th section was mounted and cover-slipped for analysis of native fluorescence
using an epifluorescence microscope and digital image capture software.

Optical Imaging

Optical imaging was conducted in multiple sessions in anesthetized (pentothal, 1-2
mg/kg/hr) and paralyzed (norcuron, 100ug/kg/hr) monkey M. Images of cortical-
reflectance change were acquired, using 630-nm illumination, by an IMAGER 3001
system (Optical Imaging, Germantown, NY). For detailed imaging methods see (Chen
et al. 2008; Lu et al. 2010; Tanigawa et al. 2010). Image acquisition (4 frames per
second) included 2 frames before visual stimulus as baseline and 14 frames during
stimulus presentation. The monkey’s eyes were fitted with contact lenses to focus on a
computer monitor. Visual stimuli were full-field achromatic gratings, presented to one
eye or the other, using electromechanical shutters placed in front of each eye. All conditions were pseudo-randomly interleaved and repeated at least 30 times. Optogenetic experiments included 4 frames before photostimulation as baseline, 3 frames during photostimulation, and 13 frames to assess the cortical response.

Brain-surface Fluorescence

Two commercial "goggles" (BLS Ltd., Budapest, Hungary) were used to monitor epifluorescence in-vivo on the brain surface, associated with virus injection sites. One pair of "goggles" had a blue excitation light and GFP/YFP emission filters. The other pair of "goggles", was used to check for non-specific fluorescence; it had a green excitation light and red-fluorescent-protein (RFP) emission filters.

Photostimulation devices

We used two different illumination systems. To activate ChR2 in monkeys A and M, we used a house-made blue LED (Osram LB W5AP-JKY-35) coupled directly to a 1- or 2-mm diameter optical fiber. We used a green laser as a control for ChR2 activation specificity in the optical imaging experiments (532 nm, 100 mW) and to photostimulate C1V1 in monkey A.

Electrophysiology

We used thin epoxy-coated tungsten electrodes (75-µm shank diameter; FHC) or glass-coated tungsten electrodes (Alpha Omega; 250-µm shank diameter). Stimulus presentation and data collection were controlled by custom-written programs.
Electrophysiological signals were amplified and stored for off-line analysis. We machined special adapters for the optical chambers to position electrodes and optical fibers.

RESULTS

Health and stability of cortex over time

Three rhesus macaques were used in this study. ADs and chambers were implanted over the primary visual cortex (V1) of each monkey. Optical-imaging data were collected in one anesthetized monkey (M) and electrophysiological data were collected in two alert monkeys (C and A). In all three monkeys, the optical window provided a stable long-term preparation: The AD remained optically clear, vessels were identifiable, and cortex was healthy for the duration of this study (a year). Figures 2A and B show the AD chamber of monkey A, one week and six months after implantation, respectively. With regular maintenance, the chamber remained infection-free. Over a span of months, a layer of neomembrane appeared beneath the window and was surgically removed under anesthesia during a procedure performed between photos A and B. As seen in Fig 2B, the cortex and vessels under the AD were healthy six months after the initial AD implant. In all our monkeys, some vessels changed size, but its location remained stable and it was easy to identify matching vessels weeks and months apart. New fine vessels appeared within the neomembrane that slowly grows above the cortex.
(compare, for example, the chamber top-right quadrant in Figs 2A and B); this neovasculature was readily distinguished from cortical vessels as it is at a different plane of focus when viewed under a microscope. These vessels do not interfere with optical imaging or photo-electrophysiological experiments.

As we have previously shown (Lu et al. 2010; Tanigawa et al. 2010), stable optical images of functional organization can be obtained over time. To illustrate, Fig. 2C and D show two ocular dominance maps in V1 of monkey M taken over 3 months apart. These maps reveal columns of left-eye (dark stripes) vs. right eye (clear stripes) responses. The ocular-dominance columns exhibit the same general size and arrangement three months apart (compare, in particular, the region within white boxes in C and D). Note that several virus injections were made over this 3-month period (across different sessions), demonstrating that neither the viral injections nor the expression of the optogenetic constructs affected the health of the cortex or the integrity of functional domains.

