Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE57220R2
Full Title:	In vivo fiber-coupled pre-clinical Confocal Laser-scanning Endomicroscopy (pCLE) of hippocampal capillaries in awake mice
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TITLE:

In Vivo Fiber-Coupled Pre-Clinical Confocal Laser-scanning Endomicroscopy (pCLE) of Hippocampal Capillaries in Awake Mice

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KEYWORDS:

in vivo fiber-optic confocal microscopy, vasospasm, mural cell, pericyte, hypoxia, neurodegeneration, hippocampus, capillary, blood flow, excitotoxicity, Kv1.1, KCNA1-null, epilepsy

SUMMARY:

Whereas multiphoton imaging is only effective at limited depths from the tissue's surface, it is possible to achieve 3 µm resolution imaging at any depth via pCLE. Here, we present a protocol to conduct pCLE imaging to measure microvascular dynamics in the hippocampus of ictal and wild-type mice.

ABSTRACT:

The goal of this protocol is to describe fiber-optic-bundle-coupled pre-clinical confocal laser-scanning endomicroscopy (pCLE) in its specific application to elucidate capillary blood flow effects during seizures, driven by mural cells. In vitro and in vivo cortical imaging have shown that capillary constrictions driven by pericytes can result from functional local neural activity, as well as from drug application, in healthy animals. Here, a protocol is presented on how to use pCLE to determine the role of microvascular dynamics in neural degeneration in epilepsy, at any tissue depth (specifically in the hippocampus). We describe a head restraint technique that has been adapted to record pCLE in awake animals, to address potential side-effects of anesthetics on neural activity. Using these methods, electrophysiological and imaging recordings can be conducted over several hours in deep neural structures of the brain.

INTRODUCTION:

In contrast with other microscopic imaging methods[1-8], in vivo fiber-optic-based confocal microscopy allows the measurement of blood flow dynamics in any brain region, at any depth, at high speed (up to 240 Hz depending on field-of-view size[9]). A fiber-optic probe enables in vivo confocal laser scanning imaging at 3 µm resolution because the tip of the probe (a lens-less objective made up of a bundle of 5000-6000 3 µm diameter individual fibers) can be positioned with a microelectrode's accuracy, within 15 µm of the fluorescent target of interest. As with in vivo two-photon imaging, fluorophores must be previously introduced into the imaging target. For example, fluorescein dextran (or quantum dots) may be injected into the vasculature, or genetically-encoded fluorescent proteins can be transfected into cells, or fluorescent dyes such as Oregon Green BAPTA-1 can be bulk-loaded into cells, prior to imaging.

Recent research using these techniques has found that mural cell motor activity leading to ictal capillary vasospasms—sudden constrictions that occur at the position of the mural cells during seizures[9]—can contribute to neurodegeneration in the ictal hippocampus[9]. Whereas previous imaging studies showed in vitro and in vivo pericyte constrictions connected to drug applications[6-7,10-12], Leal-Campanario et al. found the first evidence of in vivo spontaneous capillary constrictions in the murine brain. To establish relevance to human temporal lobe epilepsy, they studied male (P30-40 old) knockout (KO) Kv1.1 (kcna1-null) mice[14-15] (JAX stock #003532), a genetic model of human episodic ataxia type 1[15]. Pericytes drove both pathological and physiological hippocampal mural vasoconstrictions[9] in the spontaneously epileptic animals and their wild-type (WT) littermates. These observations were replicated in WT animals rendered epileptic with kainic-acid, thereby indicating their generalization to other forms of epilepsy. Leal-Campanario et al moreover determined, using novel stereological microscopy approaches, that apoptotic—but not healthy—neurons in epileptic animals were spatially coupled to the hippocampal microvasculature. Because excitotoxicity has no known spatial association to the vasculature, this result indicated that abnormal capillary vasospasmic ischemia-induced hypoxia contributes to neurodegeneration in epilepsy. **Figure 1** shows a schematic of the general setup.

PROTOCOL:

The protocol follows the NIH Guidelines for the Care and Use of Laboratory Animals. All procedures were approved by the Barrow Neurological Institute's Institutional Animal Care and Use Committee.

1. Stereotaxic positioning for craniotomy

1.1. Weigh and then anesthetize the mouse with a ketamine-xylazine (100 mg/kg–10 mg/kg i.p.) cocktail. Ensure that the animal is fully anesthetized by observing its lack of reaction to tail and/or toe pinch.

1.2. Place a heating pad under the mouse and use a rectal thermometer to continuously monitor its body temperature during the initial anesthetized implantation surgery (**Figure 2A**).

1.3. Apply ophthalmic ointment to prevent eye dryness.

1.4. Stabilize the head of the animal in a rodent stereotaxic frame, outfitted with a mouse adapter. To properly orient the mouse in the stereotaxic frame, place the animal's teeth inside the hole of the bite-bar (**Figure 2A** and **Figure 3A**, item g). Tighten the nose-clamp until it is snug (**Figure 2A,B** and **Figure 3A**, item h). The mouse's body positioning should be as straight as possible (as in **Figure 2A**).

1.4.1. Further secure the animal's head using mouse ear-bars with jaw holder cuffs (**Figures 2A** and **Figure 3A**, item i), to firmly clamp the zygomatic processes of the skull.

NOTE: Once secured, the mouse's head should have no range of horizontal motion.

1.5. Once the mouse is adjusted to the stereotaxic frame (**Figure 3B**, item j), clean and sterilize the scalp by wiping it with chlor-hexedine scrub 2-3 times, each time followed by an alcohol rinse. Then, apply topical lidocaine on top of the head.

1.6. Make an incision along the midline from posterior to anterior (start incision at the base of skull and complete it between the eyes; 12-15 mm) along the sagittal midline of the skull, using either fine scissors or a blade. Retract the borders of the scalp with four 28 mm bulldog serrefine clamps to expose the skull and maximize the working area (**Figure 2B** and **Figure 3C**).

1.7. Use a scalpel or microspatula (**Figure 3C**) to scrape the periosteum to the incision's edges. Use tissue-separating scissor or forceps (**Figure 3C**) to carefully retract the muscles at the back of the neck (**Figure 2B**).

1.8. Dry the top of the skull with a cotton-tipped applicator, moistened with alcohol or 30% hydrogen peroxide, to optimize visualization of the cranial sutures. Apply saline to the skull once the connective tissue has been removed.

1.9. Once the mouse's head is stabilized in the stereotaxic apparatus, place a syringe needle tip (21 G) in the electrode holder (**Figure 3B**, item l), and attach the electrode holder to the stereotaxic micromanipulator, lowering the needle tip to the level of the skull (**Figure 2D**).

1.10. Adjust the medial-lateral direction of the skull by measuring the height of the skull at any distance posterior to bregma (i.e., -2.0 mm), and two equal distances lateral from the midline on either side of the skull (i.e., ± 1.5 mm) (**Figure 2D**). Aim for a height difference across locations that is less than 0.05 mm in the dorsal-ventral direction.

1.10.1. If greater than 0.05 mm, rotate the mouse's skull using the bite bar, or reposition the mouse's head by loosening the ear-bars carefully, laterally shifting the head and ear-bars as needed, and then retightening the ear-bars.

1.11. Once the skull is positioned in the stereotaxic device, determine and record the stereotactic coordinates of the bregma and lambda skull sutures along the anteroposterior (AP),

medial lateral (ML), and dorsal ventral (DV) axes.

1.12. Identify the target point (right hippocampus) with the aid of a stereotaxic atlas. Then, find on the skull the target point coordinates relative to bregma (AP: -2.7 mm, ML: -2.7 mm) (**Figure 2E**).

1.13. Using a surgical marker, mark four corners of a 1.4 mm x 2 mm imaging window on the skull with dots, centered around the target point directly above the hippocampus (**Figure 2F**), for subsequent craniotomy.

1.14. Use the surgical marker to draw two additional dots: one over the top-left parietal bone (**Figure 2G**) and another over the right frontal bone (**Figure 2H**), to indicate the future placement of two bone screws (**Figure 3A**, item b) that will anchor the head-cap (**Figure 3B**, item m) to the skull.

1.15. Use the surgical marker to draw three more dots indicating where the epidural EEG recording electrodes will be inserted: one dot positioned 1 mm to either side of the midline, and one additional dot on the midline positioned 1 mm behind Lambda (**Figure 2E, Figure 4A** and **Figure 5B**).

2. Head-Cap installation

2.1. Using a 0.7 mm diameter burr (**Figure 3C**), carefully drill two small holes in the skull, at the dot positions indicating the bone screws placement (see step 1.15 above). Then, take two bone screws (stainless steel-slotted, length: 4 mm; diameter: 0.85 mm) previously disinfected with ethanol 70-80%, and screw them to the skull for additional head-cap stability (**Figure 2I-J** and **Figure 3A**, item b).

2.1.1. Ensure that the screw tips do not protrude beyond the bottom of the bone into the skull cavity, risking damage to the brain. Note that one of the two bone screws will be anterior to the two head-cap screws, and the other posterior to them (**Figure 2G-J**).

2.2. Squirt saline over the skull to cool it down and clean it. Then dry the skull completely and apply a thin layer of cyanoacrylate around the screws.

2.3. Use a custom alignment piece (**Figure 3A**, item c) clamped to the microdrive mounted to the stereotaxic device (**Figure 2F-J** and **Figure 3A**, item d), so that the position of the head-cap is aligned along the midline, with the anterior ridge overlying bregma. This custom stainless-steel piece is L-shaped (angled at 90°), with two holes separated by 4 mm (**Figure 2F-J** and **Figure 3A**, item c).

