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TITLE:

Inducing Post-Traumatic Epilepsy in a Mouse Model of Repetitive Diffuse Traumatic Brain Injury

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TBI, epilepsy, seizures, astrogliosis, EEG, mild TBI, concussion, diffuse TBI, posttraumatic epilepsy

SUMMARY:

This systematic protocol describes a new animal model of post-traumatic epilepsy after repetitive mild traumatic brain injury. The first part details steps for traumatic brain injury induction using a modified weight drop model. The second part provides instructions on the surgical approach for single- and multi-channel electroencephalographic data acquisition systems.

ABSTRACT:

Traumatic brain injury (TBI) is a leading cause of acquired epilepsy. TBI can result in a focal or diffuse brain injury. Focal injury is a result of direct mechanical forces, sometimes penetrating through the cranium, creating a direct lesion in the brain tissue. These are visible during brain imaging as areas with contusion, laceration, and hemorrhage. Focal lesions induce neuronal death and glial scar formation and are present in 20%–25% of all people who incur a TBI. However, in the majority of TBI cases, injury is caused by acceleration-deceleration forces and subsequent tissue shearing, resulting in nonfocal, diffuse damage. A subpopulation of TBI patients continues to develop post-traumatic epilepsy (PTE) after a latency period of months or years. Currently, it is impossible to predict which patients will develop PTE, and seizures in PTE patients are challenging to control, necessitating further research. Until recently, the field was limited to only two animal/rodent models with validated spontaneous post-traumatic seizures, both presenting with large focal lesions with massive tissue loss in the cortex and sometimes subcortical structures. In contrast to these approaches, it was determined that diffuse TBI induced using a modified weight drop model is sufficient to initiate development of spontaneous convulsive and non-convulsive seizures, even in the absence of focal lesions or tissue loss. Similar to human patients with acquired post-traumatic epilepsy, this model presents with a latency period after injury before seizure onset. In this protocol, the community will be provided with a

new model of post-traumatic epilepsy, detailing how to induce diffuse non-lesional TBI followed by continuous long-term video-electroencephalographic animal monitoring over the course of several months. This protocol will detail animal handling, the weight drop procedure, the electrode placement for two acquisition systems, and the frequent challenges encountered during each of the steps of surgery, postoperative monitoring, and data acquisition.

INTRODUCTION:

Every year TBI affects an estimated 60 million people worldwide. Impacted individuals are at higher risk of developing epilepsy, which can manifest years after the initial injury. Though severe TBIs are associated with a higher risk of epilepsy, even mild TBI increases an individual's chance of developing epilepsy[1-4]. All TBIs can be classified as focal, diffuse, or a combination of both. Diffuse brain injury, present in many if not all TBIs, is a result of brain tissues of different densities shearing against each other due to acceleration-deceleration and rotational forces. By definition, diffuse injury only occurs in isolation in mild/concussive non-penetrating brain injury, in which no brain lesions are visible on computed tomography scans[5].

There are currently two critical problems in the management of patients who have, or are at risk of, developing post-traumatic epilepsy (PTE). The first is that once PTE has manifested, seizures are resistant to available anti-epileptic drugs (AEDs)[6]. Secondly, AEDs are equally ineffective at preventing epileptogenesis, and there are no effective alternative therapeutic approaches. In order to address this deficit and find better therapeutic targets and candidates for treatment, it will be necessary to explore new cellular and molecular mechanisms at the root of PTE[6].

One of the prominent features of post-traumatic epilepsy is the latent period between the initial traumatic event and the onset of spontaneous, unprovoked, recurrent seizures. The events that occur within this temporal window are a natural focus for researchers, because this time window might allow treatment and prevention of PTE altogether. Animal models are most commonly used for this research because they offer several distinct benefits, not the least of which is that continuous monitoring of human patients would be both impractical and costly over such potentially long spans of time. Additionally, cellular and molecular mechanisms at the root of epileptogenesis can only be explored in animal models.

Animal models with spontaneous post-traumatic seizures and epilepsy are preferred over models where seizures are induced after TBI by less physiologically relevant means, such as by chemoconvulsants or electric stimulation acutely, chronically, or by kindling. Spontaneous post-traumatic seizure models test how TBI modifies the healthy brain network leading to epileptogenesis. Studies using additional stimulation after TBI assess how exposure to TBI reduces seizure threshold and affects susceptibility to seizures. The advantages of animal models with seizures induced chemically or with electric stimulation are in testing the specific mechanisms of refractoriness to AEDs and the efficacy of existing and novel AEDs. Yet, the degree of relevance and translation of these data to humans may be ambiguous[7] due to the following: 1) seizure mechanisms may be different from those induced by TBI alone; 2) not all of these models lead to spontaneous seizures[7]; 3) lesions created by the convulsant agent itself, with the cannula required for its delivery, or by stimulating electrode placement in depth structures (e.g.,

the hippocampus or amygdala) can already cause increased seizure susceptibility and even hippocampal epileptiform field potentials[7]. Furthermore, some convulsant agents (i.e., kainic acid) produce direct hippocampal lesions and sclerosis, which is not typical after diffuse TBI.

Until recently, only two animal models of post-traumatic epilepsy existed: controlled cortical impact (CCI, focal) or fluid percussion injury (FPI, focal and diffuse)[8]. Both models result in large focal lesions alongside tissue loss, hemorrhage, and gliosis in rodents[8]. These models mimic post-traumatic epilepsy induced by large focal lesions. A recent study demonstrated that repeated (3x) diffuse TBI is sufficient for the development of spontaneous seizures and epilepsy in mice even in the absence of focal lesions[9], adding a third rodent PTE model with confirmed spontaneous recurrent seizures. This new model mimics cellular and molecular changes induced by diffuse TBI, better representing the human population with mild, concussive TBIs. In this model, the latent period of three weeks or more before seizure onset and the emergence of late, spontaneous, recurrent seizures allows for investigating the root causes of post-traumatic epileptogenesis, testing the efficacy of preventive approaches and new therapeutic candidates after seizure onset, and has potential for the development of biomarkers of post-traumatic epileptogenesis because approximately half of the animals develop post-traumatic epilepsy.

The choice of animal model for the study of post-traumatic epilepsy depends on the scientific question, the type of brain injury investigated, and what tools will be used to determine the underlying cellular and molecular mechanisms. Ultimately, any model of post-traumatic epilepsy must demonstrate both the emergence of spontaneous seizures after TBI and an initial latency period in a subset of TBI animals, because not all patients who incur a TBI go on to develop epilepsy. To do this, electroencephalography (EEG) with simultaneous video acquisition is used in this protocol. Understanding the technical aspects behind data acquisition hardware and approaches is critical for accurate data interpretation. The critical hardware aspects include the type of recording system, type of electrodes (screw or wire lead) and material they are made of, synchronized video acquisition (as part of the EEG system or third party), and properties of the computer system. It is imperative to set the appropriate acquisition parameters in any type of system depending on study goal, EEG events of interest, further analysis method, and sustainability of data storage. Lastly, the method of electrode configuration (montage) must be considered, as each has advantages and disadvantages and will affect the data interpretation.

This protocol details how to use the modified Marmarou weight drop model[10,11] to induce diffuse injury resulting in spontaneous, unprovoked, recurrent seizures in mice, describes surgical approaches to acquire a single- and multi-channel continuous, and synchronized video EEG using monopolar, bipolar, or mixed montage.

PROTOCOL:

All animal procedures described in this protocol were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) of Virginia Tech and in compliance with the National Institutes of Health's 'Guide for the Care and Use of Laboratory Animals'.

1. Animal handling protocol

NOTE: This protocol is intended to habituate animals ordered from a vendor to the facility after arrival and to condition them to being handled by the experimenter. This improves animal well-being by reducing stress and anxiety and simplifies certain procedures that require handling animals, including inducing the TBI, post-operative monitoring, and connecting the animal to the acquisition system.

1.1. When many animals are received from the vendor, ear-tag and randomly assign them to an experimental group (TBI) or control group (sham surgery) while combining them in cages of 2−5 animals. House TBI animals separately from sham animals because sham mice occasionally act aggressively toward mice that underwent TBI.

1.2. Handling day 1 (24−48 h after ear-tagging): Prepare a chart for logging animal ear tags, date of birth, dates of handling, animal weight on the handling days, duration of the handling, and a section for comments and observations.

1.3. Gently cup the animal using both hands. Do not grab the animal by the tail as it induces defense mechanisms and a stress response.

1.4. Check and record the ear tag of the animal.

1.5. Place the animal in the container on the weight scale and record the weight.

1.6. Gently cup the animal with both hands again and handle it for 1 min, allowing it to move and explore within the hands. Perform this over a bench in the procedure room and be careful to not drop the animal on the floor.

1.7. After 1 min of handling, place the animal back in its cage.

1.8. Repeat steps 1.3−1.7 for the other animals in the cage.

1.9. Handling day 2 (the following day): Repeat steps 1.2−1.5.

1.10. Gently cup the animal with both hands again and handle it for 2 min, allowing it to move and explore within the hands. Perform this over a bench in the procedure room and be careful to not drop the animal on the floor.

1.11. After 2 min of handling, place the animal back in its cage.

1.12. Repeat steps 1.10−1.11 for the other animals in the cage.

1.13. Handling day 3 (the following day): Repeat steps 1.2−1.5.

1.14. Gently cup the animal with both hands again and handle it for 4 min, allowing it to move and explore within the hands. Perform this over a bench in the procedure room and be careful to not drop the animal on the floor.

1.15. After 4 min of handling, place the animal back in its cage.

1.16. Repeat steps 1.14−1.15 for the other animals in the cage.

1.17. Handling day 4 (control day, 1 week from handling day 1): Repeat steps 1.2−1.5.

1.18. Gently cup the animal with both hands again and handle it for 4 min, allowing it to move and explore within the hands. Perform this over a bench in the procedure room and be careful to not drop the animal on the floor.

1.19. After 4 min handling, place the animal back in its cage.

1.20. Repeat steps 1.18-1.19 for the other animals in the cage.

NOTE: The control handling day tests the retention of the calm behavior after a three-day handling protocol.

2. Weight drop procedure

2.1. Place the mouse in an induction chamber. Set the flow of oxygen and vacuum both to 1 L/min and the level of isoflurane gas to 3%−5%. Anesthetize the mouse for 5 min.

2.2. Remove the mouse from the induction chamber and place it on a foam pad. Test for the absence of a response to a toe or tail pinch.

2.3. Administer an analgesic (0.1 mg/kg buprenorphine) subcutaneously. If the EEG surgery is performed that same day, administer the buprenorphine subcutaneously in combination with the non-steroidal anti-inflammatory carprofen (5 mg/kg).

2.4. Administer the sodium lactate solution (3 µL per gram of the animal's weight) subcutaneously before or after the last impact. The sodium lactate solution can be mixed with the analgesics for quick administration in a single injection.

NOTE: The sodium lactate solution contains a mixture of sodium chloride, potassium chloride, calcium chloride, and sodium lactate in water. This step helps to replace fluids and electrolytes, aiding recovery.

2.5. Position the head of the mouse under the weight drop tube (**Figure 1A**) and place a flat stainless steel disc (1.3 cm diameter, 1 mm thick, and 880 mg weight) in the center of the head, between the line of the eyes and ears.

NOTE: This disc diffuses the impact across the surface of the skull (**Figure 1B**).

2.6. Remove the pin in the weight drop tube to release the 100 g weight rod from a height of 50 cm. To induce the sham injury for the control mice, remove the weight rod from the tube to prevent accidental release of the pin and weight drop.

NOTE: The animal's head must be positioned flat, so that the rod free-falls on the entire surface of the disc.

2.7. Place the unconscious animal on its back for recovery on a heating pad covered with a sterile polylined absorbent towel. The righting reflex recovery time (i.e., the time it takes the mouse to right itself from its back) can be measured as a readout for the time spent unconscious.

