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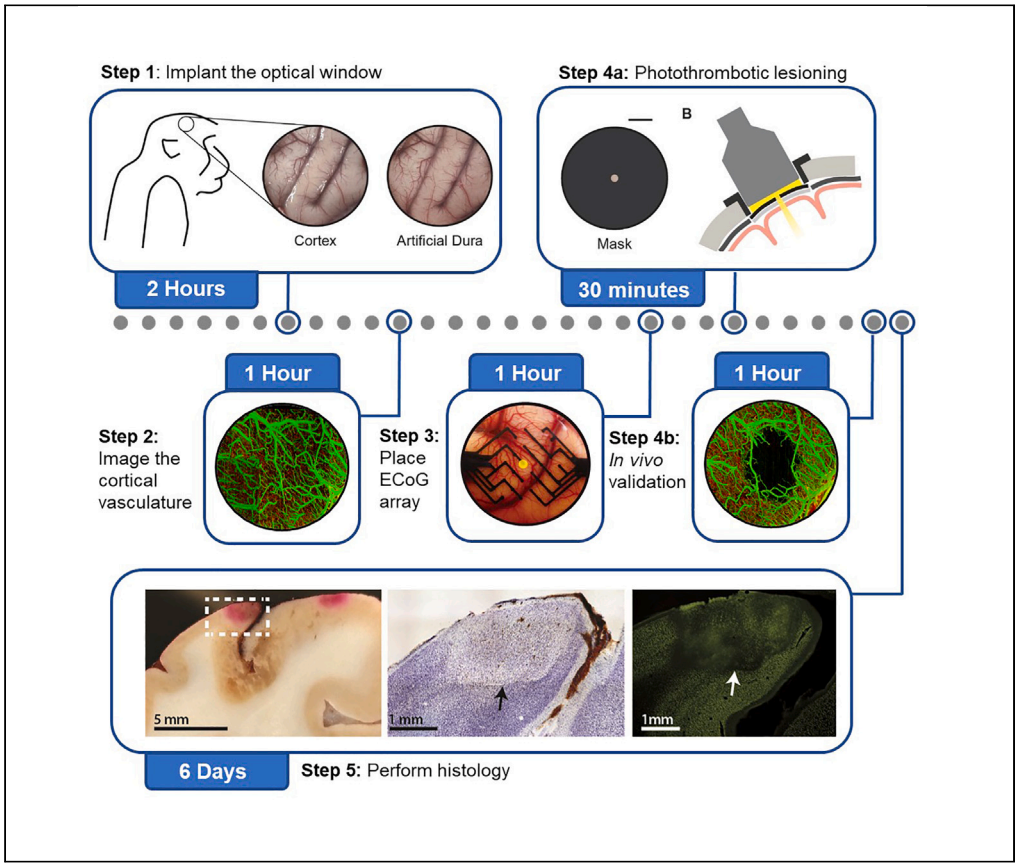
Protocol to study ischemic stroke by photothrombotic lesioning in the cortex of non-human primates



Neurorehabilitation strategies for ischemic stroke have shown promise for functional recovery, yet minimal tools are available to study rehabilitation techniques in non-human primates (NHPs). Here, we present a protocol to study rehabilitation techniques in NHPs using a photothrombotic technique, a form of optical focal lesioning. We also describe steps for simultaneous neurophysiological recording and *in vivo* validation through vascular flow imaging. This interface can examine emerging neurorehabilitation strategies in the post-stroke environment in NHPs that are evolutionarily close to humans.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Implantation of an optical interface for access to the NHP cortex

Generation of focal ischemic lesions with the photothrombotic technique

Neurophysiology recording with a semi-transparent electrocorticographic array

In vivo imaging of vascular occlusions with optical coherence tomography angiography

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Protocol to study ischemic stroke by photothrombotic lesioning in the cortex of non-human primates

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SUMMARY

Neurorehabilitation strategies for ischemic stroke have shown promise for functional recovery, yet minimal tools are available to study rehabilitation techniques in non-human primates (NHPs). Here, we present a protocol to study rehabilitation techniques in NHPs using a photothrombotic technique, a form of optical focal lesioning. We also describe steps for simultaneous neurophysiological recording and *in vivo* validation through vascular flow imaging. This interface can examine emerging neurorehabilitation strategies in the post-stroke environment in NHPs that are evolutionarily close to humans.

For complete details on the use and execution of this protocol, please refer to Khateeb et al. (2022).[6]

BEFORE YOU BEGIN

This protocol describes the implantation of an optical interface for ischemic stroke studies in the NHP cortex. The experiments in this protocol were performed in anesthetized animals; however, these methods can be implemented chronically to be used in awake-behaving animals by implanting a chronic optical interface.[1–4] This will enable the combination of our interface with sophisticated behavioral paradigms in NHPs. The acute optical interface described in this protocol offers the ability to induce lesions, record neurophysiology, and image blood flow in the cortical vasculature. For future ischemic stroke investigations, these methods can be implemented alongside additional optical methods which are discussed in this section, yet not performed.

To model ischemic stroke, a lesion is induced through the optical interface using the photothrombotic technique. This method relies on optical initiation of Rose Bengal dye in the cortical vasculature to generate microvascular occlusions and simulate ischemic stroke.[5] A photomask is used to restrict illumination of the cortical surface to an area defined by the aperture, which offers flexibility for the size and location of lesions. When designing the photomask, we suggest using our quantitative model that predicts lesion volume based on light intensity and aperture size.[6] Using light to induce lesions overcomes many of the limitations of existing surgical lesioning methods. Surgical techniques are often complex and rely on the occlusion of large, identifiable arteries, such as the middle cerebral artery, to induce damage. Since the cortical vasculature architecture is not identical between subjects, the extent of tissue damage can vary considerably. Furthermore, limiting the tissue damage to a more precise area poses a great challenge as downstream areas are

more challenging to target with these classical surgical techniques. In contrast, the photothrombotic technique enables direct targeting of a specific cortical area. Here we outline the steps for implementation of the photothrombotic technique in NHPs in concert with our large-scale neural interfaces.

We describe how lesioning can be performed with simultaneous neurophysiology recording by using a Multi-Modal Artificial Dura (MMAD)[1,2,7] which contains platinum electrodes embedded in a transparent polymer. Since our lesions are photochemically induced, this semi-transparent neurophysiology recording apparatus can be assembled directly on the cortical surface, providing the ability to monitor neural activity while the lesions are forming. While a few studies have reported disruption in network communication following stroke in rats[8–11] further investigation is required regarding neural dynamics occurring during and after stroke in a more clinically relevant model. Although the MMAD enables this paradigm, it is not required. Alternatively, these procedures may be completed with an artificial dura as outlined in the protocol.

Using the MMAD not only allows for simultaneous neurophysiology recording but also provides the ability to administer electrical stimulation. Stimulation-based techniques have shown promise for neurorehabilitation after stroke. For example, electrical stimulation administered during the acute phase of ischemic stroke using this system proved to reduce lesion volume.[12] Furthermore, the optical access provided through this interface enables optical stimulation (e.g., optogenetics)[1,2,4,6,7,13–19] in addition to electrical stimulation for more sophisticated manipulation of the network. Previous work, using both electrical[20–25] and optogenetic[17] stimulation, has demonstrated the feasibility of perturbing the neural network and establishing precise functional connectivity changes. Although not described in this protocol, the ability to manipulate the network with high spatiotemporal resolution using these stimulation modalities following stroke creates a unique interface for developing stimulation-based neurorehabilitation.