2.3.1. Use the stereotaxic manipulator to position the L-shaped alignment piece, with two Fillister Head Slotted drive screws attached (M1.6 x 0.35 Metric Coarse, 12 mm Length; **Figure 3A**, item a) over the bregma, until the base of the screws lies flat on the skull, being careful to

not exert pressure on the skull (**Figure 2I-J**).

2.4. Apply cyanoacrylate followed by dental cement to attach the base of the screws to the skull. Be careful to not obstruct the imaging window reference marks over the hippocampus (**Figure 2K**).

3. Imaging window craniotomy surgery

3.1. At each of the positions of the craniotomy reference marks that indicate the corners of the imaging window (see step 1.14 above), drill a 0.7 mm diameter burr hole, followed by gently delineating the periphery of the 1.4 mm x 2 mm craniotomy window with the drill, with iteratively deeper cuts (**Figure 2L**).

3.2. Take care to not to drill too deeply or with too much force on the skull, which is thin and fragile, having a thickness of approximately 300 µm. Once the bone flap is complete, pry the loose flap up with the tip of a scalpel, being mindful to not damage the underlying dura mater.

3.3. Cover the open window with bone wax (**Figure 2M**).

3.4. Using a 0.9 mm diameter burr, drill three holes for future placement of the epidural EEG electrodes (see step 1.15 above) taking care to not to cut through the dura mater (**Figure 2E, L**).

3.5. Cover the three holes with bone wax.

3.6. Gently apply cyanoacrylate around the edges of the bone-wax covered imaging window and EEG holes, being careful to not seal the craniotomies (**Figure 2L**).

3.7. Build a wall of dental cement that encompasses the imaging window and EEG holes and binds it to the previously cemented head-cap (**Figure 2M** and **Figure 3B**, item m).

3.8. Let the cement dry for at least 10 minutes.

3.9. Close any loose wound margins with simple interrupted sutures, using 4-0 or 5-0 black braided silk (**Figure 2N** and **Figure 3C**).

4. Post-surgery

4.1. Use a cotton-tipped applicator to apply lidocaine ointment around the exposed skin to deaden sensation around the wound's edge.

4.2. Use a sterile cotton-tipped applicator to apply Neosporin to the exposed skin.

4.3. Remove the animal from the stereotaxic frame (**Figure 2N**).

4.4. Inject ketoprofen (5 mg/kg) IP or IM for post-surgical analgesia.

4.5. Observe the animal until it is alert and moving (this will usually occur within minutes).

4.6. Place the animal in a warm cage and continue to monitor its recovery until it drinks or eats and is ready to transfer to animal housing.

4.7. Check on the animal at least once daily for approximately one week after the surgery, watching for any signs of listless behavior and/or inadequate eating/drinking (Figure 2O).

5. Dye injection and EEG recording

5.1. Wait a minimum of one day after the head-cap implantation surgery to initiate the recordings.

5.2. Place the mouse in a foam-restraining device molded to its body, within the stereotaxic frame (**Figure 3B**, items n & j).

5.3. Secure the head-cap (**Figure 3 B-I**, item m) to a custom mounting bar which is attached to the stereotaxic frame (**Figure 3A,B**, items e & j, and **Figure 4A**). The long section of item e (**Figure 3A,B**) should have a length between 9.4 – 13 mm. Fix item f (a mounting plate with dimensions 1.5 cm x 0.5 cm, with two holes separated 4 mm from center to center) to item e (**Figure 3A,B,** items e, f and k). This alignment system will position the awake mouse securely to the stereotaxic frame throughout the imaging sessions (**Figure 4**).

5.4. Insert the epidural EEG recording electrodes (**Figure 5A**), placing the ground electrodes (white wires) within the posterior central hole, and the recording electrodes (red wires) in the anterior left and right holes (**Figures 2E, 4B and 5B**). Position each of the wires at an L-shape between the skull and the dura mater (**Figure 5C**) to optimize electrical contact.

5.5. Inject the tail vein with green fluorescein lysine-fixable dextran (0.2 mL/25 g body wt. of Fluorescein 5% w/w), to visualize blood flow within the hippocampus.

5.5.1. To enhance dilation of the central vein of the mouse's tail previous to injection, use one or more of the following strategies: a) Apply 70% ethanol to the central vein using a cotton-tipped applicator; b) Dip the tail into warm water (35-40 °C) for 1-3 minutes; c) Expose the tail to a heating lamp for a few minutes.

5.5.2. Secure the mouse's tail with two fingers and locate the central vein, which lies immediately below the skin. Insert the needle (ultrafine insulin syringe with integrated needle 30G), with the bevel up, into the vein, approximately halfway to two thirds from the base of the tail. Keep the needle parallel to the tail during the injection.

5.5.3. Inject the dye slowly and steadily, taking care to avoid any changes in the position of the

needle or tail.

NOTE: There should be no resistance upon depressing the syringe plunger.

5.5.4. After completing the injection, withdraw the needle and apply gentle pressure to the puncture site to seal the vessel.

5.5.5. Allow clotting to occur.

6. In vivo fiber-optic-bundle-coupled pre-clinical confocal laser-scanning endomicroscopy (pCLE)

6.1. Directly following the tail vein injection (step 5.5.3 above), clamp the 300 µm beveled fiber-optic bundle (containing 5000–7000 3 µm fibers in each bundle) in the downward orientation to the mobile arm of the robotic stereotaxic drive.

6.2. Remove the bone wax from the craniotomy.

6.3. Remove the dura, using the bent tip of a syringe needle.

6.4. Using the robotic positioning system, first move the fiber objective to the appropriate anterior-posterior and left-right stereotaxic location, with the tip zeroed in the z-axis on the surface of the cortex.

6.5. Initiate the EEG recording (**Figure 5D**).

6.6. Initiate confocal laser-scanning of the back plane of the fiber-objective, followed directly by descending the objective slowly in the z-axis using the robotic microdrive's z-controls, until it reaches the target depth within the brain (**Figure 4C**). View the imaging results in the microscopic software while descending.

6.6.1. If the fiber tip is within the target X-Y-Z recording region, and two or more vessels enter the imaging window's FOV, stop the descent and initiate video recordings. Obtain live full-field time-lapse movies of the blood flow at 11.7 Hz using Cellvizio Lab's imaging software. (Higher speeds may be obtained with a smaller FOV). Recordings may take place for 4-5 hours at a time, in consecutive days if needed.

6.7. Using a 10 mL syringe with beveled silicon tubing fixed to the tip of the needle, feed the animal during the recordings by periodically providing paste made with mice pellets ground with water.

6.8. At the end of the imaging session, terminate the recordings.

6.9. Gently remove the EEG leads and clean them with Tergazyme.

6.10. Slowly remove the fiber-optic bundle from the animal's brain by reversing the fiber along the z-axis of entry.

6.11. Release the animal from the head post and body restraints.

6.12. If appropriate, follow the necessary euthanasia and tissue processing protocols to visualize blood vessels and to perform immunohistochemistry.

6.13. If planning to record from the same animal in a future session, cover the imaging window with bone wax or silastic.

REPRESENTATIVE RESULTS:

We developed these methods to assess whether abnormal pericyte-driven capillary vasospasms in the hippocampus—occurring as the result of seizures—could cause frank hypoxia that contributes to cell death in the ictal focus[9,13].

The development of the head-cap and its proper installation afforded high-stability in the recordings, allowing simultaneous recording of EEG and blood flow deep in the hippocampus of wild-type and epileptic awake mice during both ictal and interictal periods. Capture of blood flow events related to seizures requires recording for extended periods of time, so that aperiodic blood flow events (such as vasospasms) can be captured in response to both induced and naturally occurring epileptic seizures. The restraint system permitted stable blood flow recordings of deep brain microvessels and their proximal mural cells over long hours (**Figure 6A-R**). We found that, in entire microvessels, blood flow stoppages occurred at the positions of labelled mural cells.

We reliably located vessels in the hippocampus using a robotic stereotaxic device targeted with internal coordinates from a standard stereotaxic atlas. We verified the quality of the fiber recordings by comparing them to high-resolution two-photon imaging recordings of blood flow and equivalent vasospasms from cortical tissue, at depths that two-photon imaging can reach (**Figure 6S-AA**). We further verified the pCLE results using immunohistochemistry at recording sites, to show with traditional confocal microscopy that hippocampal mural cells were targeted correctly and that they were not only constricting but also spatially associated with strictures in microvessels far from arterioles[15] (**Figure 7**).

The published findings with these methods indeed show abnormal hippocampal capillary vasospasms driven by seizures, as well as correlated cell death in Kv1.1 epileptic mutant mice and kainate model epileptic mice[9,13]. These results indicate a role for local ictal ischemia/hypoxia in cell death during seizures, due to abnormal vasospasms, and contribute to a growing body of work that expands on the potential mechanisms of ictal cell death.

FIGURE AND TABLE LEGENDS:

Figure 1. Schematic representation of the experimental design. We recorded EEG while conducting confocal microscopy in hippocampal capillaries of both awake epileptic mice and WT littermates. After injecting the tail vein with fluorescein, we used a novel preclinical Confocal Laser-scanning Endomicroscopy (pCLE), with a fiber-objective with a tip diameter of 0.3 mm to record capillary blood flow dynamics deep in the brain. Yellow indicates increased blood flow, and red indicates steady or reduced flow.

