**Video Article**

# Large Volume, Behaviorally-relevant Illumination for Optogenetics in Non-human Primates

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## Abstract

This protocol describes a large-volume illuminator, which was developed for optogenetic manipulations in the non-human primate brain. The illuminator is a modified plastic optical fiber with etched tip, such that the light emitting surface area is > 100x that of a conventional fiber. In addition to describing the construction of the large-volume illuminator, this protocol details the quality-control calibration used to ensure even light distribution. Further, this protocol describes techniques for inserting and removing the large volume illuminator. Both superficial and deep structures may be illuminated. This large volume illuminator does not need to be physically coupled to an electrode, and because the illuminator is made of plastic, not glass, it will simply bend in circumstances when traditional optical fibers would shatter. Because this illuminator delivers light over behaviorally-relevant tissue volumes ($\approx$ 10 mm$^3$) with no greater penetration damage than a conventional optical fiber, it facilitates behavioral studies using optogenetics in non-human primates.

## Video Link

The video component of this article can be found at https://www.jove.com/video/56330/

## Introduction

Optogenetic tools, which allow for millisecond-precise, light-driven neuronal control are widely used to study functional physiology and behavior in rodents and invertebrates. However, technical challenges have limited the use of optogenetics in the non-human primate brain, which has a volume ~100x larger than the rodent brain [1].

To facilitate optogenetics studies in non-human primates, an illuminator was designed to address two competing goals: large volume illumination and minimal penetration damage. Previous attempts to address one of these concerns have come at the expensive of the other. Bundles of fibers illuminate larger volumes but with increased diameter, and, thus, damage[2,3]. Tapered glass fibers reduce penetration damage, but narrowly focus light to light emitting surface areas <100 $\mu$m$^2$ [4,5]. External brain illumination through a window in the dura circumvents the challenge of penetration damage and may allow for large volume illumination, but it can only be used for a few superficial brain areas[6].

To create a large-volume, small diameter illuminator (**Figure 1a**), the tip of a plastic optical fiber is heat tapered and the core and cladding are etched (**Figure 1b,c**). Unlike other tapered fibers that focus light to a narrow point, the etching allows light to escape evenly out the sides of the tip, thus, distributing light broadly over a large area (**Figure 1d,e**). Because penetration damage is proportional to penetration diameter, this illuminator has no more penetration damage than a conventional fiber, yet it has >100x the light emitting surface area and delivers light more broadly with 1/100th the light power density in a brain phantom (1.75% agarose) (**Figure 1e**). A Monte Carlo model (**Figure 1f**) illustrates the difference in light spread between a conventional fiber and the large volume illuminator when they have equal light power densities as their light emitting surfaces. Each illuminator is individually calibrated using an integrating sphere (**Figure 2a,b**) to ensure even light distribution along the tip (**Figure 2c**).

This large volume illuminator has been validated with optogenetic manipulation of both behavior and neuronal firing in non-human primates. The fiber tip length may be customized to any brain area and to each animal's individual receptive field map. The illuminator may be paired with a penetrating electrode for neuronal recordings that span the length of illumination. Further, because the fiber can carry any color of visible light, it can be paired with any of the available optogenetic molecules available.

Note: All animal procedures were in accordance with the NIH guidelines and were approved by the Massachusetts Institute of Technology Committee on Animal Care.

# 1. Illuminator Fabrication

1. Use a pair of sharp shears to cut a section of 250 µm diameter plastic optical fiber that is at least 10 cm longer than the desired total illuminator length.
2. Remove 15 - 20 cm of polyethylene jacket from one end of the plastic optical fiber using a 22 gauge wire stripper.
3. Secure the distal most 3 - 5 cm of the stripped end of the fiber in a table vise clamp.
4. Hold the jacketed end of the fiber taut in one hand with a constant steady pull. The fiber should be parallel to the floor, perpendicular to the vice. Maintain this constant tension on the fiber throughout heating and cooling (Steps 1.5 and 1.6 below) so that the fiber remains straight and taut as it thins.
5. Using the lowest setting of a dual temperature heat gun (570/1,000 °F,), heat the stripped section of the fiber until it is thinned to a diameter of 60 - 100 µm, or about the diameter of a human hair.
6. Maintain the constant tension on the fiber while allowing the fiber to cool. Failure to maintain tension may cause the fiber to curl.
7. Confirm the diameter of the thinned tip under a dissecting microscope. Alternately, one may use calipers instead.
    1. If the fiber is not thin enough, repeat steps 1.4 through 1.6 as needed to achieve the desired tip diameter.
    2. Optional if re-pulling, put a small mark at the narrowest portion of the fiber if it will be heated again so that the center of the heat gun targets the narrowest portion of the fiber.
    3. If the fiber is too thin, start over.