To monitor and validate the vascular damage we describe how to visualize microvascular occlusion *in vivo* by imaging blood flow dynamics through the use of optical coherence tomography angiography (OCTA).[26] The *in vivo* validation is particularly important for designing experiments and investigating the changes following stroke as it eliminates the need for histological validation in primates. Alternative methods to OCTA are possible for *in vivo* validation and are listed in the materials and equipment section.

Institutional permissions

All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals and exceeded the minimum requirements recommended by the Institute of Laboratory Animal Resources and the Association and Accreditation of Laboratory Animal Care International. All animal procedures were approved by the University of Washington Institutional Animal Care and Use Committee (IACUC). All animals were obtained through the Washington National Primate Research Center (WaNPRC) which is accredited by the American Association for Assessment of Laboratory Care (AAALAC). Animals were housed and maintained in accordance with the guidelines set by the Guide for Care and Use of Laboratory Animals. The research in this study was also conducted in accordance with the American Society of Primatologists Principles for Ethical Treatment of Nonhuman Primates. Before proceeding with this protocol, appropriate licensing and permissions for animal use must be obtained on the institutional, national, and international level, as appropriate.

Prepare the artificial dura

Timing: approximately 1 day

This protocol requires an artificial dura to protect brain tissue throughout the procedure while providing optical access to induce lesions and visualize the cortical vasculature. When

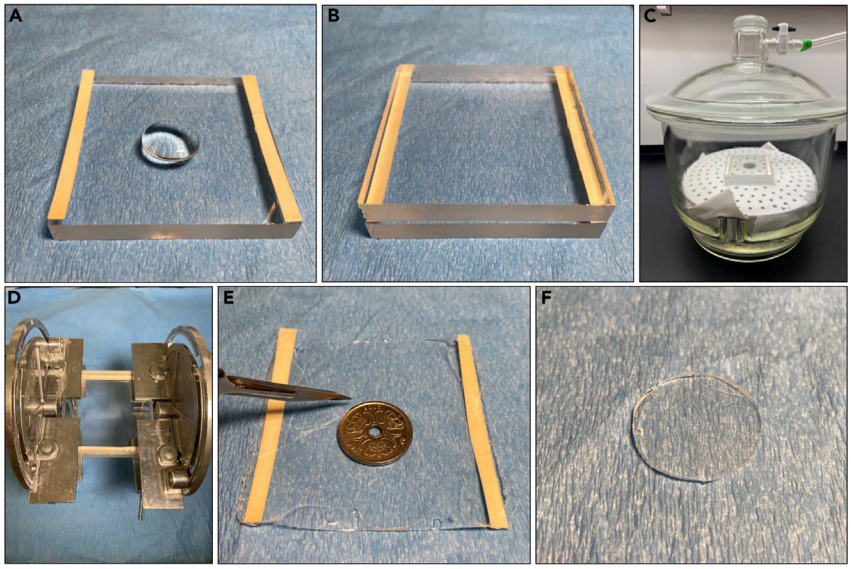

Figure 1. Steps to manufacture the artificial dura

(A) The PDMS mixture is poured over an acrylic block with cardboard spacers.
(B) Another acrylic block is placed on top to compress the mixture.
(C) The acrylic mold assembly is placed in a vacuum system.
(D) The blocks are clamped together and left to cure for 25 h.
(E) A stencil is used to cut a circular disk out of the cured PDMS.
(F) The final product is an artificial dura that is ready to be sterilized.

neurophysiology is being recorded, a MMAD will be used instead of the artificial dura. If neurophysiology is not being recorded, manufacture the artificial dura through the steps below. Other methods for obtaining transparent polydimethylsiloxane (PDMS) disks are available in the literature, and in some cases may be available for purchase. It is critical that this device is free of bubbles for optical transparency and is sterilized prior to use.

1. Pour 5 mL silicone base and polymer (10:1 Shin-Etsu KE1300-T and CAT-1300) into a 10 mL beaker.
2. Deposit 500 μL catalyst (XAT-1300) into the beaker using a pipette.
3. Mix thoroughly for a few minutes.
4. Place spacers made of non-corrugated cardboard on either side of a flat acrylic square (roughly 4 in x 4 in x 0.5 in).
5. Drip Mixture onto the acrylic square (Figure 1A).
6. Place another acrylic square on top of the mixture (Figure 1B).
7. Vacuum the fixture for 10 min (Figure 1C).
8. Clamp the fixture together (Figure 1D)
9. Allow the mold to cure for 24 h.
10. Remove clamps and top acrylic square.
11. Using a stencil, cut circles out of the PDMS sheet with a sharp cutting tool (Figures 1E and 1F).

    Note: An X-acto knife (X-acto, Z-Series #11) was used in this procedure, however another sharp edge such as a scalpel will suffice.

12. Sterilize the artificial dura.

    Note: Vaporized hydrogen peroxide gas plasma sterilization (STERRAD) was used in this procedure. PDMS is highly resistant to modification through many sterilization techniques

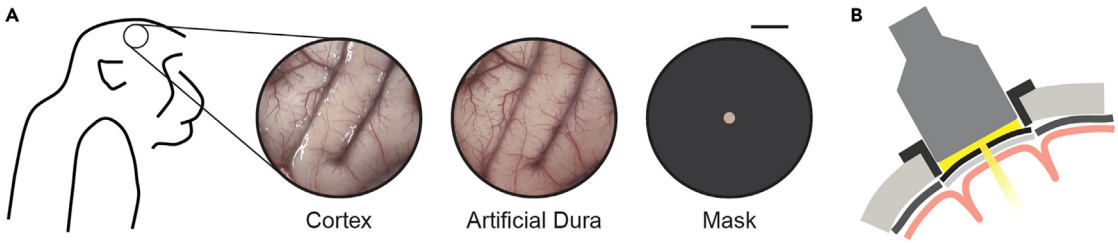

Figure 2. Preparation of the cortical surface before lesioning
(A) The artificial dura is placed directly on the cortex followed by the silicon photomask.
(B) Illumination of the cortex is controlled by the photomask aperture.
Figure adapted from Khateeb et al., 2022.[6]

enabling the use of alternative gas sterilization, such as ethylene oxide, or steam sterilization (autoclave).

Prepare the photomask

Timing: approximately 1 day

Lesioning with the photothrombotic technique begins with an injection of Rose Bengal dye intravenously. This reagent initiates thrombi formation in the vasculature when exposed to light; therefore, it is imperative that the area of the cortex exposed to light is controlled before Rose Bengal is administered. By placing a photomask over the exposed cortex and artificial dura, the area and size of lesioning can be precisely controlled (Figure 2). The photomask is an opaque, laser cut, silicon-based disk with an aperture that defines the lesion location and size.