Figure 2. Protocol for head-cap implantation surgery. A) The anesthetized animal was placed on a heating pad and positioned within the stereotaxic frame (see **Figure 3B**, item j) and secured with mouse ear-bars (**Figure 3A**, item i) and jaw holder cuffs (**Figure 3A**, items g,h), over a heating pad. **B**) Exposure of the cranial sutures. **C-D**) Measurements of Bregma and Lambda. **E**) Visualization of drilling locations over the animal's skull. The black points and square indicate the window over the hippocampus that will permit the pCLE insertion. The two red circles mark the insertion positions for the EEG recording wires. The white dot marks the insertion point of the EEG ground wires. **F**) The four black dots on the skull mark the future corners of the imaging window craniotomy over the hippocampus. The custom alignment piece (**Figure 3A**, item c), with the headpost implants (**Figure 3A**, item a) attached to the stereotactic microdrive positioner (**Figure 3A**, item d). **G-H**) Two additional dots, one posterior (**G**) and one anterior (**H**) mark the future location of head-cap anchor screws (**Figure 3A**, item b). **I-J**) Two small screws anchor the head-cap to the skull (**Figure 3A**, item b). **K**) Cyanoacrylate and dental cement are applied to the anchor screws from panels I & J. **L**) The imaging window craniotomy over the hippocampus, and the three holes for the EEG electrodes, have been drilled. **M**) The imaging chamber is built (**Figure 3B**, items i, m) by sculpting a berm with additional dental cement to encircle the three EEG holes and the imaging window. **N**) Overhead view of the finished head-cap. **O**) The recovered mouse back in its cage after the implantation surgery.

Figure 3. Materials used in the Protocol (specific items enumerated with lower-case letters). A) Materials used to build the head-cap. (**a**) Two M1.6 x 12 mm machine screws (the headpost implants). (**b**)Two small bolts to secure the head-cap to the skull. (**c**) A custom L-shaped stainless-steel alignment piece, with two holes 4 mm apart, which secures the two M1.6 screws during the surgery. (**d**) A standard microdrive mounted on a stereotaxic device holds item c. (**e**) A custom mounting bar with an (**f**) alignment piece that matches the two holes from item c. (**g**) A bite bar and (**h**) nose clamp to stabilize the position of the animal's head. **B**) Setup during the recordings. (**i**) The head-cap is secured to item f during the anesthetized implantation surgery. (**j**) A stereotaxic frame positions the microdrive (item d). (**l**) A 21G needle is attached to the microdrive (item d) and used to determine the positions of the Bregma and Lambda cranial sutures. (**m**) A simulated head-cap is pictured here, attached to item f, to demonstrate the positioning of the mouse in (**n**) the foam-lined restraining-tube (mouse not present). Item n molds itself to the shape of the mouse's body. **C**) Instruments and supplies needed to perform the surgery.

Figure 4. Recording session setup. A) The animal's head is fixed. **B**) Location of the EEG recording wires. White EEG wires are placed separately, in each side of the skull. Red EEG wires are placed together in the middle-bottom (ground) hole. **C**) The fiber-optic bundle is inserted into the target window.

Figure 5. EEG recordings. A) Epidural EEG recordings are conducted with Physiotel F20-EET transmitters, simultaneously to the fiber-optic pCLE recordings in the awake animal. **B**) Location of the drilled holes for insertion of the leads for EEG recordings. The two red leads go to the two holes indicated by red dots. The two white leads are inserted together in the hole indicated by the white dot. The red square designates the target window over the animal's hippocampus. The letter '*B*' on the mouse's skull indicates the bregma. **C**) The EEG recording wires are bent in an L-shape to enhance electrical contact between the skull and the dura matter. **D**) Examples of EEG recordings in awake mice.

Figure 6. Recordings of mural cell vasoconstrictions in mice. A-R) In vivo fiber-optic dual-band pCLE image of capillary vasospasm (vessels in green, labeled with 2MD fluorescein-conjugated dextran) colocalized to mural cells (red, labeled via intravenous vein tail injection of Alexa Fluor 647) in awake knockout mice during seizure (arrow indicates mural cell and associated vasospasm). See **Video 1** and **Video 2**. **S-W**) In vivo two-photon scanning laser microscopy (TPLSM) stack of capillary vessels (green) and mural cells (red) of a wild type mouse. See **Video 3**. **X-Z**) In vivo TPLSM of a mural-cell-localized (red) capillary constriction of kainic mice. See **Video 4**. **AA**) Quantification of vessel constriction from panels X-Z measured at white line. Scales for panels A-R= 5 μm; S-W= 25 μm; X-Z= 5 μm. Videos were recorded at 11.7 Hz. From Leal-Campanario et al.[8].

Figure 7. Immunohistochemistry of the recorded brain. A-B) Vessels at different magnifications injected with fluorescein dextran (green). DAPI-stained cellular nuclei in blue (white arrows). Scale bar for A= 200 μm; scale bar for B= 100 μm. **C**) Different section of the brain injected with fluorescein where a red blood cell (red arrow) is clearly seen. Scale bar= 50 μm.

Video 1. Vasospasm and mural cell blood flow recorded (11.7 Hz) in a KO hippocampal capillary. See frames from the same recording in **Figure 6A-H**.

Video 2. Vasospasm, leukocyte blockages, blood flow and mural cells recorded in a WT hippocampal capillary (11.7 Hz). See frames from the same recording in **Figure 6I-R**.

Video 3. Non-uniform blood flow in in vivo WT mouse parietal cortex recorded with TPLSM. See frames from the same recording in **Figure 6S-W**.

Video 4. Mural cell-driven (red) capillary (green) constriction from parietal cortex of seizing kainic animal recorded with TPLSM. See frames from the same recording **Figure 6X-Z**.

DISCUSSION:

We developed a head-cap restraint system for simultaneous electrophysiological and fiber-optic pCLE experiments in awake mice, reducing potential response contamination due to anesthetic drugs. The head-cap and mounting apparatus are straightforward to construct and are reusable for chronic awake-behaving imaging experiments. We checked the quality of the recordings against the gold-standard for in vivo microscopic blood flow imaging, TPLSM.

Proficient surgical skills are necessary to implement the protocol we describe here. The surgery must be done under aseptic conditions and always under an operating microscope, while taking care to leave the dura mater intact when drilling the window over the hippocampus. Familiarity with the underlying vasculature is essential, as cutting above a major blood vessel creates the risk of undesirable bleeding.

Placing the animal correctly in stereotaxic alignment ensures that the anterior-posterior plane is level before attaching the head-cap. This safeguard facilitates locating brain loci with the help of a brain atlas.

Mouse motion can result in damage to vessels or erroneous recordings of vasospasms (that is, fiber motion rather than true vasomotion), so it is important to reduce animal motion during the recordings. Foam padding within the restraint tube helps extend the duration of the recording sessions while minimizing stress and movement from the mouse. Mice prefer to fit snugly within the foam, so proper fitting reduces excessive motion. Without this precaution, mice may struggle. If the mouse does show signs of struggle, the recording session should be terminated. Recordings may resume the next day, once physiological stress reactions have abated. In our recordings, the animals were evidently calm most of the time.

We note that the motion that most negatively impacts the pCLE imaging method occurs when the probe moves differentially to the brain tissue. Breathing, seizures, and motion of the animal are irrelevant unless they displace the fiber-probe with respect to the tissue. That is, when the imaging probe moves differentially to the tissue the entire imaging field moves as a unit, making motion artifacts evident. Thus, it is critical to always record from two or more vessels at a time: if all vessels move at once, it may be due to fiber instability, whereas if at least one vessel does not change, the changes seen in other vessels are likely due to actual variations in blood flow, including vasospasms.

One limitation of the pCLE imaging method compared to TPLSM is that the fiber-probe punctures the pia and is thus invasive to the brain. As the probe descends, it causes some damage to the neural tissue it passes through. Thus, recording from the same vessel on two consecutive days can be exceedingly difficult, even when employing the same exact coordinates in both sessions (and often only possible if the initial recording session has a short duration). It is therefore critical, when conducting chronic recording studies, to record at increasingly greater depths in subsequent recording sessions.

The results of the blood flow experiments suggest that excitotoxicity is not the only mechanistic pathway for ictal cell death and hippocampal sclerosis[13]. The finding that capillary blood flow restriction plays a role in ictal neurodegeneration paves the way for extending the methods described here to the identification of microscopic blood flow changes at any depth of the body, and in any tissue, for clinical diagnostic purposes.

ACKNOWLEDGMENTS:

The project was funded by the Office of the Assistant Secretary of Defense for Health Affairs under Award No. W81XWH-15-1-0138, a Research Initiative Award from the American Epilepsy Society, and an award from the Arizona Biomedical Research Commission to S.L.M., as well as a challenge grant from Research to Prevent Blindness Inc. to the Department of Ophthalmology at SUNY Downstate Health Sciences University, the New York State Empire Innovation Program, and further grants from the National Science Foundation (0726113, 0852636, & 1523614), the Barrow Neurological Foundation, Mrs. Marian Rochelle, Mrs. Grace Welton, and Dignity Health SEED awards, to S.L.M and S.M.C. R.L.C. was supported by a José Castillejo fellowship from the Spanish Ministry of Education. We thank O. Caballero and M. Ledo for their technical advice and assistance.