2.8. When the animal regains consciousness, place it in a clean cage that has been warmed on a heating pad, with recovery gel and a few moistened chow pieces to recover for 45 min. Make sure there is sufficient litter so the cage does not get overheated. Overheating the animal can prove just as great an obstacle to recovery as allowing the mouse to become too cold.

2.9. After 45 min, repeat steps 2.1–2.8 twice, omitting step 2.3 (i.e., administration of analgesics and anti-inflammatory drugs).

2.10. Allow the animals to recover for 1–2 h if EEG electrode implantation surgery is performed on the same day.

3. Surgical field preparation for implantation of EEG electrodes

NOTE: Autoclave the surgical tools and screws prior to surgery. Clean the surgical gloves by spraying and rubbing with 70% ethanol before and after touching the animal, non-sterile materials, and in between handling the animals. Sterilize the surgical tools for 2–3 min in the bead sterilizer (see **Table of Materials**) between animals. Change the sterile drape before placing a new animal into the stereotactic apparatus. Ensure that the surgical field contains all the necessary components for the surgery (**Figure 2**). The absence of an invasive surgical procedure to induce the TBI in this model has several advantages: 1) implantation of the electrodes is flexible and may be performed on the same day as TBI or after a defined period of time; 2) the animal's recovery time is faster; 3) the cranium remains intact, allowing more surface area and flexibility for implanting electrodes.

3.1. Anesthetize the mouse in 3%–5% isoflurane gas in an induction chamber for 5 min.

3.2. Transfer the mouse from the induction chamber to the stereotactic apparatus and place it on a sterile drape on a heating pad with isoflurane gas and vacuum tubes connected to the nose cone.

3.3. Maintain the body temperature at 37 °C over the course of the surgery. Place the temperature sensor so that it makes contact with the chest or abdominal wall of the mouse.

3.4. Fix the animal's head in place using the ear bars.

3.5. Maintain the anesthesia at 1.5%−3.5% isoflurane or at ~60 breaths/min in the surgical plane (with no response to toe or tail pinch).

3.6. Apply an eye ointment to the animal's eyes to keep them lubricated throughout the surgery.

3.7. Administer a mixture of analgesics (0.1 mg/kg buprenorphine) and the non-steroidal anti-inflammatory drug (5 mg/kg carprofen) in a single injection subcutaneously unless the TBI was performed earlier during the day, in which case the animal already received analgesics and anti-inflammatories.

NOTE: Buprenorphine should be administered again if the time between the first TBI and EEG placement surgery exceeds 8 h or if the animal displays signs of pain 8 h after the first administration, but it should be given without the addition of carprofen.

3.8. Administer sodium lactate solution (3 µL per gram of the animal's weight) subcutaneously to replace fluids and electrolytes in the animal.

NOTE: If surgery is performed immediately after the TBI, this step has to be timed properly. Sodium lactate solution should be administered every 2 h while the animal undergoes the procedures and once after the surgery, 2 h from the previous injection.

3.9. Remove the hair from the scalp using a hair removal cream.

3.10. Before making the incision, disinfect the skin of the scalp with povidone-iodine surgical antiseptic solution and 70% ethanol in alternating swabs with sterile gauze pads in a circular motion 3x (20 s per solution each time).

3.11. Using a scalpel, make a rostral-caudal incision on the scalp midline from just above the eyes to the back of the head. This method of scalp opening is preferred over cutting the scalp off, as skin flaps can be sealed over or around the EEG-cap providing more stability.

NOTE: When preparing the skull for implantation of the 3-EEG headmount, cutting the scalp off is required, as the size of the headmount will not allow for closure of the skin flaps over the headmount.

3.12. Expand the area of incision by applying small hemostats on the opened skin borders. If any bleeding occurs after the incision, clean with a sterile cotton gauze or swab.

3.13. Gently remove the periosteum (i.e., the thin membrane over the cranial bone) with a

scalpel blade. If any bleeding occurs during this step, press on bleeding site with a sterile cotton swab until it stops.

3.14. Use sterile cotton swabs to clean the cranium with hydrogen peroxide, but avoid touching the soft tissue surrounding the exposed cranial area. Repeat this step until the cranium is cleaned from any soft tissue and has a whitish appearance.

3.15. Dry the cranium with a sterile gauze or cotton swab.

NOTE: Steps 3.12−3.15 are important for the proper fixation of the electrodes and dental cement. Any soft tissue, non-cauterized bleeding, and debris can cause infection, unstable headmount fixation, distorted or absent signal, and loss of the implant within several days or weeks after surgery.

4. Electrode placement

4.1. Implant the single EEG (1EEG) channel headmount.

NOTE: Abbreviations in the stereotactic coordinates represent spatial relationships and specify the distance in millimeters of the target from the bregma at a given orientation on the animal's head: anterior-posterior (AP) and medial-lateral (ML). Dorsal-ventral is not applicable in this protocol because all electrodes are placed into the epidural space rather than in a certain structure within the brain (**Figure 3**). Vin+ is an active electrode and Vin- is its reference electrode.

4.1.1. Use a high-speed drill with a steel bit (0.5 mm, round, ¼ in.) at ~5,000−6,000 rounds per min (rpm) to create six burr holes (three for stability screws and three for electrodes) using the provided stereotactic coordinates[12]. For the two anterior screws: AP = +1.5 mm, ML = ±1.5 mm; for the one posterior screw: AP = -5.2 mm, ML = -1.5 mm; for the ground electrode: AP = -5.2 mm, ML = +1.5 mm; for the recording electrodes: AP = -2.3 mm, ML = ±2.7 mm, with Vin+ to the right and Vin- to the left.

4.1.2. Add three screws for enhanced stability of the head stage. Using a screwdriver, turn screws 1−1.5 x each to be fixed stably in the cranium.

NOTE: Placing the screws deeper will damage the brain.

4.1.3. Insert the 1EEG headmount into a stereotactic holder arm and position the headmount so that the three electrodes are located along the cranial midline. In this configuration the ground electrode and its respective opening on top of the headmount is in the back, the Vin+ electrode in the middle, and the Vin- electrode in the front. A mark can be made on the headmount with a permanent marker.

4.1.4. Bend each electrode 90° so that the end of each wire is bent downwards and is positioned above the corresponding burr hole. Then, measure out 1 mm length of the portion of the wire

that is now perpendicular to the burr hole and trim the excess off (**Figure 3**). This will ensure epidural placement of the electrodes. The electrodes should be barely touching the dura mater surface.

4.1.5. Lower the headmount and adjust all three electrodes to match the respective burr hole. For epidural recording, the electrodes must be placed above or barely touching the dura mater.

4.1.6. Prepare dental cement for application by mixing a ½ scoop of powder with several drops of solvent. Use a mixing spatula and stir until the final mixture is putty-like, tacky but malleable, and stiff enough to be properly condensed when placed on the animal's cranium.

4.1.7. Apply dental cement mixture covering all screws and electrodes and wait ~3−5 min for it to solidify. Make sure not to cover the plastic pedestal with dental cement, because it will make it impossible to connect the animal to the commutator with a tether.

4.1.8. Release the hemostats holding the skin flaps and close the incision by connecting the skin flaps around the plastic pedestal. Apply several drops of tissue adhesive (see **Table of Materials**) to seal the skin flaps.

4.1.9. Apply chlorhexidine antiseptic to the area around the implant to avoid infection. If the animal is under anesthesia for longer than 2 h after the previous injection of sodium lactate solution, given during the TBI induction, administer another injection subcutaneously. To maintain proper hydration of the animal, repeat the injection every 2 h that the animal spends under anesthesia.

4.1.10. After the surgery, give a final injection of sodium lactate solution 2 h after the previous injection. If the surgery is less than 2 h long, administer the final recovery dose of the sodium lactate solution 2 h from the first injection.

4.1.11. Remove the animal from the stereotactic apparatus and measure the animal’s weight after the EEG surgery as a reference for future monitoring. Due to the implant, the animal’s weight will be greater than before surgery.

4.1.12. Place the animal in a clean cage on a warm heating pad for recovery.

4.2. Implant the two EEG and one EMG (2EEG/1EMG) channels headmount.

4.2.1. Use the bregma as a landmark for placement of the headmount. Apply a small amount of tissue adhesive (see **Table of Materials**) to the bottom side of the 2EEG/1EMG headmount, avoiding the four screw holes and place the 2EEG/1EMG headmount on the surface of the cranium.

NOTE: There are no specific coordinates for placement of this headmount. The headmount is 8 mm long and 5 mm wide, which covers most of the cranial surface. Positioning the headmount

with its front edge ˜3.0 mm anterior to the bregma is optimal and provides good signal quality. Quick manual placement is necessary before the drop of tissue adhesive cures. Allow approximately 5 min for tissue glue to cure completely.

4.2.2. Use a sterile 23 G needle to create pilot holes for the screws through the four openings in the headmount. To accomplish this, gently push the needle and slowly rotate until the tip of the needle penetrates the skull without damaging the brain. Remove any bleeding from the pilot holes using a sterile cotton swab.

4.2.3. Insert the 0.10 in screws in the pilot holes and rotate them until each is fixed in the skull. This can be up to half of the screw length, but not the full length, as this would damage the dura mater and cortex. If the headmount is positioned so that there is a gap between the skull surface and the rear end of the headmount use two 0.12 in screws in the posterior part.

4.2.4. Make small opening on the sides of the two-component epoxy (silver-epoxy) twin-pack pouch. Take a double-sided spatula and use each side to scoop a small and equal amount of each component from the pouch and mix them together. Use only a small amount sufficient for a single surgery, because the mixture solidifies within 20 min. Seal the sides of the pouch to prevent drying.

NOTE: The silver-epoxy allows for proper electrical contact between the screw and headmount and enhances the stability of the screws.

4.2.5. Apply a small amount of this mixture between screwhead and screw hole, then tighten each screw until its head rests on the base of the implant. Ensure that no silver-epoxy is making contact between the two screws because each screw serves as an individual electrode and, to ensure an accurate signal, it should not make contact with the other screw.

4.2.6. If the silver-epoxy mixture was misplaced, there is a few second time window to carefully scoop out the excess to separate the connection. Carefully bend both EMG leads from the posterior edge of the headmount to follow the contour of the animal's head and neck, and then insert them into the nuchal muscles.

4.2.7. Prepare dental cement for application by mixing a ½ scoop of powder with several drops of solvent. Use a mixing spatula and stir until the final mixture is putty-like, tacky but malleable, and stiff enough to be properly condensed when placed on the animal's cranium.

4.2.8. Apply dental cement mixture covering the entire headmount while avoiding covering the six pin holes, as this will make it impossible to connect the pre-amplifier. Wait ˜3−5 min for the cement to solidify. Make sure that the skin is not sealed to the headmount with dental cement.

4.2.9. Release the hemostats holding the skin flaps and close the incision by connecting the skin flaps around the plastic pedestal. Apply several drops of tissue adhesive to seal the skin flaps.

NOTE: If the skin incision was made longer to allow for straightening of the EMG wire leads, the skin can be sealed with tissue adhesive or sutured. Sealing the skin with tissue adhesive is usually sufficient. However, if during post-operative monitoring opening of the incision is observed, sutures are recommended instead.

4.2.10. Apply chlorhexidine antiseptic to the area around the implant to avoid infection. Administer sodium lactate solution (3 µL per gram of the animal's weight) subcutaneously to replace fluids and electrolytes if the animal is under anesthesia for longer than 2 h after the previous injection.

4.2.11. Remove the animal from the stereotactic apparatus and measure the animal's weight after the EEG surgery as a reference for future monitoring. Due to the implant, the animal's weight will be greater than before surgery.

4.2.12. Place the animal in a clean cage on a warm heating pad, with recovery gel and a few moistened chow pieces for recovery.