13. Design a photomask using laser cutting compatible software that specifies the diameter and the aperture size of the photomask.

Note: The diameter of the photomask should cover the entirety of the cranial window (Figure 2B).

Note: The aperture size correlates with lesion size and its location will determine where the lesion is induced. For a model that relates aperture size to lesion volume, reference Khateeb et al., 2022.[6]

14. Laser cut the silicone material (Rubber-Cal, Cat# 02-W210-36B-0032-5) into the desired shape of the photomask.
15. Sterilize the photomask before use.

Note: Steam sterilization (autoclave) is recommended.

Prepare the Rose Bengal solution

Timing: approximately 30 min

This reagent is used as the chemical initiator of thrombi formation during lesion induction. It is critical that this reagent is prepared on the day it will be used and is shielded from light exposure.

16. Calculate the volume of Rose Bengal needed for injection based on the monkey's weight with the equation below:

$$Volume\ to\ Inject\ [ml] = \frac{D}{C} * W$$

Note: Concentration (C) = 40 mg/mL

Note: Dose (D) = 20 mg/kg

Note: Weight (W) = weight of the animal in kg

17. Prepare the necessary volume of solution with United States Pharmacopeia (USP) saline (0.9%).

⚠ CRITICAL: Ensure the solution is not exposed to light while it is being prepared by wrapping in foil.

18. Sterilize the Rose Bengal solution by filtering through a 0.22 μm syringe filter into a sterile 30 mL syringe vial.
19. Wrap in foil and store at 20°C

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Saline	Fisher Scientific	Cat #Z1377
Isoflurane	Sigma-Aldrich	1349003
Silicone base and polymer	Shin-Etsu	Cat# KE-1300T/CAT-1300
Silicone photomask	Rubber-Cal	Cat# 02-W210-36B-0032-5
Rose Bengal Dye	Sigma-Aldrich	330000
Thionin acetate salt, Lauth's violet	Sigma-Aldrich	Cat# 861340-5G
Triton X-100	Fisher Scientific	Cat# BP151500
Sodium azide	Sigma-Aldrich	S8032
Normal donkey serum	Jackson ImmunoResearch Laboratories	Cat# 017-000-121
Glycerol	Sigma-Aldrich	Cat# 356350
PDMS	Shin-Etsu Silicone	KE1300-T
Sodium acetate	Sigma-Aldrich	Cat# W302406
Glacial acetic acid	Sigma-Aldrich	Ca# PHR1748
Antibodies		
Mouse-monoclonal ani-NeuN (1:500)	Millipore-Sigma	Cat# MAB377; RRID: AB_2298772
Donkey anti-mouse IgG (H + L), Alexa Fluor 488 (1:500)	Invitrogen ReadyProbes	Cat# R37114; RRID: AB_2556542
Other		
Syringe filter, 0.22 μm	Sigma-Aldrich	SLGL0250S
Syringe filter, 0.45 μm	Thermo Scientific	1680045
30 mL Syringe vial	Westend Medical	SV30ML
Stereotaxic frame	KOPF	Model 1430
25 mm Diameter trephine	GerMedUSA, Inc.	SKU: GV70-42
3D Printed connector holder	STL file	Available in "resource availability section"
3D Printed light shield disk	STL file	Available in "resource availability section"
ECoG array (MMAD)	Ripple Neuro	Custom 32-channel array
Clamp connectors	Ripple Neuro	Custom accessory
Grapevine processor	Ripple Neuro	Nomad

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
OCTA system	University of Washington, Bioengineering Department	Biophotonics and Imaging Laboratory
Cold light source	Schott Inc.	KL 2500 LCD
Sliding microtome	Leica	N/A
6D Widefield automated microscope system	Nikon	Imaging center at UCSF
Rotary pump	Qcvialz	Mec-O-Matic Peristaltic Pump, 115 V, 60 GPD
Anesthesia delivery system	Soma Technology	GE Datex Ohmeda Aisys
Anesthesia monitoring system	Soma Technology	Datex Ohmeda Compact S5
Nonin LifeSense capnography and pulse oximeter monitor	Turner Medical	Nonin Part # 9187-002
Bran matrix	Ted Pella, Inc.	Prod# 15039
10 mL beaker	Pyrex	1000-10
X-acto knife	X-acto	Z-series #11
Software and algorithms		
MATLAB	MathWorks	RRID: SCR_001622 http://www.mathworks.com/products/matlab/
Trellis	Ripple Neuro	https://rippleneuro.com/support/software-downloads-updates/
Hugin-2019.2.0	Hugin software	http://hugin.sourceforge.net/
Adobe Photoshop	Adobe	RRID: SCR_014199 https://www.adobe.com/products/photoshop.html
Experimental models: Organisms/strains		
Macaca mulatta/nemestrina	Washington National Primate Research Center	Adult/male

MATERIALS AND EQUIPMENT

Multi-modal artificial dura

For neurophysiology recording, we used a MMAD previously designed by our lab[1,2,7] and manufactured by Ripple Neuro (Ripple Neuro, Salt Lake City, UT). The electrodes are embedded in a biocompatible transparent medium (Figures 3A and 3B) which connects to neurophysiology recording equipment through custom built clamp connectors (Figure 3E). This device contains 32 platinum electrodes (500 μm diameter, 2 mm pitch, Figures 3C and 3D) covering a cortical area of approximately 3 cm^2 which can be used for both electrical stimulation and recording. Similar recording devices[15,16] can be used in place of the MMAD as long as they are semi-transparent.

Custom-built prototype OCT system

In vivo validation of vascular occlusion can be confirmed through blood flow imaging with optical coherence tomography. This validation technique is essential to confirm blood flow in the desired cortical region. This system was designed and constructed at the Biophotonics and Imaging Laboratory at the University of Washington Bioengineering department. A more descriptive report of this device can be found in previous publications.[26,28,29] OCTA images were acquired using a 200 kHz vertical cavity surface-emitting swept source (SL1310V1-10048, Thorlabs Inc., Newton, NJ) with a central wavelength of 1310 nm, and a sweeping bandwidth of 100 nm, giving an axial resolution of 8 mm in tissue. The imaging probe was a hand-held piece in which a paired X-Y galvo scanner and sample optics assembly were housed. To facilitate scanning, a monitoring screen was integrated with the hand-held probe where the OCT and OCTA cross-sectional images were displayed in real time to aid the operator during imaging. This imaging modality is not commercially available; however, alternative imaging methods may be used for *in vivo* validation.[27] Laser Speckle Imaging (LSI) has been used to monitor hemodynamics in the brain following stroke with comparable insights to OCTA.[34] These systems are available commercially and are recommended as an alternative.