DISCLOSURES:

We have nothing to disclose.

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Figure 1

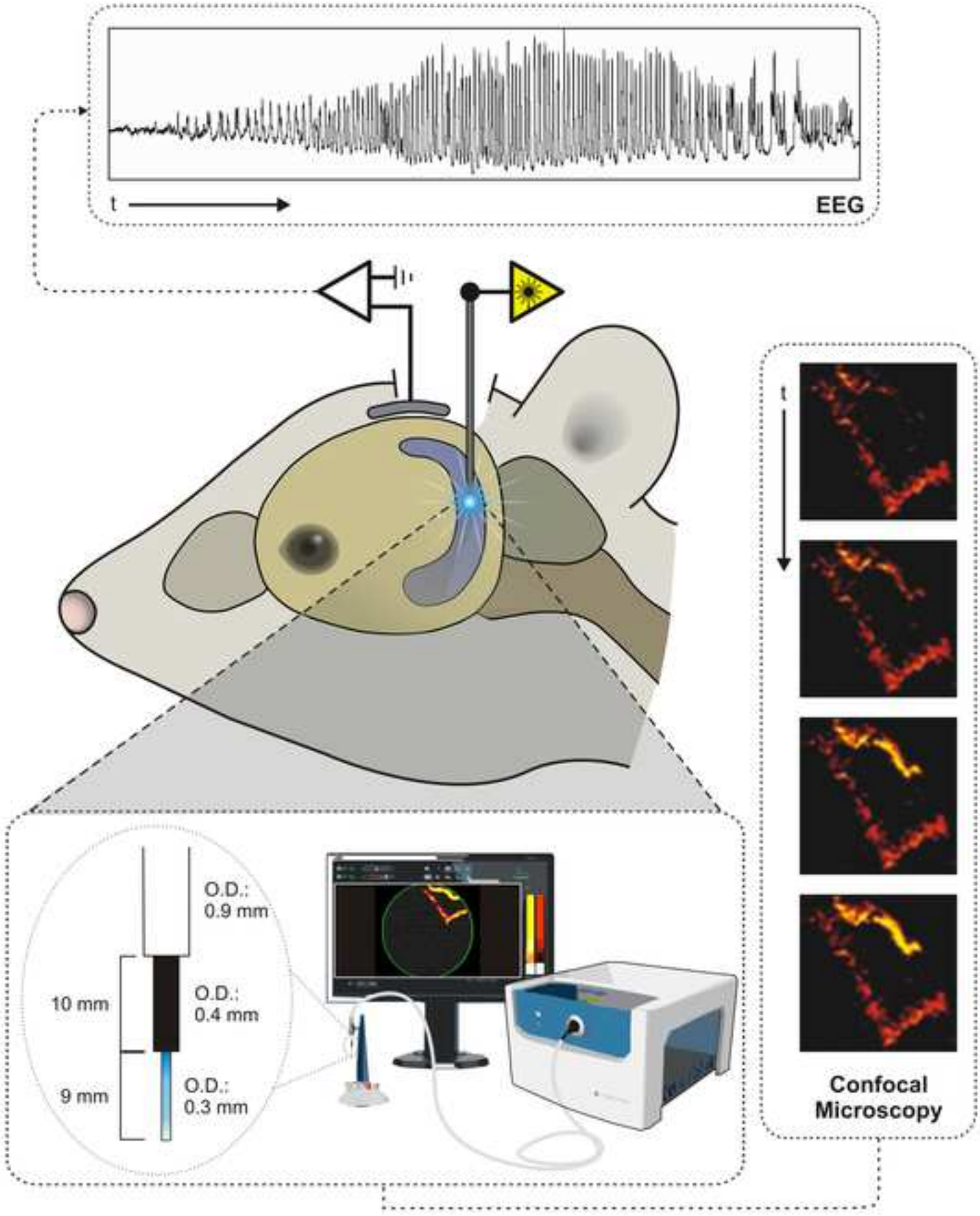

Figure 2

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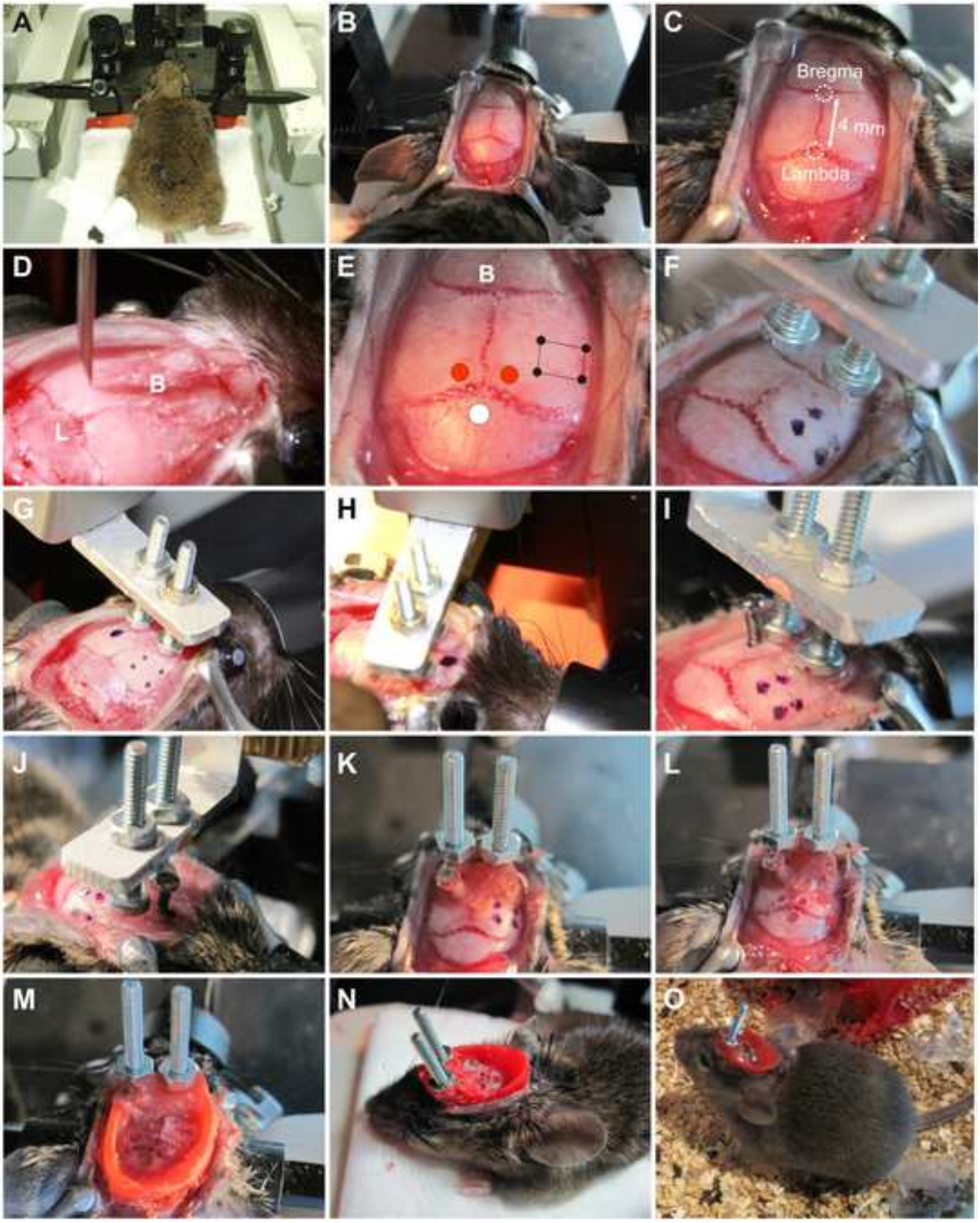