In summary, the stability of functional maps over time suggests that our approach is appropriate for long-term studies compatible with repeated imaging, electrophysiology, and optogenetic manipulations.

Window for targeted viral injections

The AD allows for precisely-targeted and minimally-invasive injections of viral vectors using fine pulled-glass micropipettes. Accurate targeting is enabled by reference to anatomical landmarks such as cortical sulci, microvasculature patterns, and functional maps obtained with optical imaging. Viral injections can be targeted, for
example, into orientation domains or ocular-dominance columns mapped with optical imaging.

Figure 3A shows a virus-filled micropipette (tip diameter ~40 µm) during a viral-vector injection in monkey M, performed under anesthesia to minimize movement. The pipette was inserted slowly via a micromanipulator. Brain pulsations were reduced with a rigid plastic stabilizer, placed against the AD. A hole in the stablizer (circle around pipette tip in Fig. 3A) allowed passage of a pipette through the AD. We paid attention to avoid surface vessels; this is critical as bleeding increases light absorption and scattering. The small size of the pipette tip, cortex stabilization, and vessel avoidance prevent tissue damage and markedly increase the likelihood of successful long-term optogenetic studies.

We injected viral vectors through the AD of monkey M in three different sessions. As lentivirus has limited spread in the cortex (Dittgen et al. 2004; Nathanson et al. 2009), injections were made in clusters of 2 to 5 penetrations, ~ 300 µm apart, to create large and dense regions of expression (Fig. 3B). For each penetration, virus was injected at three depths: 1.2, 0.8 and 0.4 mm from the brain surface. We used low-pressure injections to minimize tissue damage and prevent virus refluxing back up the injection site. Our ability to visualize vessel patterns allowed us to accurately target the injections in these clusters across sessions (for example, cluster 1 was produced in three sessions, each session one to two weeks apart). Each red dot indicates a pipette penetration that injected lentivirus carrying the CaMKIIα promoter and genes for ChR2 fused to the fluorophore mCherry (Lenti-CaMKIIα-ChR2-mCherry). Green dots show the injection sites for a similar lentivirus that carried genes for ArchT fused to the
fluorophore GFP (Lenti-CaMKIIα-ArchT-GFP. These viruses were injected into specific functionally identified ocular-dominance columns revealed by optical imaging (Fig. 3C): Lenti-ChR2 injections (red dots) were made in right-eye columns (white zones); Lenti-ArchT injections (green dots) were targeted into left-eye columns (gray zones). The cortex remained in excellent health following these multiple injections and recording sessions.

Detection of viral expression

One difficulty in NHP optogenetics is the inability to visualize expression in vivo through the native dura. Thus, successful expression is usually confirmed by epifluorescence microscopy of post-mortem tissue. The artificial dura approach provides a non-invasive means of assessing protein expression.

Post-mortem epifluorescence: monkey M

We first determined whether we could visualize optogenetic fluorescently-tagged opsins through the surface of a fixed brain. Fig. 4A shows the occipital pole of monkey M's brain, fixed for histology (paraformaldehyde) after having allowed sufficient time for the opsins to express. Figure 4B shows a photo of the same brain region taken through commercial YFP/GFP "miner-lamp goggles" (blue-excitation/green-emission filters). Two fluorescent spots are evident in the picture (blue arrows), at locations corresponding to Lenti-ArchT-GFP clusters 1 and 2 in Fig 3B. Histological analysis of cut sections, using epifluorescence microscopy, revealed neurons expressing GFP at these two cortical locations (not shown).
In-vivo epifluorescence: Monkeys C and A

Figure 4C shows Monkey C’s chamber under natural light, and Fig. 4D shows the chamber viewed through GFP/YFP “goggles” during a session 7 days after Lenti-CaMKIIα-ArchT-GFP injections (blue arrow) and 23 days after Lenti-CaMKIIα-ChR2-EYFP injections (dotted blue arrow). Two fluorescent spots, which match the location of the respective virus injection sites, are clearly visible in cortex through the AD.