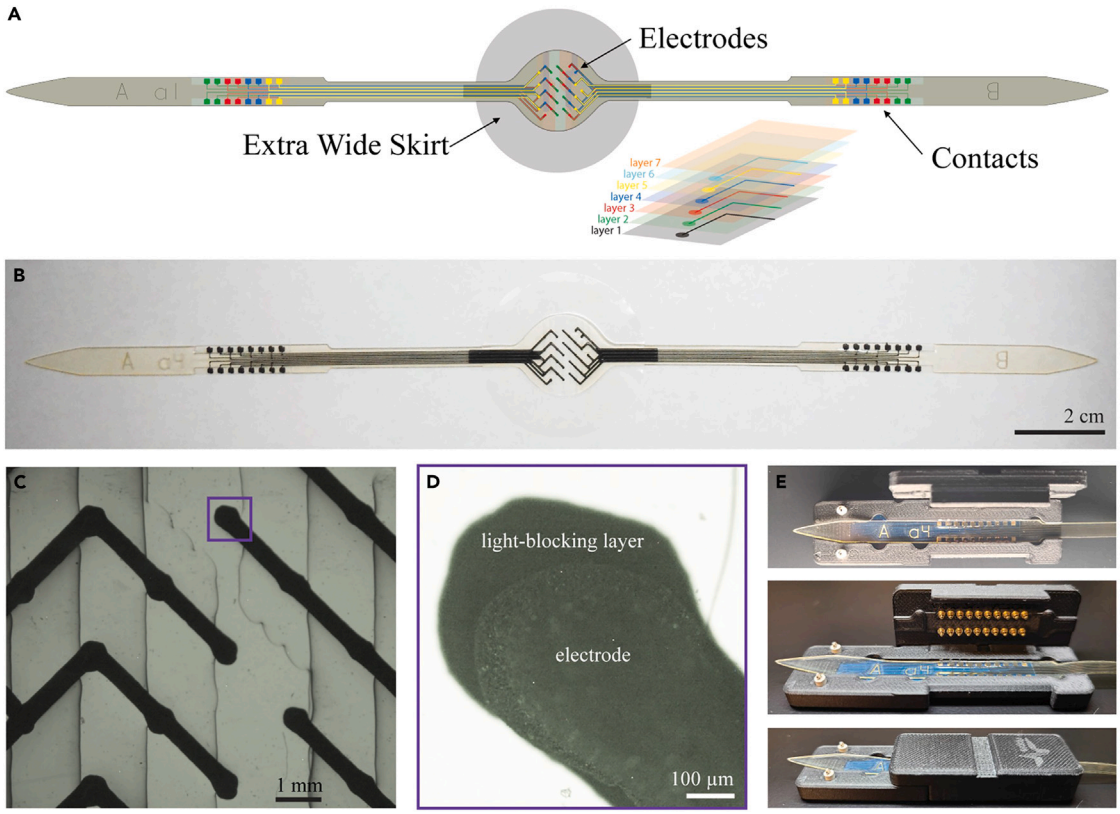

Figure 3. Schematic of the MMAD

(A) Electrodes are embedded in the transparent artificial dura. Contact points on array arms interface with the clamp connectors.

(B) Image of the fabricated MMAD.

(C and D) (C) A microscopic image of the electrodes shows polymer edges and (D) the light blocking layer of a single electrode.

(E) The clamp connecter holds the array arms in place with pads on the printed circuit board.

This figure has been adapted from Griggs et al., 2021.[2]

Anesthesia

The primary anesthetic we used was isoflurane; however, it is necessary that neural recordings are not taken while the animal is anesthetized with isoflurane due to its impacts on neural activity.[30,31] Since our experiments were terminal procedures, urethane was the optimal choice for anesthetic while recording neurophysiology. If the experiment is not terminal, barbiturates are recommended, such as pentobarbital.[17] It is important to understand the mechanism of action of the chosen drug, as neural activity will inevitably be altered in the anesthetized state.

Thionin Acetate

This reagent was used in histology procedures described later, yet should be prepared ahead of time in a glass bottle.

Thionin Acetate (~1L, 0.05%)		
Reagent	Final concentration	Amount
dH_2O	N/A	950 mL
Sodium Acetate (anhydrous)	3.7%	37 g
Thionin Acetate	0.05%	500 mg
Glacial Acetic Acid	N/A	30–37 mL

- Pour 950 mL of dH_2O into a glass bottle
- Add 37 g of Sodium Acetate (anhydrous) and mix with a glass stir rod until dissolved
- Under a chemical fume hood, add 30–37 mL of Glacial Acetic Acid to adjust pH to 4.8
- Filter twice after preparation with a 0.45 μm filter
- Store at 4°C for up to 1 year

STEP-BY-STEP METHOD DETAILS

Optical window implantation

Timing: approximately 2 h

An optical window to the cortex will be established by placing an artificial dura, or MMAD if neurophysiology recording is desired, on the cortical surface following a craniotomy and durotomy. This will protect the cortex throughout the experiment while establishing an interface for photothrombotic lesioning and *in vivo* imaging.

1. Sedate the animal with 100 mg/mL ketamine at a dose of 10–15 mg/kg delivered intramuscularly and monitor heart rate, oxygen saturation, and temperature using a capnography and pulse oximeter monitor (Turner Medical, Nonin Part # 9187-002).
2. Anesthetize the animal with 0–5% isoflurane, varying the concentration as needed to maintain an appropriate anesthetic plane using an anesthesia delivery system (Datex Ohmeda Aisys).

⚠ CRITICAL: Dosing requirements range for primates depending on species, weight, body condition, age, type of procedure being performed and length of procedure. For more information, consult a veterinary surgeon before administering anesthesia.

⚠ CRITICAL: All surgical procedures should be conducted using standard sterile technique.

3. Place the animal in a stereotaxic frame where they will remain throughout the procedure and place a drape.
4. Unpack all sterile equipment onto a sterile field.
5. Monitor core temperature, heart rate, oxygen saturation, electrocardiographic responses, and end-tidal partial pressure of CO_2 with the anesthesia monitoring system (Datex Ohmeda Compact S5) throughout the procedure.

⚠ CRITICAL: Work with veterinary staff on the specific drug administered for information about adverse effects that may require additional monitoring.

6. Mark the location of craniotomy on the skull.

⚠ CRITICAL: Ensure craniotomy coordinates are guided by stereotaxic coordinates from MRI scans or the macaque brain atlas before performing the craniotomy.

7. Note the location of craniotomy in reference notebook.

Note: For the experiments performed in Khateeb et al.,[6] the stereotaxic coordinates for the center of the craniotomies conducted were 11.55 mm anterior to the interaural line and 17.5 mm lateral to the midline.

8. Perform the craniotomy using a 25 mm diameter trephine.

Note: The right and left hemispheres of the brain can be performed simultaneously or individually with this technique.

9. Perform a durotomy to excise the opaque native dura.
10. Presoak a transparent silicone artificial dura of 0.5 mm thickness and 25 mm diameter in saline for 5 min, then place it on the cortex. If neurophysiology recording is desired, perform this step with the MMAD instead.

⚠ CRITICAL: Before implantation, the optical window must be sterilized.

⚠ CRITICAL: The optical window prevents the cortex from drying out during the procedure which can damage the neural tissue.

11. Acquire baseline OCTA images of the cortical surface through the optical window.

Note: Reference the "optical coherence tomography angiography imaging" section for more details on this procedure.

Note: OCTA imaging before inducing a lesion will establish a baseline vascular structure prior to injury.

12. If neurophysiology recording during lesioning is desired, follow the steps in the "electrical recording" section after OCTA imaging, otherwise, move to "stroke induction."