8. Once the fiber has full cooled and the desired diameter has been achieved, pinch the fiber 3 cm from the narrowest point on either side. With a quick, sharp pull along the axis of the fiber, separate the sides to create a tapered tip.
9. Examine the tapered tip under a low power (*e.g.*, 4X) on dissection microscope. If the tip is forked or curled (**Figure 1f**), discard the fiber.
10. Prepare to etch the tapered tip by placing a piece of lab tape near the end of the tip, leaving the last 5 mm exposed. The tape protects the fiber from etching above the desired length of light emission. This tip length was selected based on thickness of primate cortex in the target region. A longer or shorter length could be exposed depending on the desired length of illumination. If a different etch tip length is desired, the edge of the lab tape can be moved closer to or farther from the tapered tip.
11. Fold a small (~1 inch x 2 inch) square of 5 µm silicon carbide lapping sheet over between the thumb and forefinger. Place the tip of the fiber between the two sides of the lapping sheet and gently etch the fiber using small circular motions, as if rolling a BB between the thumb and forefinger. Frequently rotate the fiber so that all sides are etched evenly. The fiber tip will appear "roughened" to the naked eye in areas that have been etched, while un-etched areas typically appear smooth.
12. Repeat step 1.11 with a 3 µm aluminum oxide lapping sheet.
13. Affix a connector on the end of the illuminator opposite to the etched tip. This allows the illuminator to connect to an optical cable or laser.
    **Note:** This method differs from the preferred method for fixing glass / silica optical fibers in ferrules. Because the metal ferrule is harder than the plastic optical fiber, polishing the fiber and ferrule as a unit after gluing can damage the plastic optical fiber as tiny shards of metal produced during the polishing process can embed themselves in flat-cleaved fiber.
    1. Remove about 5 mm of the polyethylene jacket from the opposite end of the illuminator using a 22 gauge wire stripper.
    2. Cut the opposite end flat with a hot knife to smooth the surface.
    3. Polish the opposite end flat with successively finer lapping sheets: 5 µm, 3 µm, 1 µm and 0.3 µm.
    4. Insert the flat opposite end into a 260 µm inner diameter stainless steel ferrule until it is flush with the end of the ferrule.
    5. Visually confirm that the opposite end is smoothly and evenly polished with a fiber microscope.
    6. Remove the fiber from the ferrule and tape the ferrule vertically on the edge of a table with the fiber pointing down.
    7. Fill a 1 mL syringe with plastic epoxy and attach a blunt 18 gauge needle to the syringe.
    8. Use the needle to apply epoxy down into the ferrule. Fill the ferrule completely with epoxy.
    9. Insert the fiber into the ferrule.
    10. Wipe any excess epoxy from polished surface of the fiber with a wet lint-free wipe prior to drying if needed. Otherwise, excess epoxy can be removed after 12 - 24 h.
    11. Store the fiber gently until calibration and place a dust cap over the ferrule.

# 2. Illuminator Calibration and Quality Control

**Note:** These methods for calibration assess for light output non-linearity at different distances from the tip of the fiber. Uneven light distribution typically results from a "bumpy" or "wavy" taper.

1. Create a light shielding diaphragm for the top of the integrating sphere using light absorbing foil (**Figure 2**).
    **Note:** Wear gloves whenever handling light absorbing foil to prevent skin oils from creating light reflecting smudges.
    1. Fold a 2" by 4" piece of light absorbing foil over on itself to form a 2" x 2" square. An alternative method is to cut a 2" x 2" square with one light absorbing side and one light reflecting side (*i.e.*, a side of standard aluminum foil); however, the fold-over method is safer because it provides a dull edge for handling the diaphragm in the next step.
    2. Fold lab tape or electrical tape along the edges of the square to bind the non-folded edges. This both holds the sides together and protects against cuts from sharp edges.
    3. Puncture a hole in the center of the square using a 26 gauge needle.