Similar results were obtained in monkey A, injected with AAV5 and lentivirus, both carrying a C1V1-EYFP construct (Fig 4E,F). In this monkey, we detected fluorescence through the optical window at the injection sites a few days after the AAV5-CaMKIIα-C1V1-EYFP injection (blue arrow); fluorescence of the Lenti-CaMKIIα-C1V1-EYFP was apparent about 3 weeks after injection (dotted blue arrow). Fluorescence at both sites remained stable for several months.

Non-invasive in-vivo epifluorescence thus provides an important tool in optogenetic experiments, both by confirming protein expression and by guiding targeting of light and recording electrodes to opsin-expressing sites.

Effects of photostimulation

We used two methods to assess the success of the optogenetic transfection: intrinsic-signal optical imaging and extracellular single-unit electrophysiology.

Intrinsic signal optical imaging
When visual cortical neurons are activated by visual stimuli, this activity is accompanied by hemodynamic changes in cortex due to stimulus-induced deoxygenation events. Such events are detectable as a darkening of the blood and cortical tissue, and can be optically detected as a negative reflectance change commonly referred to as the *intrinsic signal*. Cortical intrinsic signals are on the order of 0.1% in magnitude and typically peak around 2-3 seconds post-stimulus onset (Grinvald et al. 1991; Grinvald et al. 1986). If neurons express optogenetic proteins, then wavelength-specific photostimulation of those neurons should induce neuronal activity and an accompanying local negative reflectance change.

65 days after virus injection, we located the ChR2 cluster of Monkey M using vasculature as a guide. We positioned a 1-mm diameter optical fiber over the ChR2 cluster (Fig. 3B, red dots), and coupled the other end of the fiber to either a blue LED (470 nm) or to a green laser (532 nm). The irradiances measured at the cortical end of the fiber were 20 mW/mm² (blue LED) and 40 mW/mm² (green laser). Optical-imaging frames were collected (4Hz, 250ms per frame, 5-second/trial) during optical stimulation of the cortex with the fiber optic.

As shown in Figure 5A, baseline reflectance was measured for 4 frames (top row of panel A, fiber tip visible at left side of frame), followed by photostimulation through the fiber (frames 5 -7). The blue light (pulses lasting 600 ms, pulse width = 20 ms, 24 Hz) saturated the optical imaging system during frames 5-7. Immediately following the photostimulation offset, a focal darkening appeared in the cortex (frame 8). This signal (darkening) persisted over the next 3 seconds (frames 9-20). Panel B shows, magnified,
The timecourse of the reflectance change in the focally activated area (sampled from yellow box in Figure 5B) is plotted in Figure 5C. The baseline reflectance (first 4 frames) is followed by saturating photostimulation (3 frames off scale). This artifact is followed by a ~0.1% negative reflectance change that slowly returns towards baseline, consistent with the intrinsic optical-image signal that typically follows neuronal activation.

To confirm that the observed changes in reflectance were not due to heat or other non-specific effects illumination (Cayce et al. 2011; Christie et al. 2012), we switched the light source to a green laser (much less efficient in activating ChR2) while maintaining the fiber position over the ChR2 site. As shown in Fig. 5D-F, green light produced no significant change in reflectance from baseline (compare frames 8-20 in Figures 5A and 5D; frames 4 and 8 shown enlarged in Figure 5E). The lack of an intrinsic signal elicited by green light is further confirmed by the time course of the reflectance (Fig. 5F), which does not deviate significantly from baseline after photostimulation (frames 8-20). Thus the green laser, despite having a higher irradiance than the blue light (40 vs. 20 mW/mm², respectively), produced no significant intrinsic-signal response in the ChR2 cluster. The response was wavelength specific, consistent with the absorption spectrum of the ChR2 opsin, and hence was not due to non-specific effects of light on the tissue.