Figure 3

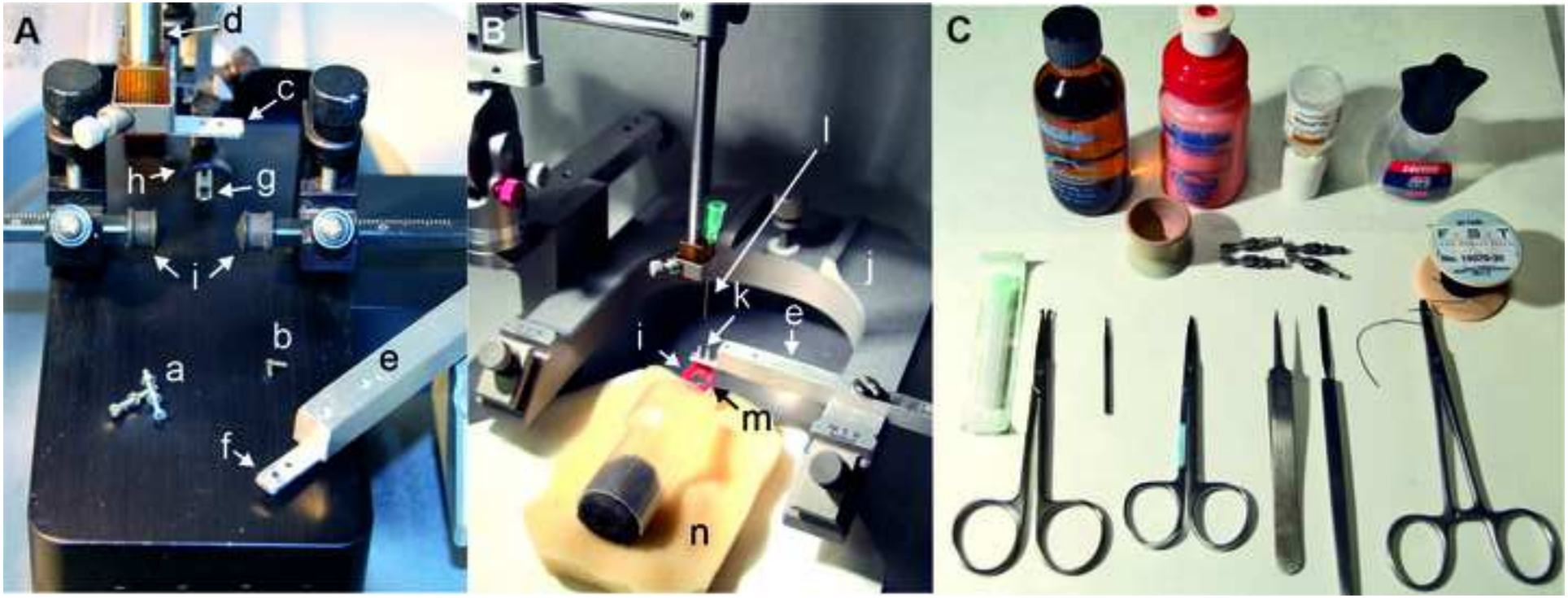

Figure 4

Figure 5

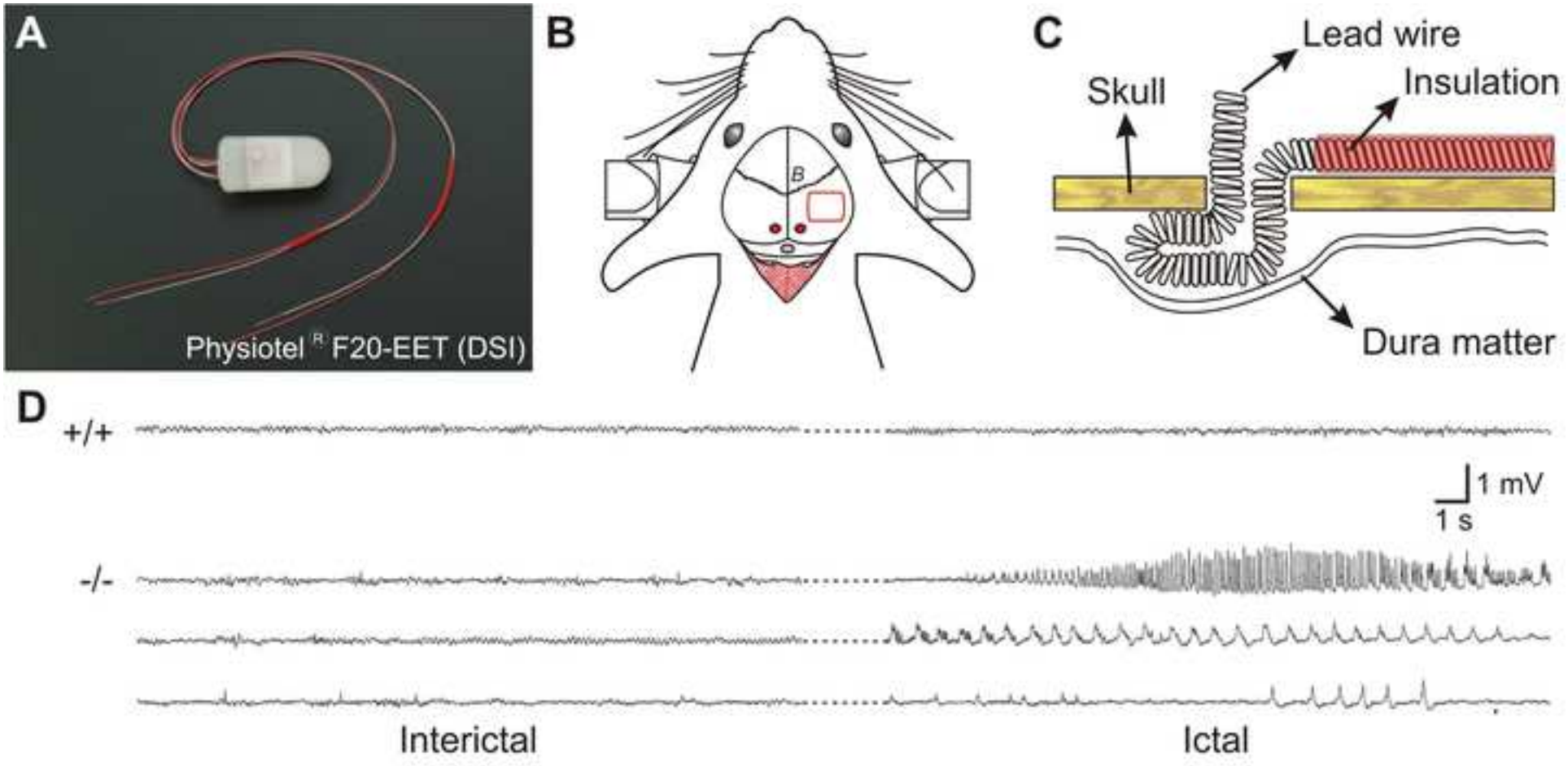

Figure 6

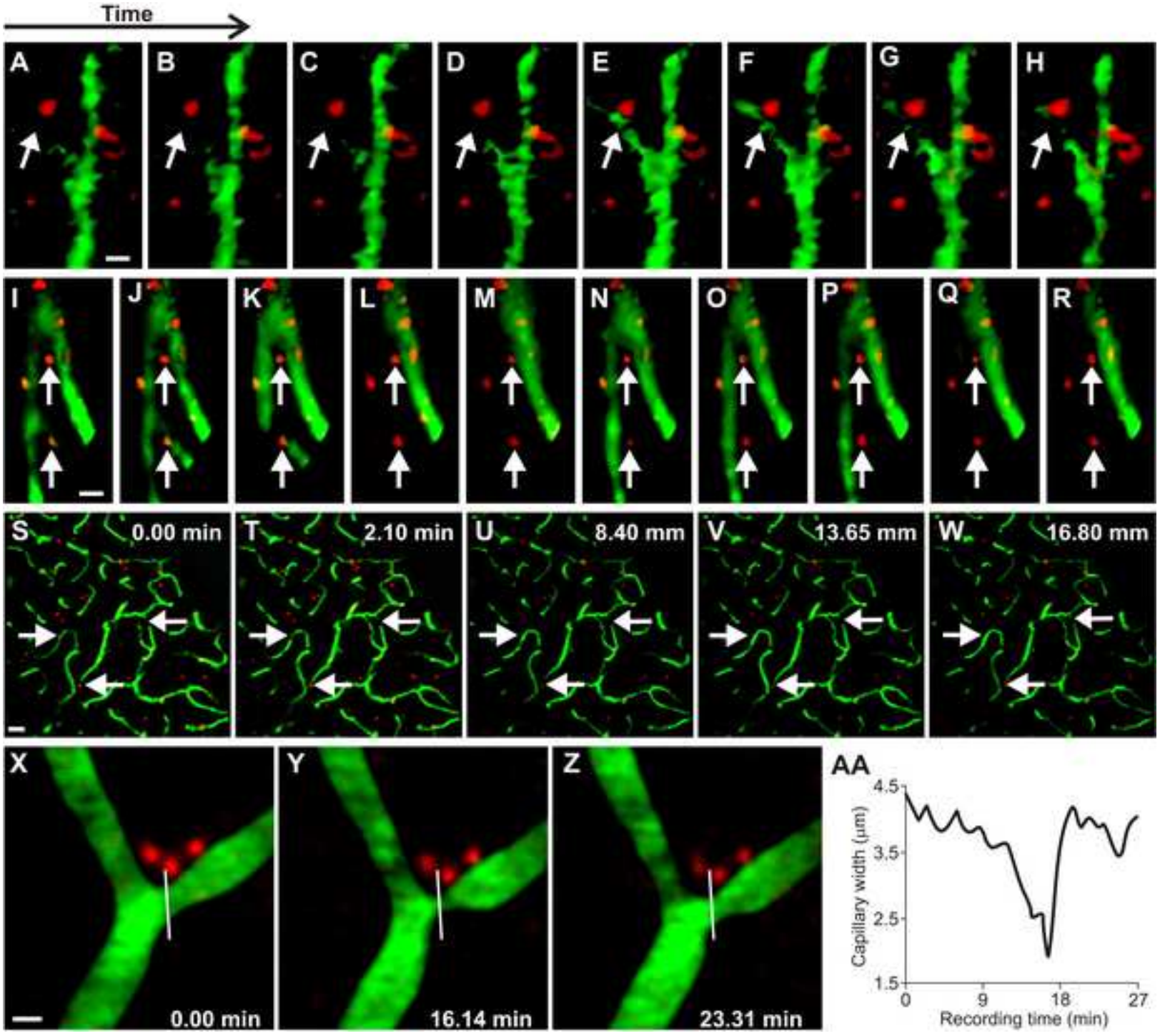

Figure 7

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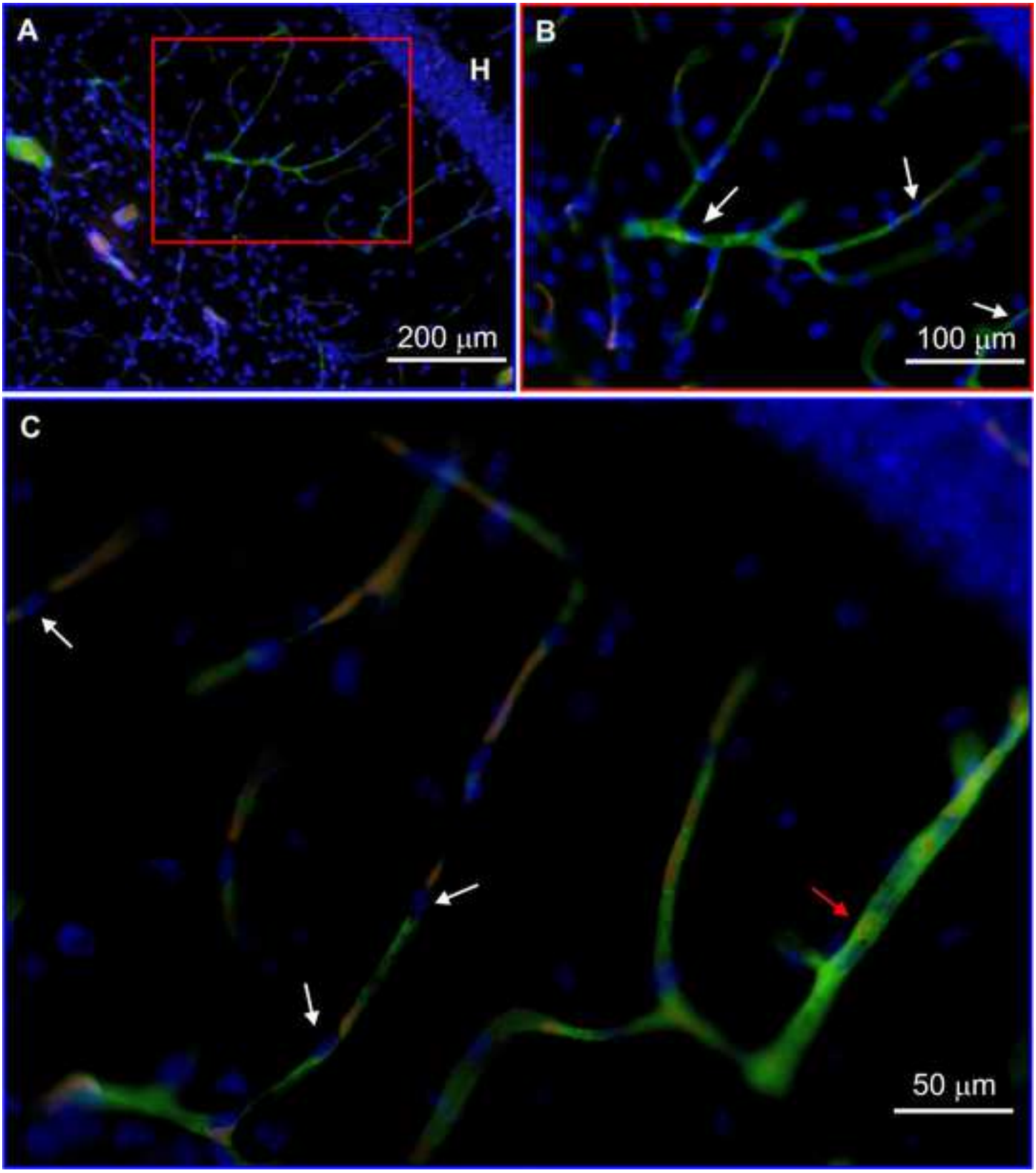

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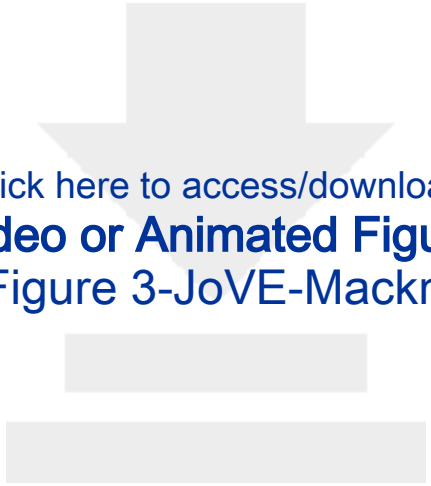

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.7 mm diameter burr	Fine Science Tools	19007-07	For Screws No. 19010-00
0.9 mm diameter burr	Fine Science Tools	19007-09	
ASEPTICO AEU-12C TORQUE PLUS	from Handpiece solution	AEU12C	
Bull dog serrifine clump	Fine Science Tools	18050-28	
CellVizio dual band	Mauna Kea Technologies		
CellVizio single band	Mauna Kea Technologies		
Confocal Microprobe 300 microns (Serie S)	Mauna Kea Technologies		
Custom-made alignment piece	L-shaped (angled at 90 deg) and made of stainless steel with two holes drilled on it, with a 4 mm separation from center to center		
Custom-made mounting bar	The long section piece of the mounting bar should be between 9.4 - 13mm. Fixed to this piece of the mounting bar, position a stainless-steel plate 1.5 cm long and 0.5 cm wide that has two holes drilled separated 4 mm from center to center, the same distance that the L-shaped alignment piece.		
Cyanoacrylate adhesive-Super Glue			
Dumont forceps #5	Fine Science Tools	11252-20	
DuraLay Inlay Resin – Standard Package	Reliance Dental Mfg Co.	602-7395 (from patterson dental)	
Fillister Head, Slotted Drive, M1.6x0.35 Metric Coarse, 12mm Length Under Head, Machine Screw	MSC industrial direct co.	2834117	
Fine Point scissor	Fine Science Tools	14090-09	
luorescein 5% w/w lysine-fixable dextran (2ML	Invitrogen, USA	D7137	
Halsey smooth needle holder	Fine Science Tools	12001-13	
Kalt suture needle 3/8 curved	Fine Science Tools	12050-03	

lab standard stereotaxic, rat and mouse	Stoelting Co. 51704	51670	
Methocel 2%	Omnivision GmbH	PZN: 04682367	Eye ointment to prevent dryness.
Mouse Temperature controller, probe (YSI-451), small heating pad-TC-1000 Mouse	CWE Inc.	08-13000	
PhysioTel F20-EET transmitters	DSI	270-0124-001	
Stereotaxic, Manipulator Arm, ADD-ON, 3 Axis	Stoelting Co.C13	51704	
Sel-Tapping bone screws	Fine Science Tools	19010-10	
Standard Ear Bars and Rubber Tips for Mouse Stereotaxic	Stoelting Co	51648	
Suture Thread - Braided Silk/Size 4/0	Fine Science Tools	18020-40	
Tissue separating microspatula	Fine Science Tools	10091-121	

SUNY Downstate Medical Center
Department of Ophthalmology
University Hospital of Brooklyn
College of Medicine
School of Graduate Studies
College of Nursing
College of Health Related Professions
School of Public Health

Sunday, August 23, 2020

Dear Editor of the Journal of Visualized Experiments,