Optical coherence tomography angiography imaging

Timing: approximately 1 h

Images of the cortical vasculature are taken using a custom OCTA imaging system to validate blood vessel occlusion at the site of lesioning. This section should be performed both before and after lesioning while an artificial dura or MMAD is in place.

13. Using an OCTA system, mount the probe on an articulated arm to maintain a consistent distance between the objective lens and the imaging target.

Note: The OCTA system used was a custom-built prototype that is not commercially available. A description of this system is provided in the equipment section of this protocol. Alternative imaging modalities to asses neuropathology are discussed in the "materials and equipment" section.

14. Using a 5× objective lens within the probe, focus the 1310 nm light source into a beam spot on the brain surface with an incident power of 5 mW through the artificial dura.

Note: This process is similar to that of a laser pointer. Alongside the imaging light was a visual laser beam to guide OCTA imaging.

Note: Multiple images of the brain surface may be required to image an entire brain area of interest.

Note: If imaging through the transparent ECoG array, some cortical regions will be obscured by the electrodes.

15. While performing a repeated raster-scan with a lateral field of view of approximately 9 mm × 9 mm, acquire one full A-scan approximately 8 mm deep (1408-sampling-pixel) at each position.

Note: Each B-frame contained 1000 A-line scans and was repeated 8 times, then moved to the next B-frame.

16. Repeat this process to form a volume of 1408 × 1000 A-lines x 8 repeat x 1000 B-frames.

Note: The optimal resolution in the axial direction (into the tissue) was 5.5 μm and the lateral resolution was 24 μm.

Note: The volume scan was repeated at different regions within the cranial window, then processed to form a 3D OCTA data set as seen in Figure 5.

Electrical recording

Timing: approximately 1 h

The following steps in this section are optional. Here, it is described how to assemble a recording apparatus for neurophysiology recordings to be acquired in concert with stroke induction.

17. Transition the animal from isoflurane to another anesthetic appropriate for neural recording at least 30 min prior to electrical activity recording.

Note: Isoflurane causes disruption to neural synchrony that will result in noisy and disordered neural signals. We chose urethane anesthesia since this experiment was terminal; however, other drugs can be used and are referenced in the "materials and equipment section."

⚠ CRITICAL: Always prepare more urethane than is needed for a single experiment to ensure that the animal is always in an appropriate anesthetic plane.

18. Assemble the electrical recording apparatus.

Note: A skull screw located anterior and medial to the ipsilesional cranial window was used as a ground for ECoG recordings

Note: Alternative ECoG arrays may be used; however, it is critical they are transparent to allow stroke induction via the photothrombotic technique.

a. With the MMAD already in place, attach the 3D printed connector holder (Figure 4A) to the skull with 2–4 bone screws attached closest to the midline.

Note: The skull is typically thicker closer to the midline which will be less prone to damaging the underlying cortical tissue when using bone screws.

Note: An STL file of the holder is made available in the resource availability section.

b. Thread the MMAD arms through the cable slots in the holder (Figure 4A) and attach the array arms to the clamp connectors (Figure 3E).

⚠ CRITICAL: Contact between the cortical surface and the ECoG array is imperative for electrical recording.

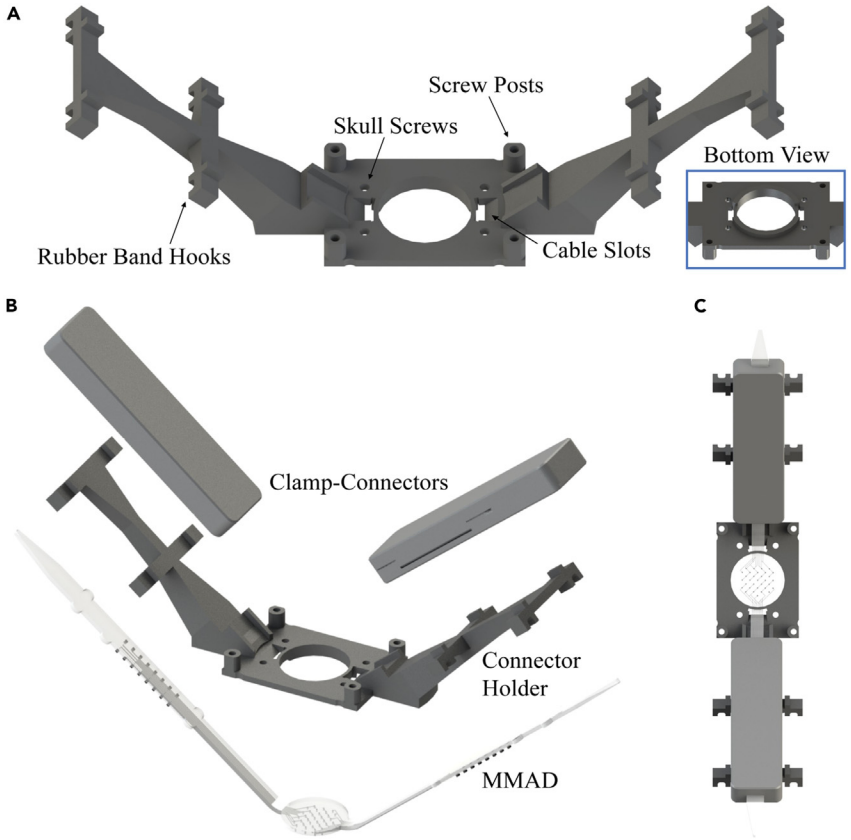

Figure 4. Assembly of recording apparatus used for acute neurophysiology monitoring

The photomask is not pictured, yet remains necessary to control the lesion location and size.

(A) 3D printed connector holder used to secure the MMAD against the cortical surface.

(B and C) Main components of the recording apparatus including the connector holder, transparent ECoG array (MMAD), and clamp connectors.

This figure has been adapted from Griggs et al., 2021.[2]

Note: To ensure good connection, swab array leads with saline before securing to the clamp connectors.

c. Secure the clamp connectors to the connector holder (Figure 4B)
d. Connect the clamp connectors to the neurophysiology recording system.

19. Initialize the neurophysiology recording software to begin recording electrical activity:

Troubleshooting: If background noise is noticeable, refer to problems 4 and 5 for solutions to minimize noise while recording.

20. Collect at least 30 min of baseline data before generating a lesion.
21. Initiate the lesion by following the steps described in the "stroke induction" section below.

Troubleshooting: Gamma power can be analyzed during or shortly after obtaining electrical recordings as described in the "analysis of ECoG recordings" section. This offers the ability to validate lesioning *in vivo* through neurophysiology. Figure 7 shows an expected outcome of reduced gamma power; however, if no reduction in gamma power is observed during lesioning, see problem 1.

22. Allow electrical recordings to continue for at least 60 min past the end of illumination.

Stroke induction

Timing: approximately 30 min

The photothrombotic technique is used to lesion the cortex. Here, it is described how to perform the photothrombotic technique and control lesion size by using a photomask. If simultaneous electrical recording is desired, ensure the steps in the "electrical recording" section are complete before proceeding.