In conclusion, the wavelength-specific induction of intrinsic signal suggests that neurons at the ChR2 injection site expressed the opsin and were appropriately activated by photostimulation. Moreover, we have demonstrated that photo-elicited activity can
be monitored via intrinsic-signal optical imaging. This combination of optical window, optical imaging, optogenetics, and external photomodulation, provides a non-invasive all-optical activation and readout assessment of cortical activity.

**Electrophysiology**

We recorded single-neuron activity elicited by photostimulation in V1 of monkeys C and A (Fig. 6A,B). In all of these experiments, illumination was directed at the cortical surface via an optical fiber placed above the artificial dura. Figure 6A shows an optical fiber and an electrode aimed at the ChR2 cluster of monkey C's brain (red dots in Fig. 4C and dotted arrow in 4D). This experiment was conducted three months after virus injections. The photo shows also a transparent rigid-plastic stabilizer, over the AD, that has a hole to permit the passage of the fine microelectrode into the AD and into the brain. The electrode was held by a microdrive on an X-Y stage, and the optical fiber was positioned using a second micromanipulator. Using the vasculature as reference, the electrode was targeted to the ChR2 cluster and advanced under microscope guidance (Fig. 6A). We were able to see dimpling of the AD and of the brain, and hence to identify exactly when the electrode penetrated the cortex; this was typically confirmed by hearing neuronal activity on the audio monitor. Fig. 6B shows a similar setup in monkey A.

Figures 6C-F show examples of single-unit recordings from monkeys C and A. Fig 6C shows data collected from a neuron in the ChR2 cluster of Monkey C. This unit was recorded ~ 0.9 mm below the cortical surface. It exhibited very low spontaneous activity (see waveforms, raster, and histogram for t < 0), and was clearly activated by blue light
directed at the surface of the cortex (blue LED, continuous 0.5-s pulse starting at $t = 0$ s, 30 mW/mm$^2$ at the cortical end of the 2-mm diameter fiber). The spike waveforms before and during the photostimulation period can be seen in the top inset (black, individual waveforms; red, average); waveforms collected in both periods are similar, suggesting that raster shows the activity of one and the same neuron.

Notice the modest irradiance (30 mW/mm$^2$) that led to robust photo-elicited responses in the experiment described in Fig. 6C. Also, although the AD absorbs very little light (transmission through a 0.35-mm thick AD was 85 to 95%), brain tissue, at 0.9 mm depth, should have reduced the irradiance of the photostimulation to about 1/100 its value at the surface (see http://www.stanford.edu/group/dlab/cgi-bin/graph/chart.php). 0.3 mW/mm$^2$ is unlikely to have driven a photosensitive neuron strongly enough to produced the robust response observed in panel C. We thus infer that this response was due either to direct activation of apical dendrites of the recorded neuron, near the cortical surface, or to indirect activation from photosensitive neurons near the cortical surface. About half of the neurons recorded in monkey C’s ChR2 site were modulated by photostimulation levels under 30 mW/mm$^2$. When displacing the electrode laterally, we never observed photomodulation of neuronal activity beyond approximately 500 µm from the ChR2 injection cluster.