4. Remove the large screw on cap from the integrating sphere and cover the opening with the diaphragm. Place the hole over the center. If the diaphragm was created with one light absorbing and one light reflecting side, place the light absorbing side up and the light reflecting side down, facing the inside of the integrating sphere.
   **Note:** To prevent dust and debris from entering the integrating sphere and to keep the hole centered, secure the diaphragm to the outside of the integrating sphere with lab tape.

2. Measure light output along the tip in 500 µm increments using a micromanipulator or stereotaxic arm and an integrating sphere.
   **Note:** Safety goggles of the appropriate wavelength should be worn whenever the laser is on.
   1. Connect the opposite end of the fiber to a laser or light source, but do not trigger light output yet.
   2. Secure the illuminator to a stereotaxic holder (preferably with lab tape) with the tip 7 - 10 mm below the bottom of the holder.
   3. Screw the stereotaxic holder into the stereotaxic arm.
   4. Align the tip of the illuminator with the hole in the center of the diaphragm. Zero the micromanipulator when the tip is at the exact same level as the diaphragm.
   5. Turn off the room lights and zero the integrating sphere.
   6. Trigger the laser (or other light source) and measure the total light power output using the integrating sphere.
      **Note:** Use the lowest light output possible for calibration testing.
   7. Lower the fiber 500 µm into the integrating sphere and repeat step 2.2.6.
   8. Continue lowering the fiber into the integrating sphere and measuring total light output in 500 µm increments until the total light power levels off.
      **Caution:** Total light power should level off once the entire tip is in the sphere. Do not continue to lower the fiber more than 1 - 2 mm beyond the etched tip length. If the tip contacts the bottom of the integrating sphere, it may melt and/or ruin the integrating sphere.

3. Confirm an evenly etched fiber by plotting total light power output as a function of how far the fiber has been lowered into the integrating sphere to confirm linearity.

# 3. *In Vivo* Illumination

 **Note:** Here, these methods are shown using a plastic model rather than a non-human primate.

1. Implant a 25 mm diameter recording chamber was implanted over the brain area of interest prior to experimentation.
2. Perform anatomical magnetic resonance imaging (MRI) prior to experimentation. Place a custom recording grid in the chamber and fill it with sterile surgical lubricant to allow for grid visualization and determination of the level of the dura and target brain structures.
3. Prepare a tower micro-drive prior to each testing session.
   1. Affix a 25 gauge guide tube with beveled tip to the middle and lower clamps on the drive. Two clamps are used to ensure that the guide tube remains straight. Both of these clamps can be moved manually if desired.
      **Note:** The guide tube may be epoxied or soldered to the clamps.
   2. Secure the large volume illuminator in the upper clamp on the same drive. This clamp is attached to the drive motor.
   3. Thread the large-volume illuminator through the guide tube fully and confirm that the tip of the illuminator extends past the tip of the guide tube.
      **Note:** The length of illuminator that extends past the tip of the guide tube equals the distance from the dura to the lower margin of the target brain region, as determined *via* MRI.
   4. Soak the guide tube and illuminator in antiseptic solution (*e.g.*, chlorohexidine) to sterilize.

4. Place a custom sterilized grid inside the clean chamber and secure it at a pre-specified orientation with a screw on the side of the chamber. The grid shown here has 1 mm spacing; however, the spacing can be customized as needed.
   **Note:** This should be identical to the grid and orientation used in the anatomical MRI.
5. Secure the stereotactic micro-drive holder on the recording chamber in a pre-determined orientation.
6. Retract the sterilized illuminator from the guide tube by pulling the illuminator up using sterile, blunt forceps. The tip of the illuminator should be 5 - 10 mm above the tip of guide tube.
   **Note:** The illuminator will bow outward between the guide tube and the clamp. This will not damage the flexible illuminator.
7. Affix the micro-drive to the holder and place the guide tube into target grid hole.
8. Lower the micro-drive stage until the guide tube is just through the level of the dura.
   **Note:** If desired, a second micro-drive with an electrode can be placed on the stage and lowered into a different grid hole. The local field potential on this electrode will show a distance-dependent light artifact, which can be used to confirm illuminator placement.
9. Attempt the lower the illuminator manually with sterile forceps.
   1. If there is any resistance, retract the illuminator 5 - 10 mm, wait 10 min to allow the tissue to settle and try again.
   2. If there is still resistance on the second attempt, retract the illuminator tip into the guide tube, lower the guide tube 0.25 mm, and try again.
   3. Repeat until the illuminator slides through the guide tube without any resistance.
   4. Lower the illuminator until it is taut.
      **Caution:** Do not push the illuminator forcefully against resistance. In these cases the guide tube typically has not penetrated the dura fully. Pushing the fiber forcefully will cause it to bend on itself, preventing the fiber from entering the brain and possibly destroying the tip (**Figure 1g**).