23. Place the photomask over the artificial dura (Figure 2A).

Note: If electrical recording is being performed, the photomask will be placed over a MMAD instead.

24. Place a 3D printed light shield disk over the photomask to block any additional light that may penetrate the circumference of the photomask.

Note: An STL file of the disk is made available in the "resource availability section."

Note: If electrical recording is being performed, the ring will not be used, and instead light will be blocked by the 3D printed connector holder (Figure 4A).

25. Position the light source over the aperture opening in the photomask using a stereotactic arm to hold it in place (Figure 2B).

⚠ CRITICAL: Shield the cranial window from light sources before proceeding to prevent extraneous light from inducing unintended lesions.

⚠ CRITICAL: Avoid targeting large blood vessels on the surface, as this can result in highly variable lesion sizes due to differences in the topography of the vasculature across animals.

26. Intravenously inject the calculated volume of Rose Bengal dye (40 mg/mL) through the saphenous vein over the course of 5 min while simultaneously initiating illumination.

⚠ CRITICAL: A quantitative model relating light intensity and aperture diameter to lesion volume has been developed and should be used to choose illumination parameters.[6,18,33]

Note: The light source temperature setting was adjusted to 3200 K.

Note: To calculate the volume of Rose Bengal dye needed for a specific animal, reference the guidance in the "before you begin" section.

27. Sustain illumination for 30 min.
28. Turn off the light source and wait 3 h before removing the photomask.

⚠ CRITICAL: Removing the photomask too soon may cause residual Rose Bengal dye in the cortical vasculature to activate; therefore, inducing unwanted lesions.

29. Acquire OCTA images of the cortical surface through the artificial dura.

Note: Reference the "optical coherence tomography angiography imaging" section for more details on this procedure.

Note: OCTA imaging after inducing a lesion will be used to visualize microvascular occlusions through reduced blood flow at the site of lesioning.

Troubleshooting: If lesioning is unsuccessful, refer to problem 1.

Histology and immunohistochemistry

Timing: approximately 6 days

NeuN and Nissl staining are performed to validate tissue damage following lesioning. Cortical regions lacking the Nissl staining validate cellular death while regions lacking NeuN staining validate neuron specific death.

30. Place the animal in a fume hood and begin the perfusion process.

Note: The animal remains heavily sedated at this point in the procedure which is confirmed by a corneal response test.

a. Perform transcardiac perfusion with 1.5–2L of 20°C saline using a rotary pump (Mec-O-Matic Peristaltic Pump, Qcvialz).

Note: After the 2L of saline is delivered, the fluid should become clearer, signaling the transition point to the fixative

b. Transition perfusion solution to 4% paraformaldehyde and deliver 4L at a medium to high speed followed by 2L at a slower speed over the course of 30–45 min

31. Extract and immerse the brain in 4% paraformaldehyde solution for 24–48 h at 4°C.
32. Dissect the brain into a single 25 mm thick coronal block containing the region of interest using a matrix (Ted Pella Inc., Prod# 15039) to guide dissection.

⚠ CRITICAL: This step should be performed in a chemical fume hood.

33. Cryoprotect the blocks by submerging them in a 30% sucrose in PBS solution at 4°C until the tissue has sunk (minimum of 2–3 days).

Pause point: The brain may be stored in the sucrose solution for prolonged periods of time.

34. Freeze the block and section into 50 μm coronal slices using a sliding microtome.
35. Store cut sections in 1X PBS solution with 0.02% sodium azide at 4°C.
36. Perform Nissl staining on mounted serial coronal sections using Thionin Acetate.

⚠ CRITICAL: Perform in a chemical fume hood.

⚠ CRITICAL: Filter Thionin before each use.

Note: Cresyl violet can be used as an alternative.

a. Create thionin acetate solution according to the recipe in the materials and equipment section. Store solution at 4°C for up to 1 year.
b. Mount the coronal slices onto gelatin-coated glass slides.
Troubleshooting: If mounting results in tissue folding, refer to problem 2.
c. Place each slide into a slide rack and safely store to air dry for 24 h.
d. Defat sections in a 1:1 mixture of Chloroform and 100% ethanol for 3 h minimum.

e. Wash and stain tissue sections using the following protocol:
   i. 100% ethanol: 30 s
   ii. 100% ethanol: 30 s
   iii. 95% ethanol: 30 s
   iv. 70% ethanol: 30 s
   v. 50% ethanol: 30 s
   vi. dH2O: 30 s
   vii. Thionin acetate: 1 min
   viii. 50% ethanol: 1 min
   ix. 70% ethanol: 1 min
   x. 90% ethanol: 1 min
   xi. 100% ethanol: 1 min
   xii. 100% ethanol: 1 min
   xiii. Xylene: 5 min
   xiv. Xylene: 5 min
   xv. Xylene: 5 min
f. Immediately coverslip with mounting medium.
g. Clean off the excess mounting medium on the slide with q-tips soaked in xylene.

Note: To avoid inhaling any xylene, let the staining trays dry within the fume hood before cleaning.

h. Once the mounting medium is dry, clean off excess xylene and store slides in boxes.
   Troubleshooting: If the contrast between lesioned and non-lesioned areas is unclear, see problem 3.

37. Immunostain adjacent slices for selective neuronal marker NeuN.
   a. Rinse with 1X PBS (3 × 5 min) at 20°C on a rocker.
   b. Make 1% $NaBH_4$ (sodium borohydride) solution (1 mL per tissue slice).
   c. Incubate the tissue slices in the $NaBH_4$ solution for 1 h at 20°C on a rocker.
   d. Rinse with 1X PBS (3 × 5 min) at 20°C on a rocker.
   e. Incubate in 10% normal donkey serum in 1X PBS containing 0.1% Triton X-100 at 4°C for 24 h
   f. Make anti-NeuN (Millipore-Sigma, Cat# MAB377) primary antibody mixture in normal donkey serum at a concentration of 1:500 (1 mL per tissue slice).
   g. Incubate the tissue slices in the primary antibody solution at 4°C for 72 h on a rocker.
   h. Rinse sections with 1x in PBS (3 × 10 min) at 20°C on a rocker.
   i. Make Alexa Fluro 488 (Invitrogen ReadyProbes, Cat# R37114) secondary antibody mixture in normal donkey serum at a concentration of 1:500 (1mL per slice).
   j. Incubate tissue slices in the secondary antibody mixture for 24 h at 4°C on a rocker.
   k. Rinse sections with 1x PBS (5 × 5 min) at 20°C on a rocker.
   l. Mount the sections using a 1:1 glycerol:PBS solution.
      Troubleshooting: If mounting results in tissue folding, refer to problem 2.
   m. Image using Nikon 6D wide-field automated microscope system

Note: Alternative imaging systems may be used with consideration of differences in spatial resolution.

EXPECTED OUTCOMES

This protocol provides tools to study ischemic stroke including focal ischemic lesioning with the photothrombotic technique, neurophysiology recording during and after stroke with a semitransparent ECoG array, vascular imaging with OCTA for *in vivo* lesion validation, and Nissl and NeuN staining for histological validation.