4.3. Implant a three EEG channels (3EEG) headmount.

4.3.1. Use high-speed drill with a steel bit (0.5 mm, round, ¼) at ~5,000–6,000 rpm to create six burr holes (three for stability screws and three for electrodes) using the provided stereotactic coordinates[12]. For ground and common reference for EEG1 and EEG2: AP = 5.2 mm, ML = ±1.5 mm; for EEG1 and EEG2: AP = -3.0 mm, ML = ±3.0 mm; for independent EEG3: AP =-1.4 mm, ML = ±1.5 mm.

4.3.2. Place the six screw electrodes into the burr holes.

NOTE: Placing the screws deeper will create significant damage to the brain. Screw electrodes provide better stability of the headmount.

4.3.3. Prepare dental cement for application by mixing a ½ scoop of powder with several drops of solvent. Use a mixing spatula and stir until the final mixture is putty-like, tacky but malleable, and stiff enough to be properly condensed when placed on the animal's cranium.

4.3.4. Apply dental cement mixture covering the entire exposed surface of the cranium and each screw electrode. Make sure that skin is not sealed to the headmount with dental cement. Wait ~1–2 min for the cement to mildly solidify. There is no need to wait until full solidification before proceeding to the next step.

4.3.5. Turn on the soldering iron to heat it up. Place the 3EEG headmount in a stereotactic holder arm.

NOTE: Position the headmount so that the six wire lead positions match the position of the wire leads of each screw electrode.

4.3.6. Lower the headmount so that its ventral part rests on top of the dental cement.

4.3.7. Twist the wire of each lead from each of the screw electrodes with the corresponding wire lead of the headmount.

NOTE: Twisting the wrong wire leads will make data interpretation complicated or impossible.

4.3.8. Carefully trim the excess wire off using scissors. Solder each twisted pair of wire for proper signal conduction.

NOTE: Each pair of wires must make contact with another pair, otherwise signal quality and data interpretation will be compromised.

4.3.9. Bend each soldered pair of wire leads around the headmount, avoiding contact between each pair.

NOTE: If the wire leads are not trimmed short enough it can be difficult to bend them around the headmount without touching another wire. In this case, bend one pair first, cover it with dental cement mixture, wait ~1–2 min to solidify, then proceed with the next pair in the same fashion.

4.3.10. Finish covering all the wire with dental cement leaving only the black portion of the headmount exposed.

NOTE: Be careful to not apply any dental cement powder or mixture to the top of the exposed portion of the headmount as any debris or cement in the holes will block the contact and will lead to either signal absence or noise.

4.3.11. Release the hemostats holding the skin flaps. Apply chlorhexidine antiseptic to the area around the implant to avoid infection.

4.3.12. Administer sodium lactate solution (3 µL per gram of the animal's weight) subcutaneously to replace fluids and electrolytes if the animal has been under anesthesia for longer than 2 h after the previous injection.

4.3.13. Remove the animal from the stereotactic apparatus and measure the animal's weight after the EEG surgery as a reference for future monitoring. Due to the implant, the animal's weight will be greater than before surgery.

4.3.14. Place the animal in a clean cage on a warm heating pad, with recovery gel and a few moistened chow pieces for recovery.

5. Connecting animals to the acquisition system

5.1. Cup the animal with both hands to remove it from the acquisition cage and transfer it to a clean area with a flat surface, like an Animal Transfer Station (ATS).

5.2. Gently grab the mouse by the skin of its back. Do not grab the animal by the tail, as this causes distress.

5.3. Identify the opening in the EEG headmount corresponding to the ground electrode and match the respective pin of the tether for proper connection.

NOTE: Reverse connection of the tether from the commutator to the animal headmount will result in a different reading from the electrodes and potentially distorted waveforms.

5.4. Return the animal to the acquisition cage and connect the other end of the tether (EEG System 1) or pre-amplifier (EEG System 2) to the commutator.

NOTE: When connecting the pre-amplifier (EEG System 2) to the tether from the commutator, match the white marks on the ends of both tethers. Reverse connection will result in permanent damage of the amplifier and requires repairs by the manufacturer, which are expensive.

5.5. Gently rotate the tether connecting the animal to the commutator to ensure the mechanism works properly and the animal can move freely.

6. EEG data acquisition settings

6.1 Set EEG System 1 acquisition parameters.

6.1.1. Set sampling rate to 500 Hz; gain 5,000; mode Norm 35 Hz; LPN off. Set high pass filter to 0.5 Hz.

NOTE: 100 Hz (low pass) is built-in and does not require manual input.

6.2. Set EEG System 2 acquisition parameters.

6.2.1. Set sampling rate to 600 Hz; preamp gain 100; gain 1 (EEG1,2). Set low pass filter to 100 Hz.

NOTE: 1 Hz (high pass) is built-in and does not require manual input.

7. Video data acquisition settings

7.1 Set acquisition parameters for EEG System 1.

NOTE: A third party video acquisition system is needed for obtaining simultaneous video data.

7.1.1. Set frame rate between 15 (minimum recommended) and 30 (maximum available) for appropriate video quality. Set the resolution to 640 x 640 pixels. Set type of compression to H.264H.

7.2. Set acquisition parameters for EEG System 2.

NOTE: This EEG system offers a video system and software which synchronize video and EEG data together in a single file for up to four animals (see **Table of Materials**).

7.2.1. Set frame rate between 15 (minimum recommended) and 30 (maximum available) for appropriate video quality. Set the resolution to 640 x 480 pixels. Set the type of compression to the WebM file format.

REPRESENTATIVE RESULTS:

The protocol outlined here describes the method for induction of a diffuse injury in isolation (e.g., in the absence a focal lesion) using a mouse model of repetitive diffuse TBI (**Figure 1**). **Figure 1A** depicts the weight drop device and its components (**Figure 1A, a1**–**a5**) used for induction of TBI in this model and crucial steps during the procedure (**Figure 1B, b1**–**b5**).

Characteristics of this model include the lack of a focal lesion to the brain as a result of the TBI, loss of consciousness, a high survival rate, the emergence of late seizure onset (>1 week of the TBI), and spontaneous, unprovoked, recurrent seizures in a subset of TBI mice after a latency period of at least three weeks following TBI.

This protocol demonstrates detailed procedures for setting up a clean surgical field (**Figure 2**), provides a step-by step approach to implanting different electrode arrays (**Figure 3**), and includes a detailed guide on using two different EEG acquisition systems (see the **Table of Materials**) for detecting seizures (**Figure 4** and **Figure 5**) in this model. The spectral power of a typical seizure indicates highest density in the frequency range of 20−40 Hz (**Figure 4**). The majority of the seizures in mice are convulsive, with an average duration of 12−15 s. Only a small fraction of seizures is non-convulsive. A thorough comparison of the advantages and disadvantages of using either system is detailed in the Discussion section. Furthermore, this protocol demonstrates the timelines for seizure onset in animals after repetitive weight drop TBI, showing the seizure clustering in some animals (**Figure 6**) which emphasizes the importance of acquiring continuous rather than intermittent recordings, as this will ensure an accurate stratification of animals that develop spontaneous seizures after TBI from those that do not. Importantly, this protocol also discusses the advantages and disadvantages of rodent models of PTE and their ability to represent a specific population of humans after TBI.

FIGURE AND TABLE LEGENDS:

Figure 1. The mouse model of repetitive diffuse TBI. (**A**) Weight drop device. (**a1**) Weight drop tube. (**a2**) A 100 g weight rod. (**a3**) Pin holding the rod. (**a4**) String to raise the rod up if changing the height or removing the rod from the weight drop tube. (**a5**). Foam pad for placing the animal under the weight drop tube. (**B**) Weight drop procedure. (**b1**) The stainless steel disc is positioned

in the center of the head between the line of the eyes and ears. (**b2** and **b3**) After visual confirmation that the animal's head is in the flat position and the foam pad is moved, placing the animal's head under the weight drop tube. (**b4**) Release of pin holding the weight rod, hitting the center of the stainless steel disc. (**b5**) Mouse is placed on a sterile towel immediately after the impact and loss of consciousness is assessed by measuring the time it takes for the animal to recover and right itself.

Figure 2. Surgical field preparation and EEG electrode placement scheme. Autoclaved tools and necessary materials for surgery and electrode implantation are prepared before anesthetizing the animal to ensure availability of all required parts. This is a sterile zone and it is imperative to not contaminate this zone with non-sterile materials.

Figure 3. Stereotactic landmarks and schematic representation of electrode placement using EEG System 1 and 2. The top panel depicts the methods of implanting the three different headmounts described in this protocol. (**A**) Single EEG channel, bipolar montage. (**B**) Two EEG channels with common reference, bipolar montage and one EMG-channel. (**C**) Three EEG channels, using monopolar (channel 1−2) and bipolar (channel 3) montage. The bottom panel depicts the headmounts and screws implanted as in the top panel. The three types of screws used in this protocol for two purposes: as stability screws (EEG System 1) or both stability and as electrode (EEG System 2).

Figure 4. Spontaneous seizure acquired using EEG System 1. The top panel depicts a spontaneous seizure in a mouse 23 days after repeated weight drop TBI using data acquired using 1EEG headmount. (**A**) Pre-ictal (pre-seizure) activity. (**B**) Ictal (seizure) activity. (**C**) Post-ictal (post-seizure) depression. Bottom panel: Power spectrum density is calculated using custom script and software (see **Table of Materials**). Mean power = average power of the power spectrum within the epoch (units: V^2/Hz). Median frequency = frequency at which 50% of the total power within the epoch is reached (units: Hz). Mean frequency = frequency at which the average power within the epoch is reached (units: Hz). Spectral edge = frequency below which a user-specified percentage of the total power within the epoch is reached (units: Hz). Peak frequency = frequency at which the maximum power occurs during the epoch.

Figure 5. Spontaneous seizures acquired using EEG System 2. (**A**) Spontaneous non-convulsive (electrographic) seizure in a mouse 65 days after repeated weight drop TBI. Data acquired using 2EEG/1EMG headmount. (**B**) Spontaneous convulsive seizure from a non-weight drop experiment. Data acquired using 3EEG headmount.

Figure 6. Seizure incidence timeline in mice after repeated weight drop TBI. The earliest seizure was observed three weeks post-injury. Some animals develop clusters of seizures within the same day followed by several weeks without seizures. Animals were recorded up to four months after TBI.

DISCUSSION:

In contrast to CCI and FPI models inducing either focal or combination of focal and diffuse injury,

the model of repetitive diffuse TBI described in this protocol allows for the induction of diffuse injury in the absence of focal brain injury and does not require scalp or cranial openings and the associated inflammation. An added benefit of the absence of craniectomy in this model is that it allows to not only implant the electrodes for chronic continuous EEG recording, but also the creation of a thinned-skull cranial window for chronic in vivo 2-photon imaging of the animals before, immediately after, and repeatedly for days, weeks, and even months following TBI as described in Shandra and Robel 2019[13].

Regardless of which animal model is chosen, the data acquisition approach adopted is a crucial element of any successful and comprehensive study. In rodent models of post-traumatic epilepsy the frequency of seizures is low[14], ranging between 0.3−0.4 seizures per day[9,15], and the latent period before the first seizure can last anywhere from days or weeks to even months after the initial TBI procedure. Lastly, in contrast to non-traumatic models, which have a generally higher incidence of seizures over a shorter period of time, on average only 9%−50% of animals with TBI will have spontaneous seizures over a period of up to six months[8,16]. This suggests that meaningful studies require continuous long-term video-EEG recording.

The overarching goal of each animal model of TBI is to reproduce as closely as possible the different forms of TBI found in human patients, in order to better investigate the cellular and molecular mechanisms underlying PTE. Techniques in this protocol will help to facilitate the discovery of therapeutic targets, the testing of the efficacy and tolerability of new preventive and therapeutic candidates, and the development of reliable biomarkers or predictors of epilepsy following TBI.