Please consider our revised paper **"Preparation for in-vivo fiber-coupled laser-scanning confocal endomicroscopy of hippocampal capillaries in awake mice"** for publication in *JoVE*.

We have now addressed all specific comments from your editorial team including rewriting those parts of the manuscript which had significant textual overlap with previous publication(s).

Thank you very much for your consideration.

Sincerely,

Stephen L. Macknik

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Responses to Reviewers and Editorial Comments

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Done.

2. The ALA is signed for open access while editorial manager suggests the manuscript to be standard access. Please review and sign a new ALA if needed and upload it onto the editorial manager.

Done.

3. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.

Done.

4. Unfortunately, there are a few sections of the manuscript that show significant overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please see lines: 56-60, 74-79, 95-96, 102, 112-115, 119-120, 156-157, 219-238-239, 318-319, etc.

We have done a very significant rewrite of most manuscript sections, within the constraints pointed out by the reviewer as to the limited number of ways in which one may describe techniques and procedures.

5. Please remove the embedded figure(s) from the manuscript. All figures should be uploaded separately to your Editorial Manager account. Each figure must be accompanied by a title and a description after the Representative Results section of the manuscript text.

Done.

6. Please refer all the figures in order. Also, please ensure that all figures are referred in the text – For example different panels for Figure 5.

Done.

7**. Figure 1: Please label the parts in mouse schematic and also provide scale bars for confocal images. For confocal images please specify what is meant by red and yellow color in the images. Please label the x-axis and provide units for y-axis for EEG data. Please provide a detailed explanation in the figure legend.**

Done.

8**. Figure 2 and 3: Please provide better and clear image.**

Done.

9. Figure 4: Please have a better focus on the head.

Done.

10. Figure 5: Please label the parts in figure 5B schematic.

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Done.

11. Figure 6: Please indicate scale.
Done.

12. Figure 7: Please explain what does red fluorescence and arrows represent.
Done.

13. Please rephrase the Short Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"
Done.

14. Please rephrase the Long Abstract to more clearly state the goal of the protocol.
Done.

15. Please revise the Introduction to include all of the following:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application
Done.

16. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Fillister Head - 304 Stainless - Slotted screws (M1.6; Small Parts, 84 Logansport, IN, USA, part number SC016-012-F-SL-S4, 200 Mesh T316 Stainless .0016 90 48" Wide, TC-1000, CWE Inc., USA, Fine Science Tools, The PhysioTel F20-EET mouse telemetry transmitter. DSI (™), Cellvizio Lab (Mauna kea tech), StereoDrive, Neurostar GmBH, Germany, etc.
We have removed the commercial language from the enclosed revision, and instead listed all such information, if relevant, in the Table of Materials.

17. Please use S.I. abbreviation throughout the manuscript.
Done.

18. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.
Done.

19. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions

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should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Done.

20. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

Done.

21. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

The Protocol no longer includes personal pronouns.

22. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

Done.

23. **Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.**

Done.

24. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

Done.

25. 1: Please move this section to introduction since this cannot be presented as numbered protocol step. This is to ensure that the protocol only contains action items which direct readers to do something.

Done.

26. 2: Please provide the details about the animal used: strain, sex, age etc.

Done.

27. Line 116: Is shaving performed after adjusting the mouse on the stereotaxic frame? Is shaving done with scissors or razors?

Now clarified.

28. Line 117: How many times is the scrubbing performed?

Now clarified.

29. Line 118: How big is the incision?

Now clarified.

30. Line 129: Please provide the size of the syringe.

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Now provided.

31. Line 138: Please provide more details on craniotomy or reference the section.
Now provided.

32. Line 183: Please provide details on the sutures. What size and kind, how do you suture it?
Now provided.

33. Line 198: Please explain do you restrain mouse for tail vein injection.
Done.

34. Line 212: Please explain is there a waiting period before performing endomicroscopy after tail vein injection?
Now clarified.

35. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. The highlighted steps should form a cohesive narrative with a logical flow from one highlighted step to the next. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
Done.

36. Please expand the representative result section by providing more details.
Done.

37. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
The figures we have contributed are original (our own art) and owned by the corresponding author (Stephen Macknik). We have published modified versions of Figure 1 and Figure 5b,c (in citations 9 & 13): per both publishers' policy, we retain the right to republish our text and figures without seeking permission from the publisher. We have indicated in the captions that these specific figures are modified from the previous publications.

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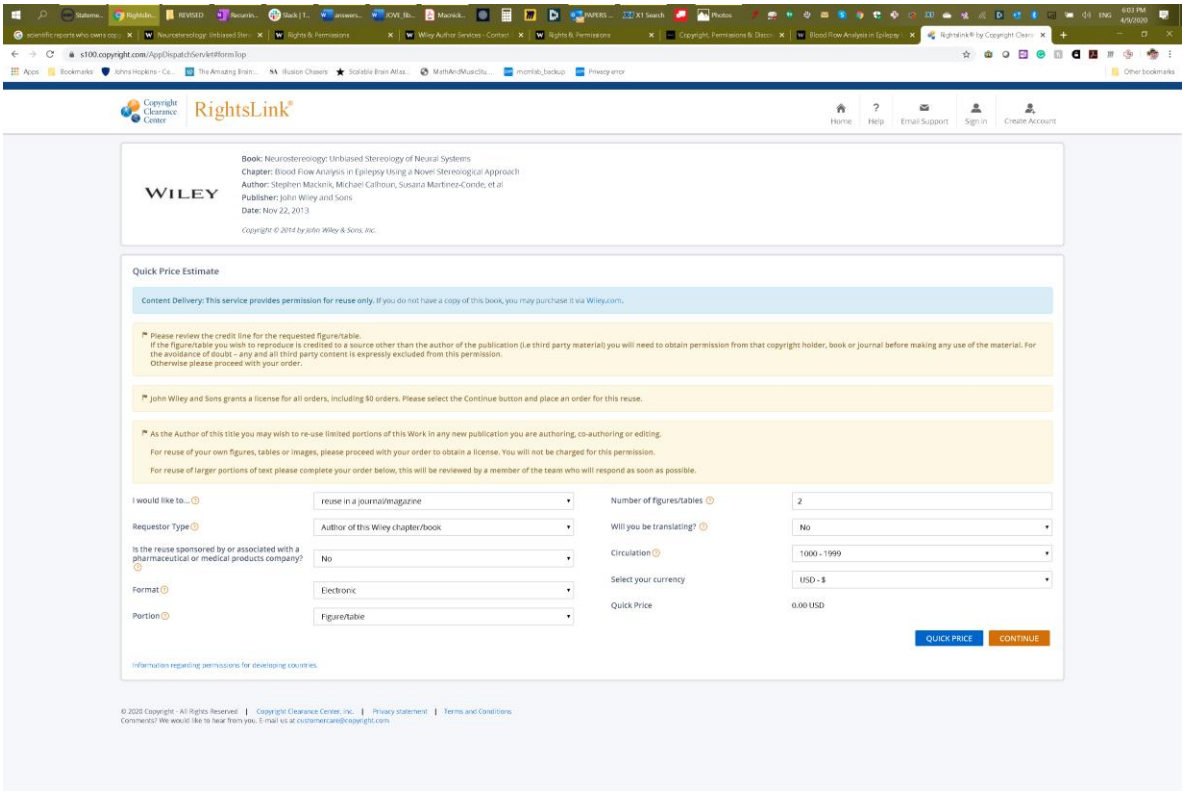

38. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
Done.

39. Please include an Acknowledgements section, containing any acknowledgments and all funding sources for this work.
Done.

Reviewer #1:

Major Concerns:
1. Since the method is a survival preparation, it is important that the animals are comfortable with the head-cup. Please provide the approximate weight of the head-cup and additional screws.
Done.

2. It is not clear from the description and figure 2b what the L-shaped metallic mesh look like and how it is used.
We removed the protocol instructions about the mesh as it is not a necessary step in the protocol and it added significant confusion.

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3. The 3. Head-cup Installation step is confusing. This part needs to be organized better and consistent:
(1) Line 149-155 says the two small screws to secure the head-cup are placed before the head-cup screws. However, Figure 3 seems to show that the head-cup is placed before the two small screws. Please clarify the steps, as some screws may interfere with the others or drilling if the steps are wrong.
(2) Please zoom in to show how and where the L shape mesh is placed.
(3) It looks like one of the small screws goes anterior to the head-cup and the other one is posterior. Please state clearly the locations of these two small screws.
Done.

4. During all the skull drilling steps, saline should be constantly applied to avoid heating of the bone.
Now added.

5. The instrument information for endomicroscope and EEG is not provided. ¿?—indicate the recording equipment information.
Fiberoptic Cofocal Microscopy: FCM system (Cellvizio, Mauna Kea Technology, Paris, France).
EEG recordings: F-20 EET (DSI ^{TM}) PhysioTel transmitter
Both are now listed in the 'table of materials.'

6. Please provide the stereotaxic coordination for the imaged hippocampus region.
Done.

7. Line 221 states that "recordings can be made for 4-5 hours even in consecutive days". It sounds like that the animals can be re-used for imaging and recordings. However, the authors didn't explain the post-imaging procedures. Will the probes be removed after imaging? Will the craniotomy be covered? How long an animal can be kept for imaging and recording? And how to prepare the animal before a second imaging?
Done.

8. As the animals can be imaged in consecutive days, can the same vessel be located on different imaging days by using the same coordinate? This will be important for people who want to track the changes of a single vessel, and will be a good indicator for the reliability of the preparation and apparatus design.
Done.

9. Since awake restrained animals are imaged, it is important to show the stability of the preparation for imaging and the physiological state of the animal during imaging. The authors mentioned that "Proper fitting reduces excessive motion, and in the event that the mouse show signs of struggle, the recording session should be terminated" (line 321). However,
(1) In terms of restrained awake animal preparation, motion of the animal from time to time is unavoidable. The animal tends to press the forelimbs against the body tube from time to time, which may affect the dynamics of blood vessel and may distort the images. The motion of the animal may be detected by a press sensor on the tube or shown on the

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EEG signals. But, the authors didn't state how they identify the period of movement or how they deal with the data during the period of movement.
The animal was placed on a foam pillow, which gently restrained its limbs. Also, motion of the animal is irrelevant for our recordings, unless it results in imaging instability (in fact, future directions for our research will include using this method to image freely roaming animals). The stability of the imaging itself is indeed important, and the most sensitive instability sensor we possess (3 μm resolution) is the imaging data itself. We can clearly see when the vessels move due to differential motion of the animal vs. the fiber-probe, because the entire field of view moves in the image in the same direction, at the same time and speed. In such scenarios, which did occasionally occur during our experiments (especially during seizures), we did not analyze the data. The only images we analyzed included a minimum of two vessels in the FOV, in which at least one vessel was stable.
(2) Besides animal movement, there is also movement caused by breathing. It is not shown how those movements affect the imaging, which will be a good indication of the stability of the preparation. The authors should show the measurements of the xyz motion artifact of the images during animal breathing and movement.
Please note that the only motion that negatively impacts this kind of imaging occurs when the probe moves differentially to the brain tissue that is being recorded. Breathing, seizures, motion of the animal, etc, are irrelevant unless they move the fiber-probe with respect to the tissue. In other words, when the imaging probe moves differentially to the tissue, the entire imaging field moves as a unit, making motion artifacts evident. Thus, we always recorded from two or more vessels at a time: if all vessels moved at once, it could be due to fiber instability, whereas if at least one vessel did not change, the changes seen in other vessels were considered to be analyzable data. We have clarified this issue in the revised MS.
(3) It is critical to ensure the animal is calm and is at natural behavioral state during awake imaging, as stress may affect the dynamics of blood vessels and neurons. The authors should show the physiological measurements (e.g. heart rate) of the animal under the restriction during imaging and compare with that when the animal is at home cage. We did not compare the heart rate during the recordings to that in the cage, as this procedure was not indicated by our IACUC-approved protocol. Having said this, we did monitor (but did not record) heart rate and SpO_2 online throughout the experiments, as part of our protocol. In the event of any spikes in heart rate, recordings were stopped, and the comfort of the animal checked.