10. Connect the ferrule on the other end of the illuminator to the larger optics set up or laser.
11. Ensure that no light is visible to the non-human primate.
    1. Use black-out shielding or light absorbing foil to fully encompass the recording towers.
    2. Place all optical connections in shielded envelopes made of light absorbing foil.
    3. Shield all patch cables with either light absorbing foil or black electrical tape.

4.  As an extra precaution, place a decoy light-emitting diode (LED) bank of the same light color used in the experiment behind the non-human primate and flash the LEDs continuously at 2.5 Hz. This prevents the eyes from adjusting fully to darkness. If there were inadvertent light leakage, this flashing light would help to prevent the leak from serving as a behavioral trigger.

## Representative Results

The illumination of large brain volumes in non-human primates allows for behaviorally-relevant optogenetic manipulation. Acker *et al.* (2016) used this large volume illuminator with the red-shifted Halorhodopsin, Jaws [7] to study the temporal contribution of the frontal eye field (FEF) to memory-guided saccades in two rhesus monkeys. Specifically, FEF neurons were injected with a viral vector containing Jaws and then illuminated with red-light using the large volume illuminator during either the target presentation, delay period, or motor preparation period of a memory-guided saccade task [8]. **Figure 3** shows the experiment conditions. Error rates (*e.g.*, failures to execute memory-guided saccades to the proper target location) significantly increased with illumination for targets in the injected receptive field, but not for targets opposite to the site of inactivation / illumination (**Figure 4a**).

In addition to the behavioral changes induced by optogenetics, the large volume illuminator allowed for inactivation of neurons over the full 2.5 mm span of cortex (**Figure 4c**) and light delivery over 4.5 mm (**Figure 4b**), as evidenced by the optically-induced local field potential artifact [8].
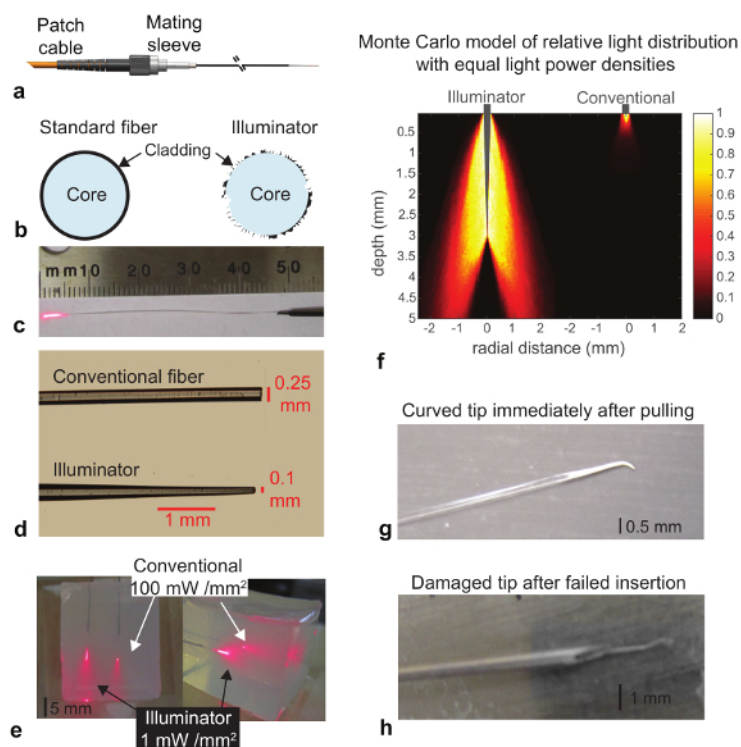