Potential challenges during the weight drop procedure

Because the head is not fixed in a stereotactic frame, extra care must be taken to ensure a flat position of the head and metal plate. If the weighted rod hits the metal plate or head at an angle or if the weight slips off to the side of the mouse head, injury biomechanics will differ, possibly resulting in a milder or no injury. In the past, the metal plate was glued to the skull to minimize variability. However, removal of the metal plate and glue from the mouse skull following weight drop, even if performed with care, induced damage to the meninges, resulting in vascular damage and subsequent damage to the brain tissue even in sham animals. Further, the incision requires healing, potentially involving a peripheral immune response, which might introduce variability. For these reasons it was chosen to omit gluing the metal plate to the skull. Animals may die with repeated (i.e., 3x in this protocol) injury. Mice with a body weight below 25 g may not tolerate repeated impacts. While single injuries almost never result in mortality, up to 7% of C57BL/6 animals die after repeated impacts[9]. Motor deficits can be observed in some animals. These deficits manifest as hindlimb paresis or gait abnormalities. This is usually a prognostic factor for poor recovery and it is recommended that the animal be sacrificed. Signs of pain or distress include weight loss, poor grooming, dehydration, increased anxiety, low or absent exploratory activity (hydrogel/recovery, chow and/or nestlet remain untouched). Rescue analgesia (0.1 mg/kg of buprenorphine) can be administered subcutaneously every 8 h for three days from TBI to alleviate the pain and prevent the animal from reaching the humane endpoint. Subcutaneous sodium lactate solution (3 µL per gram of the animal's weight) can be administered

twice a day for hydration. Animals typically recover within three days after TBI. Use of a five stage body condition score (BCS) for animal monitoring after experimental procedures is recommended. The stages include (1) Emaciated (skeletal structures are extremely prominent, vertebrae extremely segmented); (2) Underconditioned (segmentation of vertebral column is evident, dorsal pelvic bones are readily palpable); (3) Well-conditioned (vertebrae and dorsal pelvis are not prominent palpable with slight pressure); (4) Over-conditioned (spine is a continuous column, vertebrae palpable only with firm pressure); (5) Obese (mouse is smooth and bulky, bone structure disappears under flesh and subcutaneous fat). The humane endpoint is reached when BCS is 1−2, 20% or more weight loss in an adult mouse compared to its pre-TBI weight, symptoms of pain or distress are not alleviated by analgesics, signs of self-mutilation, symptoms of dehydration, hypothermia, presence of neurologic deficits (abnormal gait or motor paresis). Several possible outcomes of substance administration should be taken into consideration. Buprenorphine injected subcutaneously reaches the first peak of its analgesic effect at 10 min after injection[17]. The first impact occurs seconds after buprenorphine is administered, suggesting that the first measurement of the righting time is unlikely to be affected. However, this cannot be fully excluded as a variable. Hence, experimenters are advised to exercise their own judgement. If the weight drop procedure is followed by stereotactic surgery and carprofen is administered it is important to note that carprofen is an anti-inflammatory agent that may affect seizure incidence, hence experimenters are advised to consider its use carefully.

Potential challenges during the surgery

The risk of contamination or infection will be lowered with use of 70% ethanol, but it will not result in sterile conditions. Alternatively, sterile surgical gloves may be used. However, the stereotactic apparatus is not itself sterile, so any manual manipulation will result in loss of the sterile condition of the gloves. Hence, spraying with 70% ethanol is required after contact with any unsterile material during surgery. Drilling through the cranium into the brain creates damage to the brain tissue and may cause profuse bleeding. Creating the burr holes takes extreme care. Fixing the hand drill in the stereotactic arm and gradually lowering it is preferred over drilling the holes while holding the drill manually. Electrodes and fixation screws may sink deeper than planned, injuring the dura mater (subdural placement) or the cortex (cortical placement). This may cause profuse bleeding and a focal lesion. The experimenter must avoid overheating of the animal during the surgery. If the temperature sensor is not fixed correctly it will not maintain the required 37 °C temperature, causing overheating, burns, and sometimes the animal's death as a result. The eyes of the animal get dry, irritated, or damaged during the surgery if not lubricated as soon as the animal is placed in the stereotactic apparatus.

Postoperative monitoring

Postoperative monitoring begins immediately after the procedure or surgery concludes. Observe the animal until it wakes up from anesthesia and look for the presence or absence of any surgery-related complications, including bleeding or paresis. If bleeding is observed from the incomplete incision closure, anesthetize the animal, clean the bleeding site with chlorhexidine, perform wound closure as described above and return the animal to the recovery cage. Approximately 1−2 h after surgery, the animal should be fully awake from anesthesia, moving freely in the cage with no signs of paresis or pain. The animal will begin grooming itself, which is why sealing the

incision is necessary to prevent the animal from opening it during grooming. Once the animal has recovered, transfer it to the cage/chamber that will be used for EEG data acquisition. This will allow the animal to get habituated to the new environment. This is especially important for long-term recording (months). The animal cage must have a recovery gel (see **Table of Materials**), moistened chow, a nestlet, and a water bottle. This will allow proper recovery and will give the animal access to nutrients and water. Continue monitoring the animal daily. The assessment must include (a) Visual inspection of animal's behavior for signs of pain or distress, including weight loss, poor grooming, increased anxiety, low or absent exploratory activity (hydrogel/recovery, chow and/or nestlet remain untouched) and proper healing of the incision area around the EEG implant; (b) Assessment of the BCS for signs of dehydration and malnutrition; (c) Weight of the animal. Administer sodium lactate solution (3 µL per gram of the animal's weight) subcutaneously if the animal shows signs of dehydration (see **Table of Materials**). Administer buprenorphine (0.1 mg/kg) subcutaneously if the animal shows signs of pain or distress. If signs of pain persist buprenorphine can be administered every 8 h. Monitoring must be increased to twice a day if an animal is showing signs of pain and/or distress. Allow the animal to recover for at least three days following EEG surgery prior to connecting to the acquisition system via a tether. The humane endpoint criteria are the same as in potential challenges during the weight drop procedure above.

Advantages and disadvantages of acquisition systems and headmounts

The main advantage of the EEG System 1 with a single EEG channel headmount is the relatively low cost of the hardware, components, and service. The simple and straightforward configuration also allows users to customize the system to their preferences. Each differential amplifier provides a single EEG channel, although several differential amplifiers can be connected with each other, increasing the number of channels for each animal. In this system, a single channel configuration per animal was used to acquire chronic long-term EEG recordings of 20 animals simultaneously. Post-traumatic seizures are typically generalized, and with a bilateral bipolar montage of the electrodes it is easy to detect this type of epileptiform activity. The disadvantage of this approach, however, is that it is impossible to reliably detect focality, lateralization, or the propagation of epileptiform activity, as this would require several channels. Another potential challenge can be noise contamination of the single channel over time, rendering it incapable of acquiring useful data from the animal. This can be overcome by combining two or more differential amplifiers, which doubles the number of channels per animal. Lastly, data acquired from a single channel are harder to distinguish from potential artifacts, and epileptiform activity is best supported by video recordings of the animal's behavior. For this reason, all the recordings combined synchronized continuous video monitoring with EEG acquisition. A limitation of this system and its software is that it does not include the video acquisition system, and therefore requires a custom third-party system for acquiring synchronous video.

The major advantage of the EEG System 2 with multi-channel headmounts is the high quality of the signal due to its prefiltering of the acquired signal by the preamplifier (see **Table of Materials**) prior to being passed through the commutator to the amplifier. The amplifiers in this system allow for the acquisition of data in three channels in the following configurations: 2 EEG+1 EMG

channels or three EEG channels (see **Table of Materials**). This allows for the detection not only of generalized activity but also, potentially, focal epileptiform activity. Another major advantage is that this system was designed specifically for animal research and hence offers a video recording system and software capable of synchronizing the EEG and video channels for up to four animals in a single file, which makes analysis easier and more convenient than the EEG system 1. This system is easy to use for acquisition of data for seizure and sleep analysis without any modifications to the system other than the type of headmount used. The 2EEG/1EMG headmount allows implanting the electrodes at fixed locations only, due to the size and configuration of the circuit board. The screw electrodes with wire leads in 3EEG headmounts allow flexibility in implanting at the desired location with the possibility to do either monopolar or bipolar acquisition depending on where the reference electrode is placed. However, implanting of the 3EEG headmount requires soldering, which adds more steps to the surgery and requires extra caution and precision. The connecting tethers and preamplifiers were specifically designed for small rodents like mice and immature rats, and are thin, low weight cables that cause little pressure on the animal's head. A disadvantage of the system is the relatively high cost of the hardware, software, video license, and components (i.e., preamplifiers and headmounts).

Significance and critical steps in EEG data acquisition

The commutator has a rotating mechanism, allowing the tether to rotate depending on the direction of animal movement. If this mechanism fails, the animal's movement will be restricted, which can result in removal of the EEG cap. Repeated surgery to place new electrodes can be attempted. However, this can be challenging or impossible if removal of the previous EEG cap caused damage to the skull and brain. The sampling rate for EEG data acquisition must be at least 2−2.5 x the highest frequency of interest. Higher sampling rates result in higher resolution of the data at the price of an increase in file size, which may become difficult to store and process when continuous recordings of multiple animals is acquired. Hence, it is necessary to optimize the sampling rate to a level that allows obtaining the necessary data without loss of quality while minimizing file sizes.

Significance and critical steps in video data acquisition

In rodents, as in humans, PTE can manifest with a wide variability in associated symptomatology and electrographic correlates, making it necessary to obtain a simultaneous video during EEG acquisition in order to properly interpret and classify the observed EEG events. Interpretation of EEG data in the absence of synchronized video is particularly challenging when a single EEG channel is used. In this case, it can be difficult to determine if the EEG waveform is an artifact, unless other evidence (video) supports the classification as a seizure. Motion artifacts can appear similar to the electrographic pattern of the seizure. Hence, video with or without EMG confirmation is a requirement. While video recording is performed during both light and dark cycles, the video quality may not always be satisfactory and clear during the dark hours. In addition, if the animal is turned away from the camera during the ictal-like EEG event, it may be challenging to assess its behavior. In those cases, acquiring an electromyography (EMG) signal in addition to EEG and video can solve the challenge by providing information about the muscle activity during milder behavioral seizures (with low motor components) or to confirm the lack of animal movement during absence-like spike-and-slow-wave discharges on the EEG. The potential

challenges with the EMG channel are similar to the challenges with the EEG channels, such as noise contamination, incorrect placement of electrodes, or the electrodes becoming loose (or losing surface contact) over the prolonged time of the recording. The use of video together with EEG analysis has two purposes: to confirm that an EEG event is not an artifact caused by the animal's movement (exploratory behavior, drinking, chewing, scratching, stretching, grooming, or rapid/labored breathing) and to differentiate between convulsive and non-convulsive seizures. Use of a modified Racine scale to characterize convulsive or non-convulsive seizures is recommended. The stages include (0) Pure electrographic seizure without any identifiable motor manifestation; (1) Orofacial automatisms and head nodding; (2) Forelimb clonic jerk; (3) Bilateral forelimb clonus; (4) Forelimb clonus and rearing; (5) Forelimb clonus with rearing and falling. Each video channel must clearly show the entire surface with the animal in the cage, a label with an animal identification number, water bottle tip, food, and diet/recovery gel. To ensure video acquisition during the dark hours, use an infrared night source. (Some cameras have built-in devices or may require additional parts. See the **Table of Materials**). Adjust the frame per second rate and image resolution. The higher frame rate and resolution come at the cost of bigger file size. The main disadvantages of acquiring video during prolonged chronic continuous experiments include the need to store very large amounts of data and the technical difficulties involved in processing the large files. The proficiency of the experimenter to effectively interpret the behavioral data together with EEG must also be considered.

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DISCLOSURES:

The authors have nothing to disclose.