Monkey A was injected with both AAV5 and lentivirus, each carrying a construct for C1V1, in two separate sites in V1 (Fig 4E, F) (Table 1 specifies the C1V1 variants used). Approximately 3 weeks after the injections, we recorded from the AAV5-C1V1 site (Fig. 6B). Fig. 6D shows a neuron recorded at a depth of ~1mm), which responded to light from the optical fiber (green laser, optimal to activate C1V1; continuous pulse,
0.2-s duration, starting at $t = 0$ s, 125 mW/mm$^2$). Fig 6E and F show examples recorded from the Lenti-C1V1 site. The neuron in panel E was activated by photostimulation and shows a quiescent post-activation period (see raster; $0.2 < t < 0.4$ s). The unit in panel F was suppressed during photostimulation, an effect seen in about 20% of the neurons significantly modulated at the Lenti-C1V1 site. Since Lentivirus expressing protein under the CaMKII promoter selectively targets pyramidal neurons (Han et al. 2009), this suppression resulted, presumably, from the action of inhibitory neurons activated by optogenetically-stimulated excitatory neurons (Han et al. 2009).

In sum, reliable photomodulation of neuronal activity was obtained by focal illumination at the cortical surface. Electrophysiological recordings were obtained over months, without cortical damage.
DISCUSSION

Challenges for conducting optogenetics in the non-human primate

To date, a handful of studies have used optogenetic modulation of neuronal activity in the NHP (Diester et al. 2011; Galvan et al. 2012; Han et al. 2011; Han et al. 2009; Jazayeri et al. 2012). These studies have demonstrated proof of principle, and have highlighted several shortcomings of this approach. Two key issues make the application of optogenetics in NHPs more difficult than in other model systems: (1) the thickness and opacity of the primate dura mater, and (2) the need to affect large volumes and numbers of neurons given the large brain of primates, without causing damage to the tissue. To overcome these challenges, we aimed to achieve the following:

- **Minimize cortical damage**: Unlike the dura mater of rodents, NHP dura mater is tough and cannot be penetrated by fine glass micropipettes to inject optogenetic viral vectors. Instead, injections are made with cannulae having diameters of ~150 µm, which damage the cortex. Repeated insertion of optrodes (optical-fiber/electrode assemblies) and neuronal recording only compound the damage (Supplementary Fig. 1A,B). We aimed to achieve photoactivation without tissue penetration.

- **Achieve visually guided, precise targeting**: A key concern in previous studies is that electrodes and fiber optics can easily miss the opsin-expressing neurons, a problem particularly relevant for lentivirus vectors, which has limited spread in the cortex (Dittgen et al. 2004; Nathanson et al. 2009). The use of a chamber-mounted grids is
not sufficient to guarantee repeatability because cortex can shift up to millimeters under a cranial well (Supplementary Fig. 1C,D).

- **Photostimulating larger tissue volumes**: Previous NHP studies have only achieved modest behavioral effects (Cavanaugh et al. 2012; Gerits et al. 2012); this may be due to the small volume of tissue illuminated by optrodes inserted into cortex. Given the large size of NHP brains, photostimulating a larger volume of tissue may be needed to reliably impact behavior.

**Optogenetics in the non-human primate is aided by optical window**

The present study introduced a new approach to primate optogenetics, and showed the advantages of the optical window:

- **Tissue Health**: The artificial dura (AD) permits easy, repeated, and targeted introduction of micropipettes for injection of viruses without damage to the cortex. The use of fine glass pipettes to make viral-vector injections reduces tissue damage and likely promotes better uptake and expression (Nielsen et al. 2012; Osten et al. 2007). The ability to actively avoid major vasculature is also an advantage that cannot be overemphasized. Our use of non-tissue-penetrating optical fibers eliminates cortical damage during photostimulation. Less tissue damage translates into a better viral expression, higher effectiveness of photomodulation, response reliability, and ultimately better assessment of neuronal response.

- **Monitoring expression in vivo**: Typically, optogenetic expression takes several weeks. During this time, there is uncertainty about whether the virus has expressed and whether additional injections might be called for. We provide evidence that with the
optical window it is possible to monitor viral expression in vivo by means of non-invasive epifluorescence.

**Accurate targeting:** The AD offers the ability to accurately and repeatedly visualize the desired injection, electrode, or light-fiber locations. Injections of different viruses at different locations can easily be accommodated and photostimulation effects more readily interpreted without ambiguity. Accurate targeting of microelectrodes is also critical for interpreting the effect of photomodulation on underlying neuronal circuitry.