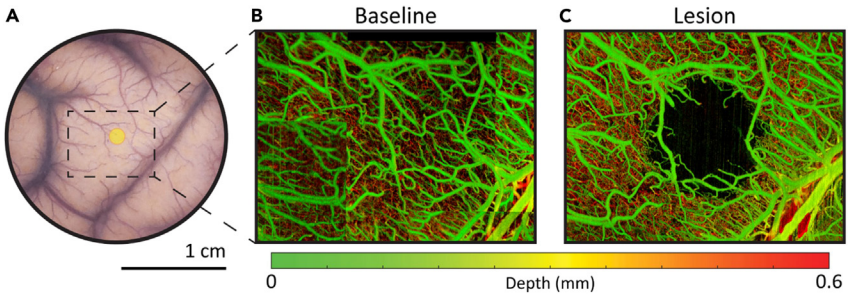

Figure 5. Optical coherence tomography angiography (OCTA) validation of lesion induction
(A) Surface of the sensorimotor cortex visualized through the artificial dura.
(B and C) (B) OCTA imaging of the rectangular area prior to lesion induction and (C) 3 h post-photothrombosis.
Figure adapted from Khateeb et al., 2019.[33]

Following photothrombotic lesioning, *in vivo* validation can be performed with OCTA imaging. Figure 5 shows OCTA imaging 3 h post-photothrombosis compared to baseline imaging of the same area. This technique allowed us to image blood flow in the cortical microvasculature, validate occlusion *in vivo*, and measure lesion sizes post hoc. Obstruction of blood flow is only expected to occur in the cortical region exposed to light as we see here. This method of *in vivo* validation allows future behavioral experiments to be carried out with an understanding of the region and extent of lesioning performed.

Histological analysis can be performed to validate neuronal loss in addition to microvascular disruption. Figure 6 shows histological staining of coronal slices. Prior to staining, bright pink areas were observed in illuminated areas (Figure 6A) suggesting thrombi occluded the vasculature, entrapping the pink-colored Rose Bengal dye. This measure shows success of the photothrombotic technique to obstruct the microvasculature. Furthermore, Nissl staining was performed and cell death was confirmed by the areas lacking pigment (Figure 6B). To identify neuronal loss, NeuN staining was performed with similar outcomes (Figure 6C). If these expected outcomes are not seen, lesion induction was most likely unsuccessful.

Continuous neural activity was recorded through the MMAD. We recorded 30 min of baseline activity, 30 min of activity during illumination, and 3 h following illumination. Expected outcomes include reduction in gamma band (30–59 Hz) signal power from LFPs in electrodes within and surrounding the illumination site (Figures 7C and 7D). This reduction began at the time of illumination and persisting past the end of illumination (Figure 7E). This reduction of gamma band signal can be monitored before, during and after lesion formation across the network (Figure 7E). Additionally, this method can be used in the future as another *in vivo* minimally invasive validation method for lesion induction.

QUANTIFICATION AND STATISTICAL ANALYSIS

Analysis of ECoG recordings

1. LFPs recorded at 30 kHz by the ECoG array were down sampled to 1 kHz in MATLAB (MATLAB R2020a, MathWorks) for power analysis
2. Signals were notch filtered at 60, 120, 180, and 240 Hz, and were bandpass filtered to isolate theta (4–7 Hz), gamma (30–59 Hz) and high gamma (60–150 Hz) bands.
3. After normalizing the discrete time series of each signal, samples with an amplitude exceeding 25 standard deviations were identified as artifacts and excluded from analysis.
4. Power spectral densities were calculated, and channels that did not exhibit the expected $1/f^x$ curve were excluded from analysis.

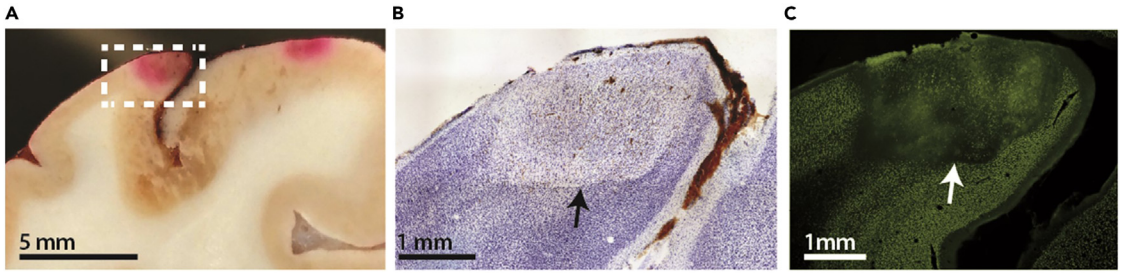

Figure 6. Histological lesion validation

(A) Unstained coronal section. Pink regions corresponding with illuminated regions indicate the presence of Rose Bengal entrapped in the cortical microvasculature.

(B) Nissl staining. Lesion is approximately 2 mm in diameter.

(C) NeuN staining of the same lesion in B.

Figure adapted from Khateeb et al., 2022.[6]

5. The signal power for each frequency band was calculated for each electrode over the course of the baseline and final 30 min of post-illumination.
   a. Signal power was calculated by squaring the filtered time-series signal and dividing by the elapsed time.
   b. Change in signal power was calculated by subtracting mean baseline power from the mean post-illumination power.
   c. A left-tailed paired t-test between the 30 min of baseline power and the final 30 min of post-illumination power was calculated at 60 s intervals.

      Note: family-wise error rate $\leq$ 0.001 was used to account for multiple comparisons.

   d. Channels were characterized in a lesioned group (decreasing power) and a non-lesioned group (failed to show decrease in power).
6. A one-way ANOVA was used to compare average change in power of channels across the two groups at a significance level of 0.05.
7. The time-varying power across all recording periods was calculated in 10 s intervals and smoothed (smoothing factor of 0.4–0.5).
   a. the power time-course for each individual channel was normalized to baseline mean and standard deviation.

LIMITATIONS

While the presented tools offer great advantages for investigating physiological dynamics in the context of a cortical lesion, there are limitations that can be addressed in later studies. Overall, the reliance on optical access for lesion induction limits this technique to the cortical surface, unless the neural tissue is compromised with penetrating optical probes.