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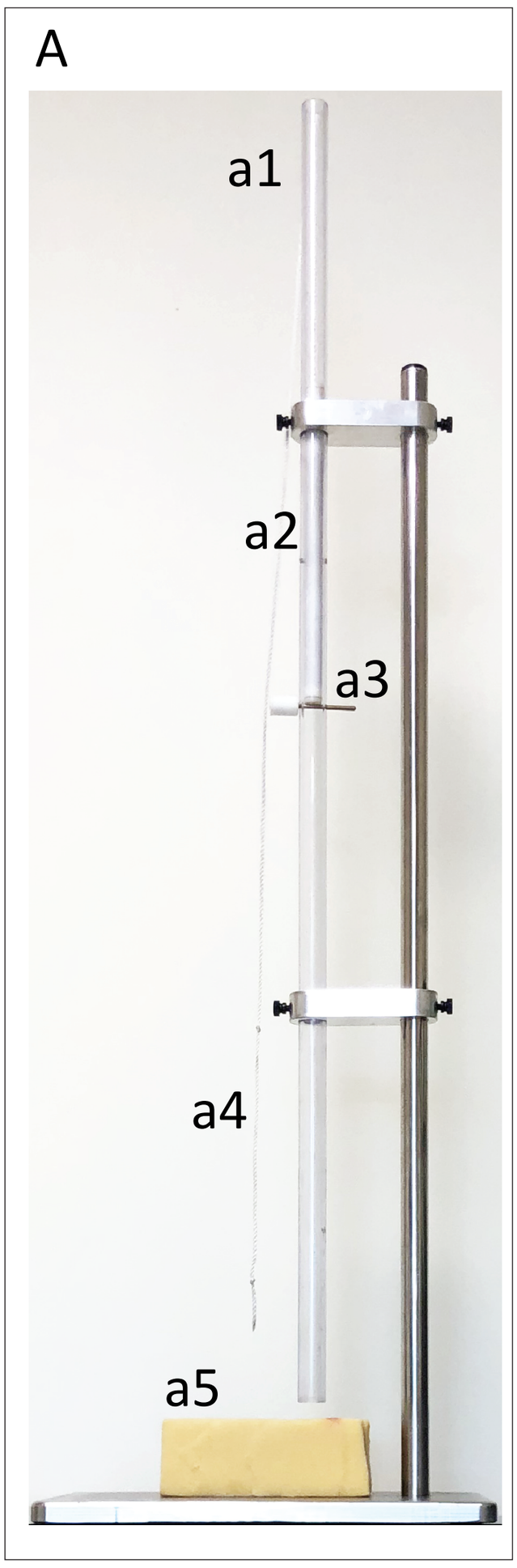
Figure 1



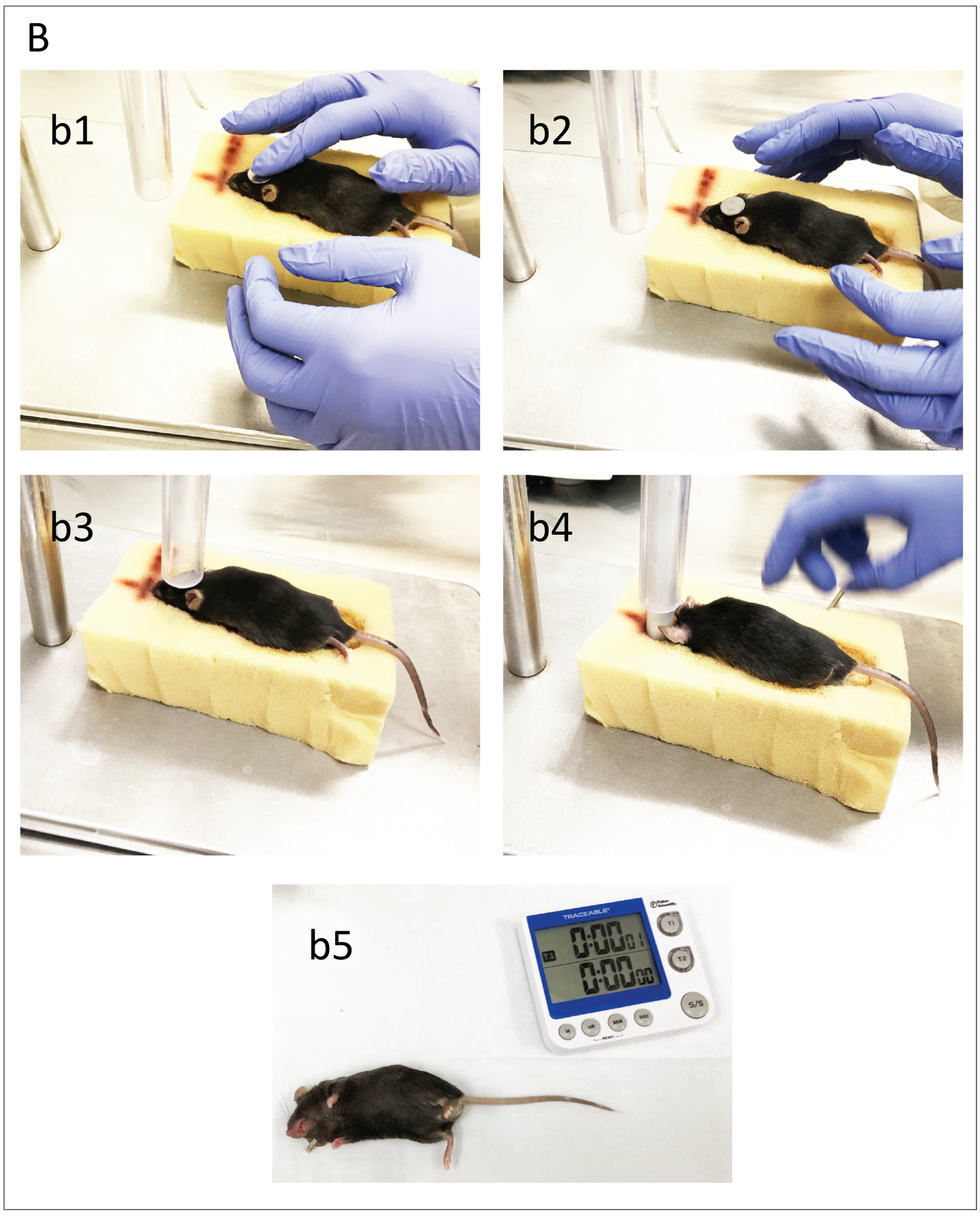
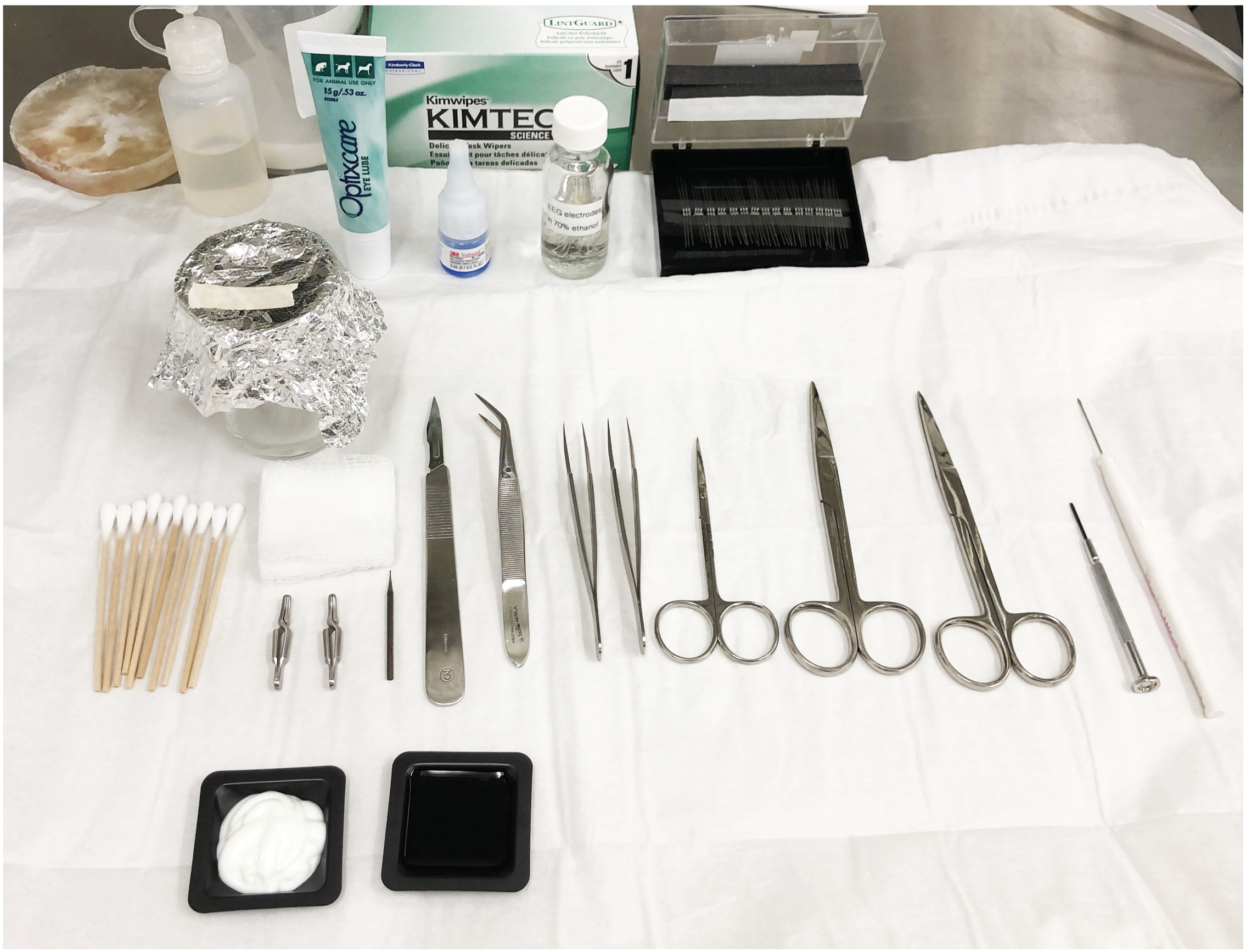
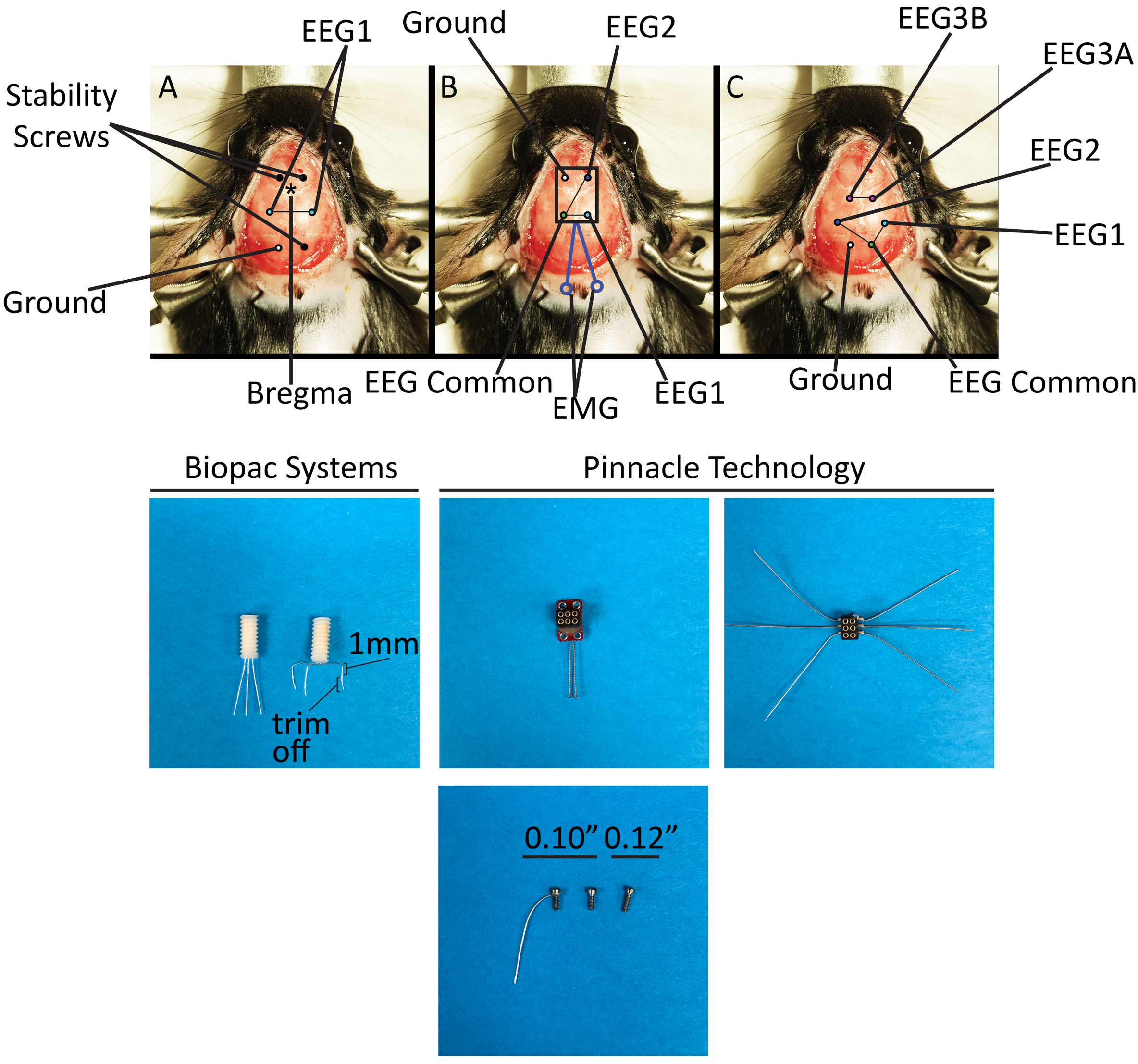
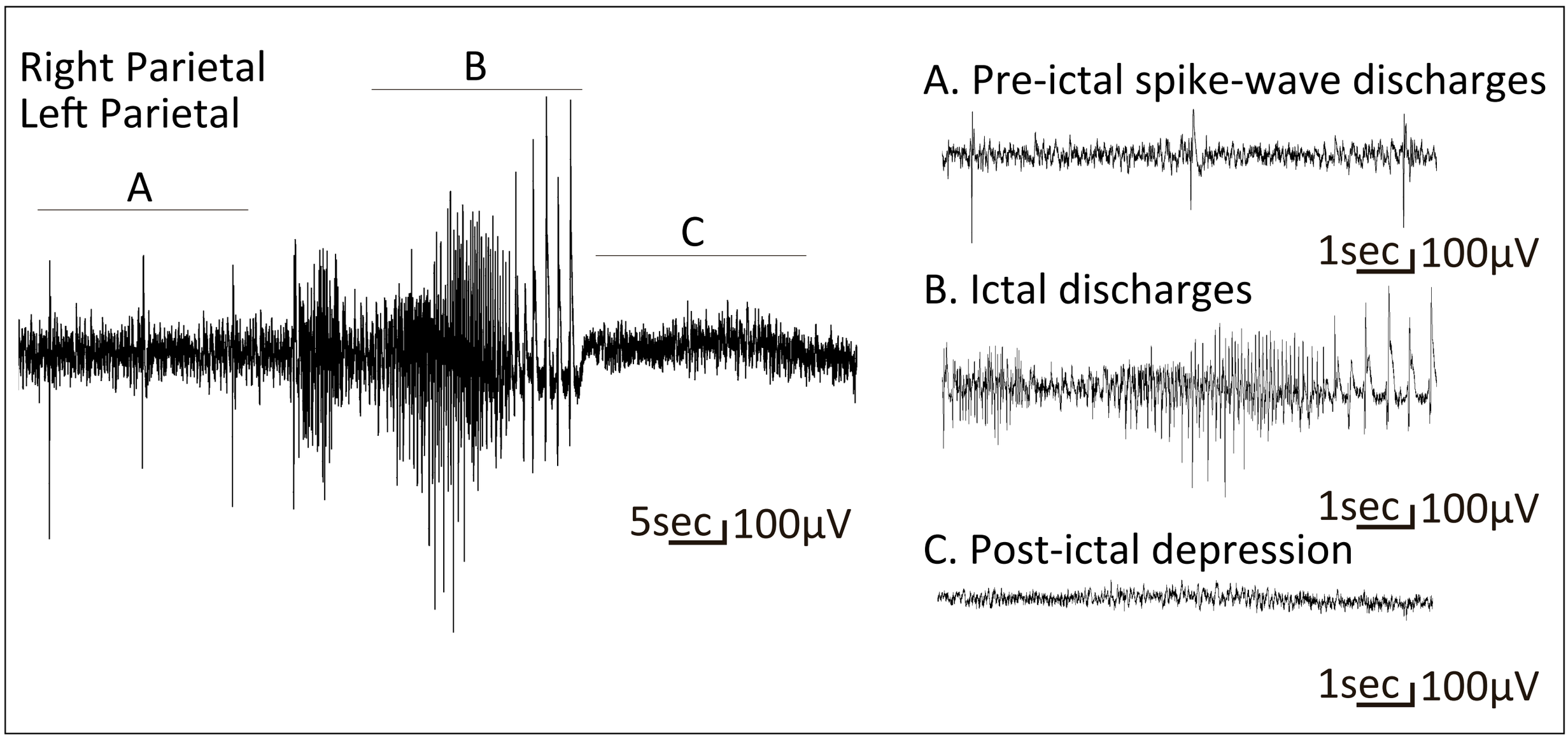