**Figure 1. Large Volume Illuminator Broadly Distributes Light**
**a)** Optical fiber/mating sleeve/illuminator interface. **b)** Etched core and cladding spread light broadly. **c)** Light-emitting 5mm-long etched tip. **d)** Comparison of tip shapes for a conventional fiber and a large volume illuminator. **e)** Illuminator and conventional optical fiber with equal total input light powers in 1" cubic brain phantom (1.75% agar). **f)** Monte Carlo models of the middle cross section of a large volume illuminator with 3 mm tip length (left) and a convention flat cleaved fiber of equal diameter with equal light power densities on their light emitting surfaces. See supplementary methods of Acker *et al.*, 2016 for the details of this model. **g)** Defective large-volume illuminator with curled tip. **h)** Damaged large-volume illuminator tip emerging from guide tube. This figure has been modified and reprinted in part from Acker *et al.*, 2016 [8]. Please click here to view a larger version of this figure.
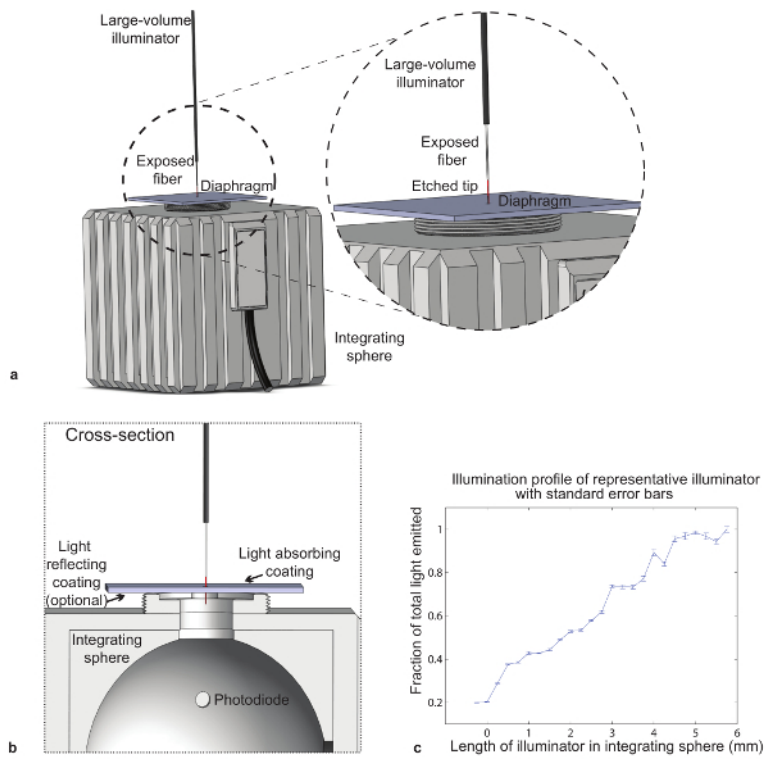
**Figure 2. Large Volume Illuminator Calibration**
This figure is reprinted from Acker *et al.*, 2016 with minor modifications. Please click here to view a larger version of this figure.
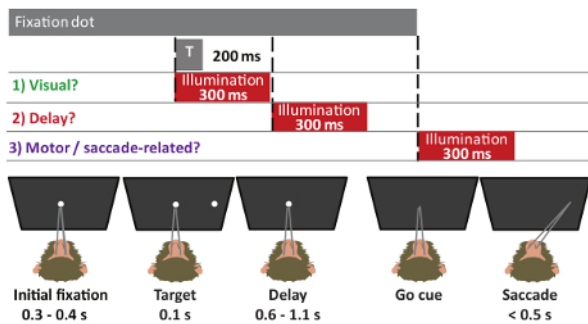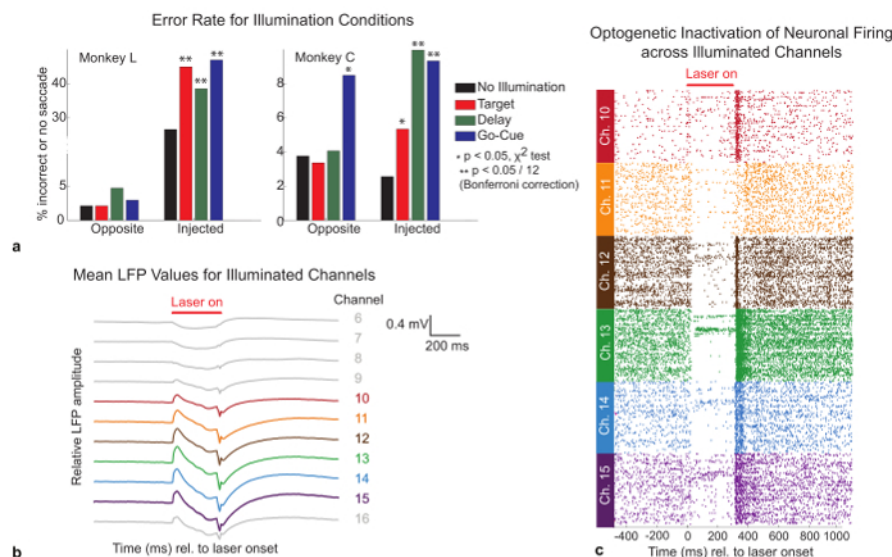


**Figure 3. Memory-guided Saccade Task with Illumination or Sham at Different Times.**
This figure is reprinted from Acker *et al.*, 2016 [8]. Please click here to view a larger version of this figure.