10. The beginning of the introduction states the uniqueness of CLE comparing to other microscopic imaging methods (line 51-52) as it can access any depths. However, the reference 1-8 refer to two-photon microscopy rather the fiber-optic based confocal. Please make accurate references.
Please note that, except for our *Scientific Reports* paper, which we cite in the present MS, no other research has used pCLE to conduct deep blood flow imaging. We have clarified this issue in the revised MS.

11. There are many animal preparation methods and imaging apparatus developed for head-fixed awake animal brain imaging by using head bar or head plate while the animal is restrained in a tube, or running on a treadmill. The authors should have a brief overview of the methods people have been using and compare with the proposed method.
Our animal preparation is fundamentally similar to other head-fixed awake mouse brain imaging studies. The main difference with our study is that we achieved 3-micron resolution blood flow

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imaging at any depth using a method that is intrinsically robust to animal motion. We have now clarified this point in the revised MS.

12. The authors should discuss the potential applications for people using other imaging methods or studying other topics to demonstrate the broader usefulness of the proposed method.
Our method allows for 3-micron high-speed fluorescence blood-flow imaging at any depth. Now clarified in the revised MS.

Minor Concerns:
1. Figure 6 A-R, time scale is missing. Please do a same vessel diameter measurement and plot as Figure 6 AA imaged by CLE to quantitatively compare. Also, clarify the type of the mice being imaged (KO? Or kainic acid injected WT?).
Now clarified in the figure legend.

2. Figure 7, state what are different color arrows point to.
Now clarified.

3. Line 99-100 sentence ("fixed to this mounting bar…") has grammar problem.
Now corrected.

4. Line 131 "move the need tip…" should be needle tip.
Now corrected.

Reviewer #2:

Major Concerns:
1. The authors specify in the title, abstracts, and introduction that this technique is used for fiber-coupled confocal laser endomicroscopy (CLE); however, they spend more time introducing the benefits of two-photon laser scanning microscopy (TPLSM) than fiber-coupled CLE. If this head-mount can be used for both fiber-coupled CLE and TPLSM, then it should be clarified within the text and the title should be updated. It is always beneficial to know that one restraint system can be used for multiple types of experiments. In addition, the differences between the two methods should be discussed, perhaps with reasons why a researcher would choose one imaging method over the other.
The MS now describes 2P methods as the current state of the art, and clarifies that our pCLE methods introduce 3-micron fluorescence blood flow imaging at any depth.

2. Instead of paragraph format - the procedure should be formatted as an outline of shorter steps to make it easier to follow.
Done.

3. The flow of the procedure becomes very confusing as the authors do not describe figure panels in an ascending order (i.e. the figures are not referenced in order). In addition, some of the figure components, such as Figure 5C and 5D are not referenced in the text at all. If unreferenced images are not needed, then they should be removed. This is a presentation of the procedure in a style different from that of JoVE and should be addressed to improve the clarity of the manuscript.

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Done.

4. References 1-8 are cited for the first sentence of the introduction, and all eight articles reference TPLSM, not fiber-coupled CLE. Though some of the eight citations describe two-photon imaging of blood flow dynamics, none of them refer to use of fiber-coupled CLE.
To the best of our knowledge, no other paper describes using pCLE to conduct deep microscopic blood flow imaging (besides our *Scientific Reports* paper that introduces this method, which is cited).

5. Lines 127-136: The description of leveling the head is difficult to follow. If the authors think that including a description of standard head leveling technique is necessary, then perhaps a diagram depicting the sutures and the points to measure labeled in the order they should be measured would help.
Done.

6. Line 190-191: It is surprising that the authors only wait one day post-surgery to begin experiments. While I agree that most mice recover from implant and head-bar attachment surgeries within 24 hours, in our experience IACUCs require a three-day post-operation check to ensure full recovery from surgery. Several laboratories using such techniques typically wait a full week post-surgery before beginning experiments to make sure that mice are fully habituated to their new circumstances prior to obtaining baseline data.
Done.

7. Lines 217-231: Is the fiber-optic bundle chronically implanted? If not, how can the authors image during consecutive days? The authors should comment on this matter as these implants are invasive - therefore, brain damage is expected if placing and removing such implant during consecutive days.
Done.

8. Lines 225-228: If this procedure can be repeated over multiple days, then include this in the protocol and describe that holes are filled with bone wax or any method performed to protect the craniotomies.
Done.

Minor Concerns:
1. Why is this called a head-cup instead of the traditional head-cap or head-bars?
We apologize for the confusion. We now use the term head-cap throughout.

2. Figure 1 includes an image of the Cellvizio Lab laser scanning system from the Mauna Kea Technologies website, but credit is not given for the image. Also, higher magnification images of the components would help for visualization, as well as more description on the figure legend. Done.

3. The custom-made alignment piece (Fig 2A-d) and/or the parts required to make this custom piece need to be listed in the Table of Materials with a description of how to make this custom piece.
Already included in the table of materials.

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4. Line 116: Shaving the scalp can be expedited by using corded or cordless trimmers, which can be purchased from most veterinary or laboratory surgical supply vendors.
Since trimming the hair on the scalp is technically not required, this step is no longer included in the revised MS.

5. Lines 123-124: It should be described why it is important to allow the muscle to tear
This is not an important detail, so we have removed it from the revised MS.

6. Figure 3 should include arrows denoting the referenced descriptions in some of the images, namely C, D, E, F, and H. This will draw attention to the main point of the image. Some of these images are difficult to see, even when magnified on the screen. If possible, higher magnification images would be more instructional to demonstrate how each component attaches to the skull. It is unclear from the description and the image how the L-shaped metallic mesh is used.
Done.

7. Line 198: Is the amount of fluorescein 1 mg/kg instead of 1 ml/kg?
The dose is 1% fluorescein, 0.2 ml/25 g body wt; now indicated in the revised MS.

8. Figure 7 does not include descriptions for A, B, or C.
Now clarified.

Reviewer #3:

Major Concerns:
1. The key ingredient of this method is the microscopy, and there appears to be no description whatsoever of the microscopy. Hence, it would be impossible for me or anyone else to reproduce what the authors did, based on the description they provided. The authors are implanting a fiberoptic bundle, but what does this bundle consist of? How is it made, and/or where is it purchased from? How is the laser coupled to the fiberoptic cable, i.e. what kind of optics and/or electromechanical apparatus? Who is the manufacturer of the laser? What are the various laser parameters, e.g. light intensity, and method of scanning? The introduction briefly mentions that the bundle is composed of several thousand smaller fibers, but no further details are given about this, or any other aspect of the microscopy.
Since this is a methodology paper, we have emphasized the methods themselves rather than the analysis, which is described in 'Leal-Campanario et al, *Scientific Reports*, 2017'. Here we have provided a description of how to prepare the animal to record with the CLE. We have now clarified in the MS the microscopy hardware (which comes with proprietary software) used for our study, so replication will be straightforward.

2. Many other methodological details are missing. For example, when is the Alexa 647 injected? Just before the recordings? Or is it hours, days or weeks before?
Details now provided.

3. The scientific findings are at best extremely preliminary. For example, the Alexa 647 is claimed to label mural cells, but no specific evidence is given for this. Are these the only

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cells labeled? What proportion of them are labeled? Overall, it is not clear what conclusions are being drawn from any of the observations.
The scientific results have been peer-reviewed and published elsewhere (Leal-Campanario et al, *Scientific Reports*, 2017). The purpose of the present paper is to provide a how-to procedure for replicating our methods, not to defend our prior findings. We welcome this discussion, but it is not the issue at hand here.

Minor Concerns:
1. At least two of the parts in the Table of Materials are severely outdated. The two stainless steel screws are said to be from Small Parts Inc., but this company was purchased by Amazon more than a decade ago, and the original part numbers discontinued shortly thereafter. Somehow, the authors still list an address for Small Parts, Inc., but it is an ENTIRELY DIFFERENT company (based in Indiana, whereas the original one was based in Florida). This creates two unpleasant impressions. First, that little checking of details was done. Second, that details that were unknown were simply replaced with inaccurate information that would look plausible to an uninformed reader.
Thank you for pointing out this issue. Whereas the parts we originally listed were those that we used in our study, we have now updated the "list of materials" to include equivalent parts from an extant company: Fine Science Tools.

2. The microscopy equipment should be included in the Table of Materials.
Done.

3. There are a lot of spelling errors, e.g. lines 92, 122, 131.
Thank you for pointing out these mistakes. We have now corrected them and redoubled our editing efforts throughout the revised MS.

4. Many portions appear to be hastily written, and not entirely clear. For example, in the paragraph from lines 127 to 136, what exactly do the authors mean by "Read coordinate from behind, back, and top"? The next phrase, "write on Bregma", is also curiously worded - I assume they mean "write down the Bregma coordinates, for eventual comparison with lambda" but the wording makes it sound like they are writing on the skull itself.
We agree. Now revised and clarified.

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