**Large field of view:** Most previous optogenetic studies in monkeys have relied on electrophysiological unit recording to assess photomodulation effects. Combining optogenetics with optical imaging provides the ability to examine the response of large neuronal populations across a cortical expanse. Optical modulation of known functional networks (e.g. ocular dominance, orientation, color, or motion-processing networks) as well as global networks across cortical areas opens the door to many studies of intra- and inter-areal cortical neuronal circuitry.

**Long-term viability:** Our study indicates that neither viral injections, protein expression, nor the replacement of the native dura mater with an artificial dura was deleterious to the health or function of the cortex. The long-term (months to years) health and viability of this preparation makes it amenable for use in behavioral studies in awake, behaving monkeys (Lu et al. 2010; Tanigawa et al. 2010).

In sum, the optical window permits the combination of optogenetics with traditional methods, such as electrophysiology and optical imaging, in both anesthetized and awake, behaving nonhuman primates. Coupled with imaging methods (optical imaging
or fMRI) in the awake behaving monkey, our approach holds great promise for studying the effects of targeted cell-type specific modulation on behavior and, at the same time, mapping the underlying circuitry.

ACKNOWLEDGEMENTS

We are grateful to Dinh Diep, Aaron Cortez, Anita Disney and Katie Williams (Salk Institute), and to Reuben Fan and Jonathan Cayce (Vanderbilt University) for experimental assistance.

GRANTS

This work was funded by R21 EY022853 (GRS, AWR), R01 EY021827 (JHR), Gatsby Charitable Foundation and R21 EY020673 (JJN), R01 EY020673 (EMC), and NIH NS044375 and P30EY008126 (AWR).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.
References


**Figure Captions**

**Figure 1.** Artificial-dura implant. (A) Schematic illustration of the implant, showing the relations between chamber, skull, native dura, artificial dura, and cortex. The brim of the "hat", or flange, is inserted under the native dura and above the cortex. The artificial-dura window allows for a clear view of the cortical surface, and provides access for imaging, injections, and electrode penetrations. (B) Optically-clear silicone artificial-dura. This hat-shaped artificial dura is composed of a cylindrical wall and a base. The area of the base surrounded by the wall is the window. (C) View of macaque visual cortex through the optical chamber (monkey C). M: medial; A: anterior. Scale bars: 5 mm.

**Figure 2.** Long-term cortical health. (A,B) View of cortex in monkey A through the optical window one week (A) and 6 months (B) following chamber implant. Approximate locations of V1, V2 and V4 are indicated. Scale bars: 5 mm. L: lateral; A: anterior. (C,D) Ocular dominance maps, measured with Optical-Imaging, in monkey M on Day 0, just prior to AD and chamber implant (C), and 104 days after implantation (D). Ocular-dominance maps are unaltered by the presence of the artificial dura (D), and were stable not only after hundreds of days, but after several virus injections in cortex (see text for details). Boxed areas indicate corresponding regions within the two fields of view, aligned according to the vessel maps; notice the stability of the ocular-dominance domains measured more than three months apart. Scale bars: 1 mm.
Figure 3. Targeted injections of optogenetic viral vectors. (A) Micropipette inserted through artificial dura under anesthesia (monkey M). Circular opening is a hole in a cortical stabilizer placed on top of artificial dura. (B) Location of virus injections in primary visual cortex of monkey M, superimposed on a blood-vessel map (image acquired during an optical imaging session). Green dots indicate Lenti-ArchT injections, grouped in two separate clusters (1 and 2). Red dots represent Lenti-ChR2 injections, in cluster 3. These injections were done in different sessions. For example, Cluster 1 was made in three sessions; penetrations 1-3 were made the day of the AD and chamber implantation; penetration 4 was made in a different session, 8 days later; and penetration 5, 14 days later. (C) Ocular dominance map (same field of view as in B) obtained with Optical Imaging. Dotted line: V1/V2 border. The Lenti-ChR2 injections (cluster 3, red dots) were made in a right-eye column (lighter stripes); Lenti-ArchT injections (clusters 1 and 2, green dots) were made predominantly in left-eye columns (darker stripes). Scale bars: 1 mm.