**Figure 4. Behavioral and Electrophysiological Effects of Optogenetic Inhibition with Large Volume Illumination**
**a)** Error rates increased significantly with illumination. **b)** Raster plots of showing inhibition of neurons spanning the 2.5 mm thickness of cortex. **c)** Local field potential showing a light artifact spanning 4.5 mm. For **b)** and **c)**, contacts are spaced 0.5 mm apart over the depth of cortex, n = 426 trials. This figure is adapted and reprinted from Acker *et al.*, 2016[8]. Please click here to view a larger version of this figure.

## Discussion

While optogenetic tools are widely used to study disease and physiology in rodents, the technical challenge of illuminating large brain volumes has limited the use of optogenetics in non-human primates. Pioneering studies in monkeys used large light power densities (~100 mW/mm$^2$ to 20 W/mm$^2$) to illuminate small volumes, perhaps < 1 mm$^3$, and reported modest behavioral effects with excitatory opsins in the cortex[4,9,10,11] and an inhibitory opsin in the superior colliculus[12].

Therefore, a large-volume illuminator was developed to allow for light delivery to large tissue volumes. With this illuminator and the red-shifted halorhodopsin, Jaws, robust, optogenetically-drive behavior was observed in non-human primates[8].

The protocol described here can be altered depending on the purpose of the experiment and geometry of the target tissue region. For example, the etched tip of the fiber can be lengthened or shortened depending on the size of the area to be illuminated. The tip can be pulled with a thicker tip than described or a larger diameter fiber could be used to create a more robust illuminator. While this protocol describes an etched fiber tip, it is feasible to etch the fiber both at the tip and at a more distal segment for non-contiguous illumination.

Quality control checks are a critical part of this protocol. The tip of a large volume illuminator can become forked or curled (**Figure 1g**), particularly if it is mechanically damaged or if it is pulled too thin initially. To pull the fiber with the proper amount of force consistently, most experimenters need a few hours of practice. Given the low cost of making fibers (the recommend plastic optical fiber costs less than $0.03 / meter), many experimenters only affix fibers with perfect tips to ferrules and, therefore, discard at least 30% of fibers for minor tip imperfections. Uneven light distribution discovered during calibration can be corrected by re-polishing the fiber tip and re-calibrating the fiber.

Further, the tip of the illuminator should be checked after each experiment. If the experimenter forces the illuminator through the guide tube before the guide tube has penetrated the dura, the illuminator will bend back on itself and it will not enter the brain. Typically, the illuminator tip is destroyed in these cases (**Figure 1h**). Aside from the resistance to illuminator insertion, the local field potential serves as an in-experiment check for proper illuminator placement. If the illuminator is bent up above the dura, the local field potential will not show the characteristic light artifact, which serves as a check for proper illuminator placement during the experiment.

While the large volume illuminator is designed to delivery light to multiple cortical layers simultaneously, it is not well-suited to illuminating the most superficial cortical layers while sparing deeper layers or to illuminating a single layer of cortex individually. If very spatially specific illumination is desired, traditional fibers that focus light more narrowly or superficial illumination through windows in the dura [6] may be more appropriate. Further, the flexibility of the large volume illuminator is both an advantage and a limitation. Unlike glass optical fibers, this illuminator cannot shatter in brain tissue, however, this flexibility also makes it difficult to advance the illuminator over large cortical distance (*e.g.*, 10 mm or more). Therefore, to target a very deep brain structure (*e.g.*, pulvinar), a guide tube would need to be advanced past the dura and into brain tissue to provide additional mechanical reinforcement.

Overall, this method offers a substantial advantage over prior methods because it allows illumination of behaviorally-relevant brain volumes in non-human primates, a key to adapting optogenetics to non-human primate studies. While this method has been shown in the FEF of rhesus monkeys, it would work in many other brain areas and even in other similarly large brain species.

## Disclosures

None

## Acknowledgements

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