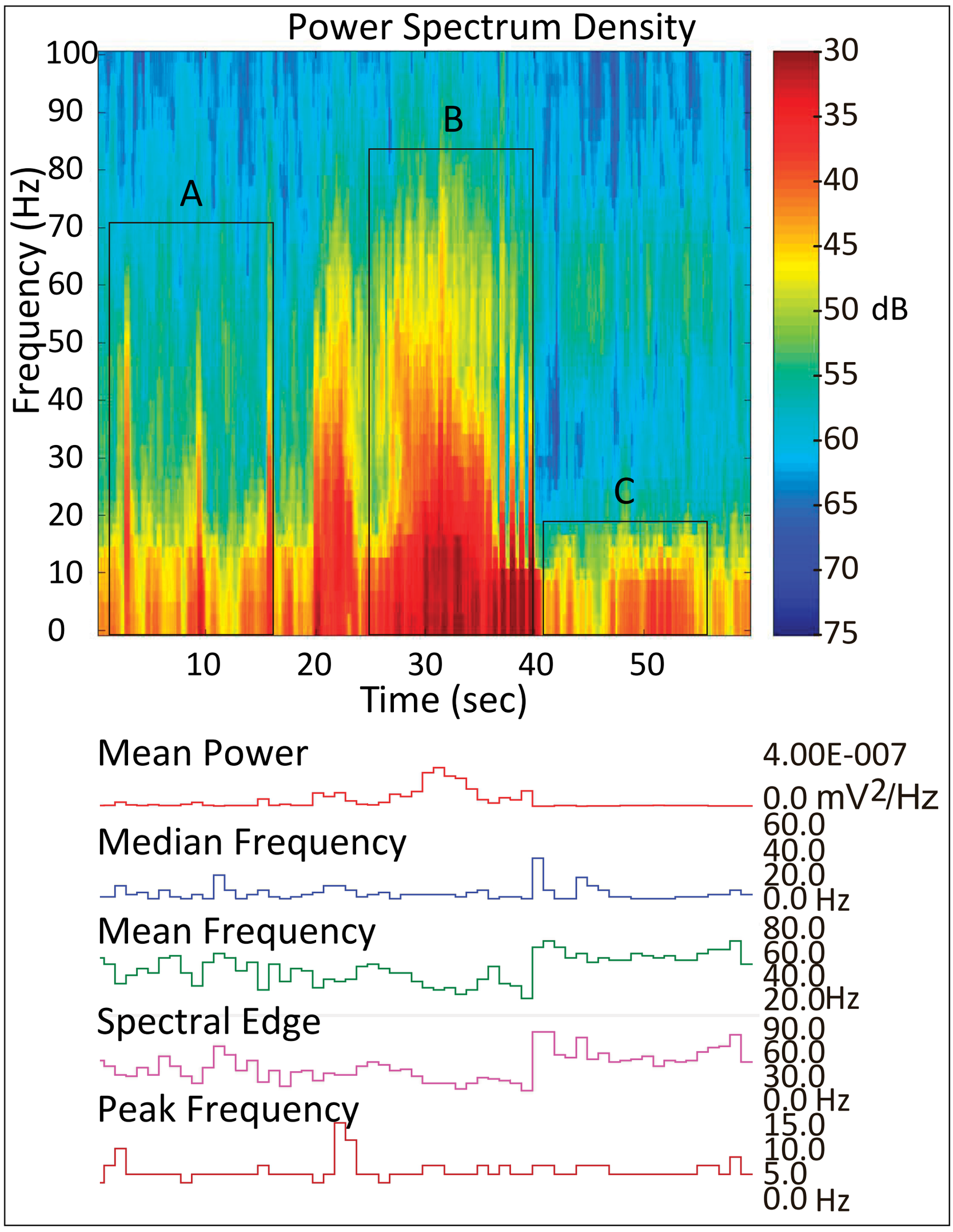
Figure 2

Click here to access/download;Figure;Figure 2 Surgical Field.ai

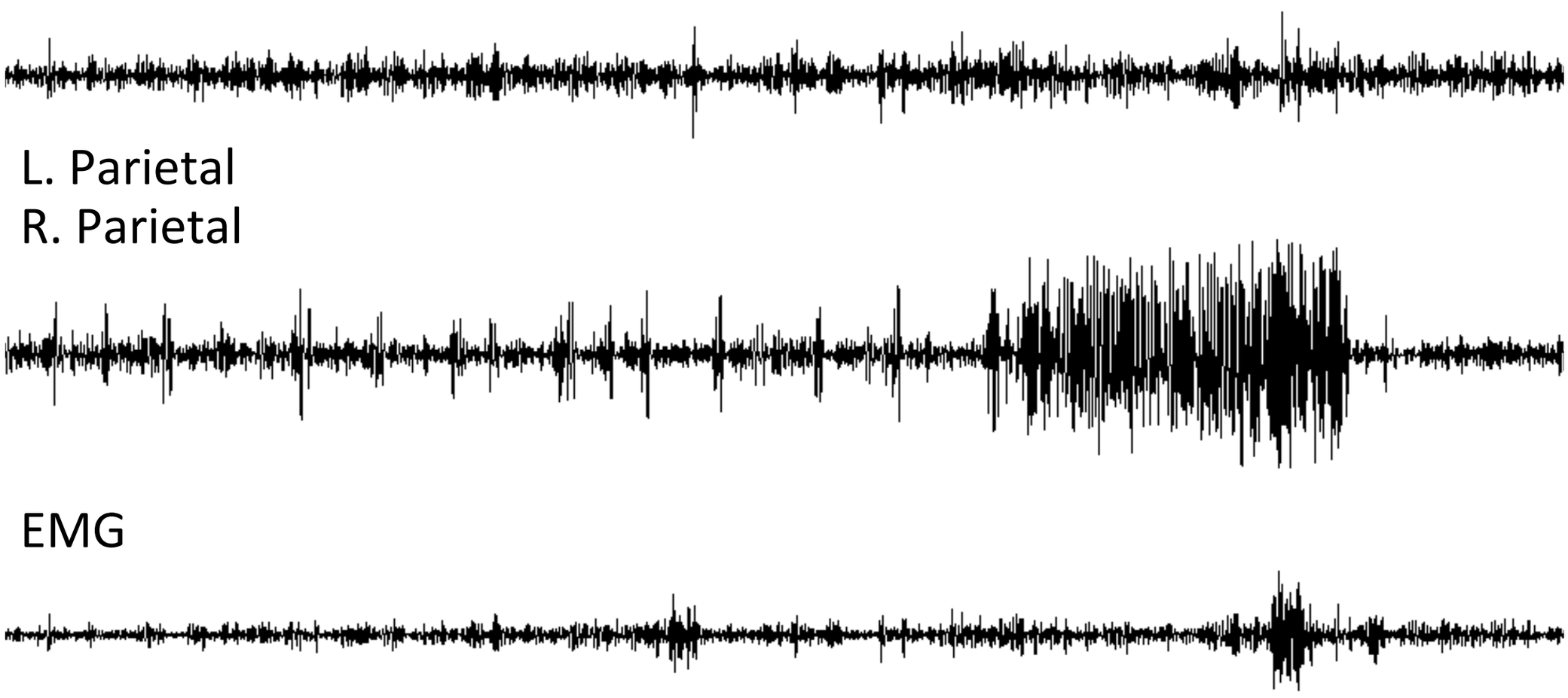








A Non-convulsive seizure



B Convulsive seizure

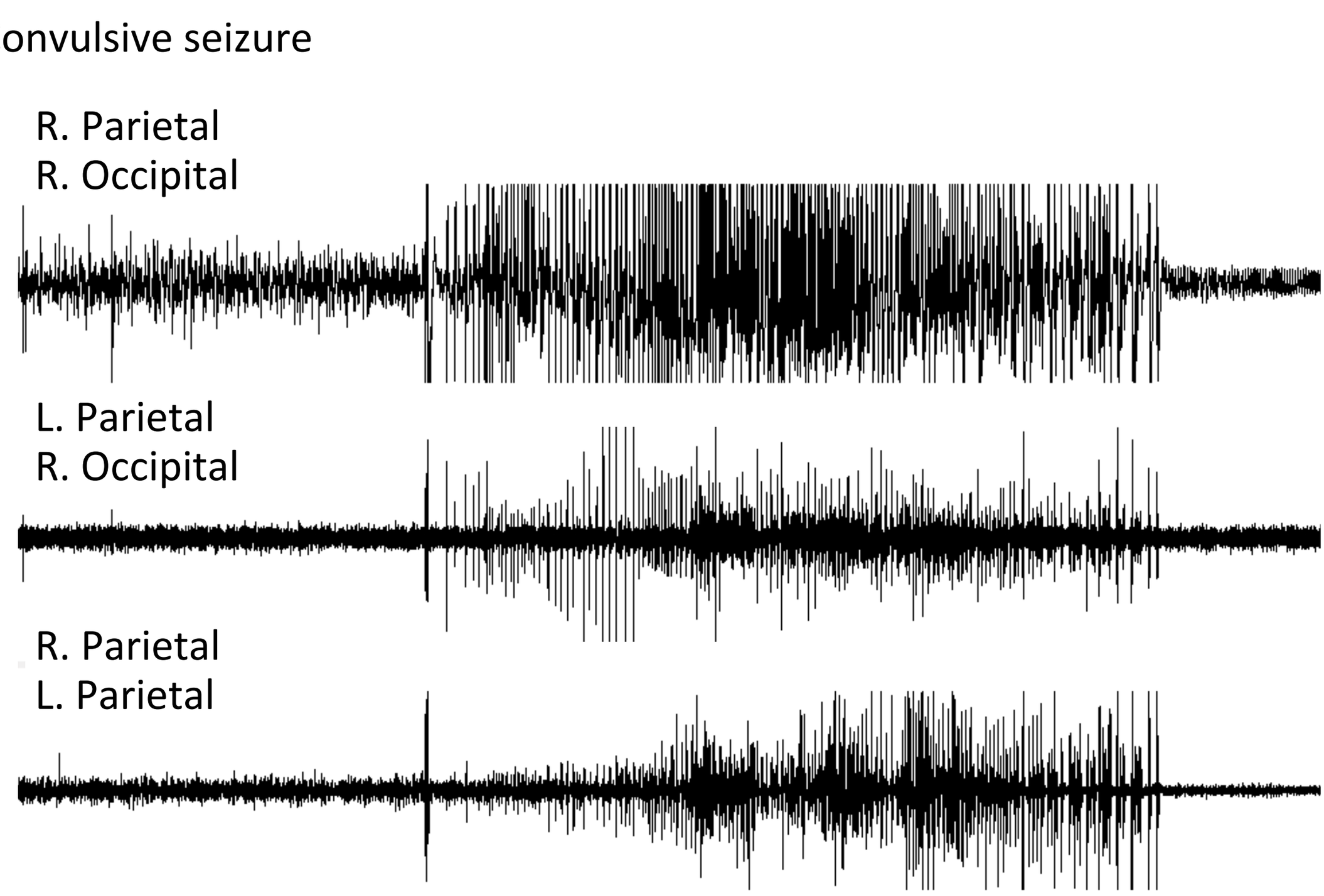


Figure 6

Click here to access/download;Figure;Figure 6 Seizure timeline.ai

Expimental group	Animal ID	days post-injury																								
		4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
Sham	7776																										
Sham	7779																										
rdTBI	123																										2
rdTBI	127																									5	1
rdTBI	129																		1		1						
rdTBI	1105																										
rdTBI	1106																										
rdTBI	1107																										
rdTBI	1108																										
rdTBI	1336																										
rdTBI	1822																										
rdTBI	1878																										

Expimental group	Animal ID	days post-injury																								
		30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55
Sham	7776																										
Sham	7779																										
rdTBI	123																										
rdTBI	127																										
rdTBI	129						1							2							1						
rdTBI	1105																										
rdTBI	1106																										
rdTBI	1107																										
rdTBI	1108																										
rdTBI	1336																										
rdTBI	1822																										
rdTBI	1878																						1*				

Expimental group	Animal ID	days post-injury																								
		56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81
Sham	7776																										
Sham	7779																										
rdTBI	123																										
rdTBI	127																										
rdTBI	129																										
rdTBI	1105																										
rdTBI	1106						1*	1*			1*		1*	1*					2*	1*	3*						
rdTBI	1107																										
rdTBI	1108																										
rdTBI	1336																										
rdTBI	1822																										
rdTBI	1878																										

Expimental group	Animal ID	days post-injury																								
		82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107
rdTBI	1822																										
rdTBI	1878																										

Expimental group	Animal ID				
		108	109	110	111
rdTBI	1822				
rdTBI	1878				

Analyzed
No Recording
Interrupted recording
Recording not started or terminated
* Non-convulsive seizure

Table of Materials

Click here to access/download;Table of Materials;Revised table of materials.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.10" screw	Pinnacle Technology Inc., KS, USA	8209	0.10 inch long stainless steel
0.10" screw	Pinnacle Technology Inc., KS, USA	8403	0.10 inch long with pre-soldered wire lead
0.12" screw	Pinnacle Technology Inc., KS, USA	8212	0.12 inch long stainless steel
1EEG headmount	Invitro1 (subsidiary of Plastics One), VA, USA	MS333/8-A/SPC	3 individually Teflon-insulated platinum iridium wire electrodes (twisted or untwisted, 0.005 inch diameter) extending below threaded plastic pedestal
2EEG/1EMG headmount	Pinnacle Technology Inc., KS, USA	8201	2EEG/1EMG channels
3% hydrogen peroxide			Pharmacy
3EEG headmount	Pinnacle Technology Inc., KS, USA	8235-SM-C	custom 6-Pin Connector for 3EEG channels
Buprenorphine	Par Pharmaceuticals, Cos. Inc., Spring Valley, NY, USA	060969	
Buprenorphine	Par Pharmaceuticals, Cos. Inc., Spring Valley, NY, USA	060969	
C57BL/6 mice	Harlan/Envigo Laboratories Inc		male, 12-16 weeks old
C57BL/6 mice	The Jackson Laboratory		male, 12-16 weeks old

Carprofen	Zoetis Services LLC, Parsippany, NJ, USA	026357	NOTE: this drug is added during weight drop only if stereotactic electrode implantation will be performed on the same day
Chlorhexidine antiseptic			Pharmacy
Dental cement and solvent kit	Stoelting Co., USA	51459	
Drill	Foredom	HP4-917	
Drill bit	Meisinger USA, LLC, USA	HM1-005-HP	0.5 mm, Round, 1/4, Steel