All experiments described in this protocol were limited to anesthetized animals. Recording neural activity in anesthetized animals is inherently affected by anesthesia which can alter neural communication. The neural recordings obtained during lesion induction may not be representative of neural activity after stroke in an awake, behaving animal. Although this protocol describes the implementation of these techniques in an acute setting, the same procedures can be used to induce a focal lesion for chronic stroke studies using previously described chronic optical interfaces.[1–4]

The MMAD presented here exhibits limited spatial resolution with 32 opaque electrodes across an area of approximately 314 mm^2 and an electrode pitch on the order of millimeters. An array with electrodes in a more compact arrangement would achieve greater spatial resolution, yet would

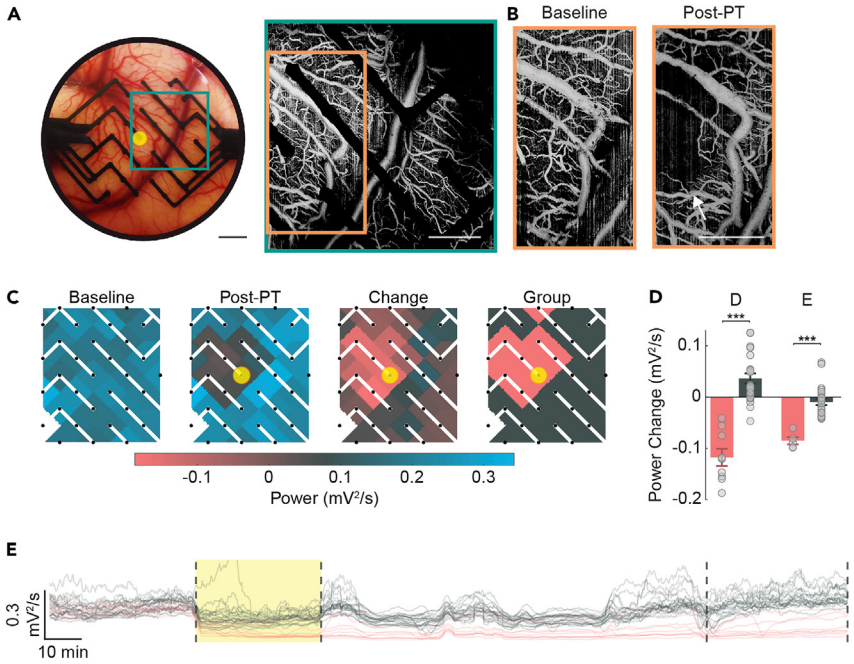

Figure 7. Large-scale MMAD recording of neural activity before, during, and after photothrombotic (PT) lesion induction

(A) Location of lesioning (yellow dot) during neural recording with the MMAD. Scale bars are 3 mm (right) and 2 mm (left).

(B) OCTA images before and after lesioning. Scale bars are 3 mm (right) and 2 mm (left).

(C) Gamma-band power (30–59 Hz) calculated from 30 min before and 3-h post-photothrombosis for each channel across the array. The yellow dot indicates location of stroke induction.

(D) Average change in power for electrodes in the lesioned area (red) versus non-lesioned area (black) in two animals (animal D and animal E) (error bars denote ± standard error of the mean [SEM]; one-way ANOVA, monkey D: p = 5.2x10^-9 , monkey E: p = 1.0x10^-5).

(E) Example of neural recording (Gamma power) through the ECoG array as the lesion was induced. Illumination period is shown in yellow. Red traces show electrodes in the lesioned area while black traces show non lesioned areas. This figure has been adapted from Khateeb et al., 2022.[6]

obscure a greater percentage of cortical area. Although the electrodes are embedded in a transparent medium, the electrodes themselves are opaque, which can occlude optical penetration of cortical tissue for OCTA imaging and photothrombotic lesion induction. This concern establishes the need for smaller electrodes to be used as electrode density increases.[23] Alternatively, transparent indium-tin oxide electrodes can be considered.[16] The size of our electrodes also limits our recording capabilities to only capture LFPs; however, smaller electrodes would allow for multi-unit activity to be recorded.[32]

TROUBLESHOOTING

Problem 1

Lesioning may be unsuccessful if aperture sizes were too small or light intensity was too low to initiate photothrombosis. A model of lesioning with this technique has been made available and should be used when considering aperture sizes.[6,18,33] Failure to create a lesion can be elucidated at the time of surgery *in vivo* only by analysis of gamma power through electrical recording as defined in step 21. Alternatively, OCTA imaging is able to show unsuccessful lesions as described in step 29, however image processing time may restrict this validation technique until after the surgery has been completed.

Potential solution

Lesions can be re-induced by reinjecting the animal with Rose Bengal and applying a higher intensity of illumination, increasing the aperture diameter, or both. If the lesion is identified to be unsuccessful

at the time of surgery, reinduction of the lesion can be performed immediately in the same subject. If a chronic window is being used, lesioning can be re-attempted through the optical window at a later date.

Problem 2

During histology and immunohistochemistry procedures, tissue mounting on glass coverslips can result in folding during steps 36-b or 37-L. This occurs when transferring the floating tissue section to the glass slide and must be resolved prior to analysis.

Potential solution

Apply excess PBS to the area of interest to ease manipulation using the brush. Determine if the tissue folded under or over itself. Use a smaller brush to straighten out the tissue. If the tissue is folded under itself, gently brush the tissue inwards and then outwards repeatedly until the tissue is straightened out. If the tissue is folded over itself, gently brush the tissue outward until the tissue is straightened out.

Problem 3

During the histology staining procedure at step 36-h, the contrast between lesioned and non-lesioned areas in Nissl-stained sections may not be clear. Leaving the tissue immersed in thionin acetate for an inadequate amount of time will either cause the staining to be too dark or too light. Both errors result in unclear boundaries between dead and alive tissue.

Potential solution

Altering the time of tissue immersion in Thionin Acetate during Nissl staining will alter the extent to which the tissue is stained. If the tissue is too lightly stained, increasing the immersion time will help increase the contrast between lesioned and non-lesioned areas. Alternatively, if the tissue is stained too heavily, reducing the immersion time may also help increase the contrast.

Problem 4

During electrical recording at step 19, line noise may negatively impact the quality of signals obtained through the MMAD. This noise can come from a variety of sources, of which may be unclear.

Potential solution

Unplug all equipment in the operating room from wall outlets that is not required for the procedure or animal care. This can include unplugging the animal heating pad and using heated rice bags instead, or relying on battery-powered equipment.

Problem 5

Signal artifacts may arise for various reasons during ECoG recording at step 19. Signal artifacts are typically marked by stark increases in LFP amplitude that can last up to a few seconds.

Potential solution

Signal artifacts may arise by coming in physical contact with the recording equipment or the animal during recording. This can be avoided by reducing the number of times physical contact is made with the recording equipment and the animal. To aid with artifact detection *post-hoc*, it is helpful to note when contact is made.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead and technical contact, Azadeh Yazdan-Shahmorad (azadehy@uw.edu).

Materials availability

STL file for the 3D printed connector holder and light shield disk for non-ECoG recording stroke induction are provided. The MMAD, which was custom made for this study, was manufactured by Ripple Neuro. The OCTA equipment used in this study was made available by the Biophotonics and Imaging Laboratory at the University of Washington Bioengineering department lead by Ruikang (Ricky) Wang.

Data and code availability

Code for power analysis and the photothrombotic quantitative model have been published to Zenodo. Zenodo: https://doi.org/10.5281/zenodo.5963338.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102496.

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AUTHOR CONTRIBUTIONS

K.K. drafted the original manuscript. A.Y.-S. conceptualized the study. A.Y.-S. and K.K. designed the experiments. A.Y.-S., K.K., and J.Z. performed the experiments. R.K.W. performed the optical imaging setup and data collection. K.K. and J.Z. performed data analysis. N.S. drafted the experimental protocol presented here. All authors contributed to the writing and editing of the experimental protocol presented here.

DECLARATION OF INTERESTS

R.K.W. discloses intellectual property owned by the Oregon Health and Science University and the University of Washington. He is a consultant to Carl Zeiss Meditec.

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