Figure 4. Epifluorescence of optogenetic agents, seen through the surface of the brain. (A-B) Paraformaldehyde-fixed brain of monkey M, 6 months after Lenti-ArchT-GFP injections. (A) Operculum of the right hemisphere. (B) Same view as A, seen through commercial "Miner-Lamp goggles" (blue-excitation/green emission filters). The two fluorescent spots (blue arrows) co-locate with the two Lenti-ArchT-GFP injection clusters (compare with Fig. 3B; clusters 1 and 2). (C-D) Epifluorescence in vivo, monkey C. (C) Image of cortical vasculature through the artificial dura of monkey C,
with superimposed virus injection locations. (D) Epifluorescence image showing fluorescent-reporter protein expression at the two spots, matching the injection sites indicated in C. Photo D was taken 7 days after the Lenti-CaMKIIα-ArchT-GFP injections (blue arrow) and 23 days after the Lenti-CaMKIIα-ChR2-EYFP injections (dotted blue arrow). (E-F) Virus-injection sites and epifluorescence in monkey A (same description than C-D). Photos E and F were taken from a slightly different perspective, making the AAV site appear closer to the right border of chamber in panel F. Blue arrow in F: site of AAV5-CaMKIIα-C1V1-EYFP injection. Dotted blue arrow: site of Lenti-CaMKIIα-C1V1-EYFP injection. Photo F was taken 31 days after injections.

Figure 5. Intrinsic-signal optical imaging of cortex, through the artificial dura, during an optogenetic-activation experiment in monkey M. (A) Sixteen frames (250 msec/frame) of the optical-imaging acquisition. The first four frames (1-4; top row) were captured before photostimulation, the next three frames (5-7) during photostimulation, and the rest (8-20) after the photostimulation was turned off. Each image is blank- and first-frame subtracted. Blue light pulses (train duration = 600 ms, pulse duration = 20ms, 24Hz; 470 nm, 20 mW/mm²) were directed to the ChR2 site via a 1-mm diameter optical fiber placed over the artificial dura. The fiber tip can be seen by the left border of the first frame in panel A. (B) Enlarged view of frames 4 and 8. Yellow box represents the region of interest (ROI) selected to calculate the time course of the optical signal at the photoactivated ChR2 site. (C) Time course of the reflectance change at the photostimulation site. There is a negative reflectance change of roughly 0.1% associated with the photostimulation consistent with the time course and magnitude of
intrinsic signals. Photostimulation artifact in frames 5-7 is off scale. Error bars: standard error of the mean. (D-F) Control data, same conventions as in A-C: green light (523 nm, 40 mW/mm²; same temporal pattern than blue light). (D) Green light did not elicit a significant reflectance change in the ChR2 site: notice absence of darkening in cortex in the region illuminated by the optical fiber (frames 8-20). (E) Enlarged view of frames 4 and 8, that shows minimal or no reflectance change associated with the green light. (F) Time course of the signal in ROI; the signal does not deviate significantly from baseline. The 600-ms photostimulation train (applied during frames 5-7) is indicated at the bottom of panels C and F. All images are the sum of 40 trials. Scale bars in A and B: 1 mm.