Dry sterilizer	Cellpoint Scientific, USA		Germinator 500
EEG System 1	Biopac Systems, CA, USA		
EEG System 2	Pinnacle Technology Inc., KS, USA		
Ethanol ≥70%	VWR, USA	71001-652	KOPTEC USP, Biotechnology Grade (140 Proof)
Eye ointment	Pro Labs Ltd, USA		Puralube Vet Ointment Sterile Ocular Lubricant available in general online stores and pharmacies
Fluriso liquid for inhalation anesthesia	MWI Veterinary Supply Co., USA	502017	
Hair removal product	Church & Dwight Co., Inc., USA		Nair cream
Isoflurane	MWI Veterinary Supply Co., USA	502017	
Povidone-iodine surgical solution	Purdue Products, USA	004677	Betadine
Rimadyl/Carprofen	Zoetis Services LLC, Parsippany, NJ, USA	026357	
Solder			Harware store

Soldering iron	Weller, USA	WP35	ST7 tip, 0.8mm
Stainless steel disc			Custom made
Sterile cotton swabs			
Sterile gauze pads	Fisher Scientific, USA	22362178	
Sterile poly-lined absorbent towels pads	Cardinal Health, USA	3520	
Tissue adhesive	3M Animal Care Products, USA	1469SB	

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Author(s):	Oleksii Shandra, Stefanie Robel

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The Journal of Visualized Experiments July 12, 2019

RE: Revision of manuscript # JoVE60360

Dear Drs. Berard and DSouza,

Enclosed, please find a copy with the requested editorial changes made to the manuscript JoVE60360 entitled "**Inducing post-traumatic epilepsy in a mouse model of repetitive diffuse traumatic brain injury**" authored by Oleksii Shandra and Stefanie Robel.

We'd like to sincerely thank you and the reviewers for your positive feedback on the manuscript and have made the requested changes to include all comments regarding the text structure, language, additional technical details and clarification of protocol steps.

We are confident that you will now find the manuscript suitable for publication in The JoVE and are looking forward to your final decision.

With our best regards,

Oleksii Shandra and Stefanie Robel

Point by point response to the comments by the reviewers and editor

Changes in the text are indicated using red font.

First we would like to address changes made with regards to Editorial Comments:

1. Please reduce the summary to 50 words.

We reduced the length of the summary to 49 words.

2. Please rewrite lines 246-264, 277-281, using original text.

We understand that the phrasing used in these steps are identical to phrases in the methods of our previous publications and made an attempt to paraphrase. However, these are basic technical steps, which can only be described in so many different ways. If the editors have suggestions of how to further change the wording, we will be happy to include those.

3. Please ensure that ALL text in the protocol section is written as steps in the imperative voice/tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

The text was revised and now is in imperative voice/tense in protocol steps. Text that either addresses a specific recommendation or is describing our personal experience is marked as a 'NOTE' or was moved to the discussion section.

4. Section 7 needs to be rewritten in its entirety.

We rewrote this section and it now includes only specific steps and settings as requested.

5. Protocol details:

1) 3.1, 4.1, 4.5: Mention oxygen flow rate.

This information was added.

2) 5.3.1, 5.5.1: Mention drill bit size and drill speed.

This information was added.

6. Protocol Numbering: Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary

and all steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step.

The numbering in each section was fixed as per author instructions and editorial comments.

7. Protocol highlight and length

The overall length of the protocol was revised and reduced to fit the 10-page limit. Sections highlighted for filming were written to form a cohesive story and include all necessary steps crucial for reproducibility of the approach. Notes were excluded from the highlighted narrative.