Figure 6. "Photo-electrophysiology" of V1 neurons in two alert monkeys through the artificial dura. (A) Optical fiber and electrode arrangement at ChR2 site in Monkey C. (B) Arrangement for neuronal recording of C1V1 site in Monkey A. (C) Extracellular single-unit recording at the Lenti-ChR2 site in Monkey C, in response to a 500-ms photostimulation pulse of blue light from an LED. Top insets show the waveforms of the extracellular action potentials before (left), and during the photostimulation period (right). Black: individual waveforms; red: averages. Raster shows the time of action potentials in different trials. The photostimulation pulse is represented by the blue bar, starting at \( t = 0 \) and lasting 500 ms. Continuous trace shows the peri-stimulus time histogram of the spikes. (D-F) Extracellular single-unit recordings at C1V1 sites in Monkey A, in response to a 200-ms pulse of green light (optimal wavelength for C1V1 activation; 532nm, 125mW/mm²). The light pulse is represented by the green bar,
starting at $t = 0$ and lasting 200 ms. All other conventions as in C. Photostimulation excited the units in D and E, and suppressed the unit in F.
Figure A1. Conventional optogenetic injections and recording, and shift of primate cortex under a cranial well.

(A) The dura mater of non-human primates (NHPs) is tough and cannot be penetrated by fine glass micropipettes to inject optogenetic viral vectors. Optogenetic virus are usually injected with metal cannulae (without or with an attached electrode; "injectrode") having diameters of ~150 µm, which damage the cortex. A single penetration causes damage to the tissue, and that cortical damage is compounded by repeated injections (necessary to achieve infection of sufficient cortical volume).

(B) In NHPs the opaqueness of dura mater and granular tissue make it necessary to insert optical fiber, sometimes inside a metal cannula, through the dura to reach and
illuminate the opsin-expressing neurons. The fiber is inserted blindly through the dura mater into the brain. The fiber produces damage to all neurons in their trajectory, including potentially the targeted photosensitive neurons themselves. Again, this damage is compounded by repeated fiber insertions in multiple experiments. Moreover, blind insertion of fibers can fail to target a previously-injected site and the region of opsin expression.

(C,D) Example of brain cortex shift under a cranial well. C shows a photo of monkey C's primary visual cortex, near the border of the chamber right after implantation (top of the photo is medial, right is anterior). D shows the same area, months later, when the monkey was seated in his chair, during an experiment. A comparison of the vasculature in both photos shows that cortex, is slightly displaced laterally (towards the bottom of photo) relative to C, (compare the position of the vessel branchings relative to the border of the implant). Similar shifts have been observed in other monkeys, used for optical imaging, in different sessions. Note that, if injections and subsequent optical-fiber positioning are done blindly (using an X-Y manipulator, or guide tubes through a chamber-mounted grid), electrodes and optical fibers would miss the virus injected locations.
Figure 1

A

Chamber
Cap
Cement
Skull
Cortex
Artificial dura
Dura mater

B

Wall
Base

C

Scale bar
Figure 3

A. Monkey M

B. Cluster 1: 1, 2, 3; Cluster 2: 4, 5; Cluster 3: 6, 7

C. LV-ChR2; LV-ArchT
Figure 4

monkey M

Operculum

Cluster 1
Cluster 2

monkey C

V1
LV-ArchT-GFP
LV-ChR2-EYFP

monkey A

V1
V2
LV-C1V1-EYFP
AAV5-C1V1-EYFP

LV-ArchT-GFP
LV-ChR2-EYFP
LV-C1V1-EYFP

AAV5-C1V1-EYFP
Table 1

Optogenetic agents and tests reported in this study

<table>
<thead>
<tr>
<th>Viral vector</th>
<th>Promoter, opsin and fluorescent marker</th>
<th>Source and preparation</th>
<th>Opsin action</th>
<th>Used in this study for ... (monkey - experiment)</th>
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<td>Depolarize</td>
<td>M - Optical Imaging</td>
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<td>B, C, S</td>
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<td>C - In-vivo epifluorescence</td>
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<td>C - Electrophysiology</td>
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Code for sources of constructs and preparations:  B - Boyden lab (MIT);  C - Ali Cetin and Ed Callaway, Salk Institute;  D - Deisseroth lab, Stanford University;  S - Salk Viral Vector Core.