8. Discussion

We revised the structure of this section to address 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol. The numbered listing style was removed and transformed into organized paragraphs. Given the complexity of the crucial background information for the first-time users of the TBI, EEG surgery or video-EEG acquisition and description of different surgical approaches and acquisition system configuration in this protocol, it is challenging to limit the text to 6 paragraphs without losing context. Hence, we re-organized it in a way to give a reader sufficient information about advantages, limitations and troubleshooting of each of the technical sections.

9. References. Please spell out journal names.

We used the Endnote style for JoVE with journal names spelled out.

10. Avoid using commercial language

All commercial names for systems, hardware, components and reagents used in this protocol were replaced with either generic or neutral term and the reader is referred to see the table of materials for clarification.

11. Table of materials

All listed materials and reagents were sorted alphabetically; names and information on company and catalog number were included.

To address the comments, suggestions and concerns from the reviewers we revised the manuscript as follows:

Reviewer 1

Minor Concerns:

1. Inflammation plays a major role in the development of post-traumatic epilepsy. It is understandable to prevent pain and distress. However, the inclusion of carprophen (an NSAID) would potentially prevent the development of epilepsy in some animals, which is what the model is designed to study.

We agree that the inflammation plays crucial role in seizures and epilepsy. However, inflammation can be a cause and a consequence of seizures. We administer carprofen acutely on the day of TBI and EEG surgery in a single injection to prevent surgery-related complications in accordance with our institutional IACUC protocol.

A study by Thau-Zuchman et al. 2012 reported potential neuroprotective effect of carprofen after TBI when administered repeatedly for 7 days. Epilepsy in our TBI animals occurs 3-4 weeks after TBI in the absence of pronounced astroglial or microglial activation (see Shandra et al., J Neurosci 2019). While we cannot exclude that the single dose of carprofen as a variable, it does not appear to prevent epileptogenesis given that one third of the animals present with recurrent seizures.

2. Administration of buprenorphine prior to injury would significantly alter the righting reflex of rats and would render this measurement completely untrustworthy. If righting reflex is to be used, it would be more appropriate to administer buprenorphine after the righting response is observed.

In accordance with our institutional IACUC guidelines we are required to provide analgesia to animals undergoing a procedure which may inflict pain such as weight drop traumatic brain injury. Hence it is not acceptable to administer buprenorphine after all impacts are delivered. Animals undergo three impacts (with righting times taken after each) separated by 45 minute intervals. If buprenorphine was administered after the last righting time animals would be in pain for 3 hours before administration of the analgesic.

According to the reference by Gades et al. 2000 (also included in section 3, the first peak analgesic effect of buprenorphine is reached 10 minutes after a subcutaneous injection. The first impact occurs seconds after buprenorphine is administered suggesting that the first measurement of the righting time is unlikely to be affected.

To address the reviewer's concern we added a statement in lines 712-715 making the reader aware that buprenorphine might affect righting times.

3. Spraying surgical gloves with 70% ethanol is not an acceptable means of disinfecting or maintaining sterility.

We agree that 70% ethanol is not creating sterility, which is why we used the term "to disinfect". Areas for rodent surgeries are typically not sterile. For example, the stereotactic apparatus is not sterile. Manipulations of the apparatus will render sterile surgical gloves non-sterile. We use 70% ethanol to disinfect gloves after manipulating non-sterile equipment, which is appropriate to reduce the risk of contamination of the close-by sterile field. In contrast, the incision site is not touched with gloves or any instruments or equipment that were not autoclaved or sterilized using a bead sterilizer.

4. The procedure indicates to weigh the animal then remove it from the stereotaxic frame (sec. 5.3 steps 10 & 11; Sec 5.4 steps 12 & 13; sec 5.5 steps 14 & 15). this order should probably be reversed.

Sections 5.3, 5.4, 5.5 were modified as suggested by the reviewer.

5. The discussion identifies CCI and FPI as a models of penetrating injury. I would disagree that FPI represents a penetrating injury. FPI produces diffuse injury as well as focal injury at a site distant from the pressure pulse.

This is a very valid point and we revised the sentence about the CCI and FPI as follows: "Until recently, only two animal models of post-traumatic epilepsy existed: controlled cortical impact (CCI, focal) or fluid percussion injury (FPI, focal and diffuse. Both induce large focal lesions alongside tissue loss, hemorrhage and gliosis in rodents".

6. There has some confusion in the literature regarding the use of craniotomy and craniotomy. The word craniotomy is used in the discussion referring to CCI and FPI models. The bone flap is typically not replaced in these models. Therefore, the correct term is craniotomy.

We now refer to technique used for CCI as 'craniectomy' since the bone flap is not replaced in experimental animals.

Reviewer 2

Major Concerns:

1. In the Discussion section, the authors emphasize the necessity of video/EEG monitoring to ensure that the electrographic events detected in the EEG are actually

seizures. Since video monitoring is such an important component of the data collection, this should be fully described in the Methods section and not just mentioned in the Discussion.

Section 8 was added addressing specific video acquisition parameters for both EEG systems described in the protocol. We also commented on the significance and critical steps for video data acquisition in the discussion section.

2. The authors should comment further on the clinical relevance of this model to TBI in humans, given that the model involves repeated injuries within a fairly short time frame. According to Section 3, animals are subjected to three weight-drops within a 90 min period, which would correspond to multiple TBIs within the acute (if not hyper-acute) injury/post-injury period. In what types of injury scenarios would this occur in human TBI patients?

We understand the reviewers concern, however it is beyond the scope of this protocol paper to discuss the challenges of aligning timelines of pathology in humans and rodents or the complexity of inter-injury interval modeling. The timeline in humans is certainly different but so is the lifespan, which appears to affect timelines of pathology. Our goal for this manuscript was to provide a systematic protocol for the procedures used in Shandra et al., J Neurosci 2019, which demonstrates that diffuse closed-head TBI without focal injury is sufficient to induce post-traumatic epilepsy.

3. In Figure 5 (panel B), the authors should highlight the fact (i.e., in the figure labels) that the EEG of the convulsive seizure was taken from a non-weight drop experiment. The recording in 5B seems to be saturated, and the duration of ictal/seizure events recorded from weight-drop TBI animals (Fig. 5A) appear to be significantly shorter than the spontaneous convulsive seizure in Fig. 5B. It is somewhat misleading to include the Fig. 5B convulsive seizure traces in this manuscript, since these did not occur in an animal from the repeated weight-drop model. The figure legend already specifies this word to word.

The example in Fig. 5B is indeed recorded in a different TBI model, which is mentioned in the figure legend. The point of this figure is to compare different EEG configurations

and to show a typical convulsive seizure using multi-channel recordings. While we recorded weight drop TBI animals using the Pinnacle Technology system, it so happened that we did not catch a convulsive seizure in the 3-EEG channel configuration in this model.

Minor Concerns:

1. In the Introduction (line 87), there is a typographic error: "ASD" should be "AED."

The typo was corrected.

2. Section 3.1 (Weight Drop Procedure, line 191): What method is used to verify that mice have reached a sufficient plane of anesthesia with "5 minutes" of isoflurane exposure, in order to ensure that they are unresponsive to pain (e.g., toe-pinch withdrawal)? This information should be included.

In this section step 2 was corrected as follows: "Remove the mouse from an induction chamber and place it on the foam pad. Test for the absence of a response to toe or tail pinch."

3. Section 4.7 (Surgical Field Preparation, lines 263 - 269): What are the routes of administration for Buprenorphine and NSAIDS? These should be indicated.

This sentence was corrected as follows: "Administer a mixture of analgesics (0.1 mg/kg buprenorphine) and the non-steroidal anti-inflammatory (5 mg/kg carprofen) in a single injection subcutaneously unless the TBI was performed earlier during the day, in which case the animal already received analgesics and anti-inflammatories."

4. Section 5.3 (Electrode Placement, lines 312, 318): (a) How are the burr holes created?; (b) What is the drill bit size that is used?; (c) Please define Vin+ and Vin-.

a-b) This information was added: "Create 6 burr holes (3 for stability screws and 3 for electrodes) with a steel drill bit (0.5 mm, round, ¼) using the following stereotactic coordinates".

c) This information was added: “NOTE: Vin+ is an active electrode and Vin- is its reference electrode”.

5. Section 5.3.8 (Single EEG, line 356): This section states to "repeat the injection every 2 hours..." What is the agent or solution that is injected? This should be indicated.

This comment was addressed as follows: “If the animal is under anesthesia for longer than 2 hours after the previous injection of sodium lactate solution (given during the TBI induction), administer 3µl per gram of body weight subcutaneously. To maintain proper hydration of the animal, repeat the injection every 2 hours that the animal spends under anesthesia. After the surgery, give a final injection 2 hours from the previous injection. If surgery is less than 2 hours long, administer the final “recovery” dose of sodium lactate solution 2 hours from the first injection”.

6. Figure 3: (a) Legend refers to A, B and C. These letters are not labeled on figure. (b) Images of Biopac pedestal are not visible. These should be taken against a black background. (c) Images of screws and head mounts are too small to see.

Figure was corrected as suggested.

7. What is the average duration of seizures in this model, and are they mostly non-convulsive? This information should be included.

The majority of seizures in our model are convulsive and occasionally non-convulsive seizures were observed as presented in the example. The average seizure duration is 12-15 seconds but is occasionally 30 seconds or longer. The average duration information was added as requested. More detailed data on the seizure phenotype is presented in Shandra et al., J Neurosci. 2019.

Reviewer 3

Two suggestions:

1. Repeated weight drop was used and is mentioned, but what this entailed is a bit hidden (Line 227-228). When mention repeated weight drop was used, why not

indicate it was done three times in other portions of the paper such as the Discussion section?

The information was added throughout the text where it is referred as "repeated diffuse" injury. It now appears as "repeated (3x) diffuse injury".

2. For Figure 4, I found myself having to search which figure was being described. While not difficult to do, please include mention in the legend that the figures on the upper right are similar to those on the left; just with a different time scale. When the power spectrum data are discussed, mention the location of the figure (left, middle), etc.

Figure was revised as suggested.

Minor Concerns:

3. The "body condition score" (Line 724) was not clear. Are the three items of point values of one each; i.e., "mouse under-conditioned, segmentation...."? This should be explicit. Should the mentioned humane endpoint then mean 2 or more and not 2 or less (Line 724)?

This section was revised as follows: "Use a five stage body condition score (BCS) for animal monitoring after experimental procedures: Stage 1 - mouse is emaciated (skeletal structures extremely prominent, vertebrae extremely segmented); Stage (2) - mouse is underconditioned (segmentation of vertebral column is evident, dorsal pelvic bones are readily palpable); (3) - mouse is well-conditioned (vertebrae and dorsal pelvis not prominent palpable with slight pressure); (4) - mouse is over-conditioned (spine is a continuous column, vertebrae palpable only with firm pressure); (5) - mouse is obese (mouse is smooth and bulky, bone structure disappears under flesh and subcutaneous fat). The humane endpoint is reached when the following is observed: BCS 1-2, 20% or more weight loss in an adult mouse compared to its pre-TBI weight, symptoms of pain or distress are not alleviated by analgesics, signs of self-mutilation, symptoms of dehydration, hypothermia, presence of neurologic deficits (abnormal gait or motor paresis)".

4. Line 776: What does passive behavior mean? How is this evaluated?

This and similar sentences were corrected as follows: "Signs of pain or distress include weight loss, poor grooming, dehydration, increased anxiety, low or absent exploratory activity (hydrogel/recovery, chow and/or nestlet remain untouched)".

5. Line 832-833: Please give a bit more description for how the videos are used. Are they referring to using the videos for the Racine scoring mentioned in Lines 910-918?

A modified Racine scale is used to characterize the behavioral seizures. The scoring details and the reference were added.