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Juxtacellular monitoring and localization of single neurons within sub-cortical brain structures of alert, head-restrained rats --Manuscript Draft--

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Abstract:	There are a variety of techniques to monitor extracellular activity of single neuronal units. However, monitoring this activity from deep brain structures in behaving animals remains a technical challenge, especially if the structures must be targeted stereotaxically. This protocol describes convenient surgical and electrophysiological techniques that maintain the animal's head in the stereotaxic plane and unambiguously isolate the spiking activity of single neurons. The protocol combines head restraint of alert rodents, juxtacellular monitoring with micropipette electrodes, and iontophoretic dye injection to identify the neuron location in post-hoc histology. While each of these techniques is in itself well-established, the protocol focuses on the specifics of their combined use in a single experiment. These neurophysiological and neuroanatomical techniques are combined with behavioral monitoring. In the present example, the combined techniques are used to determine how self-generated vibrissa movements are encoded in the activity of neurons within the somatosensory thalamus. More generally, it is straightforward to adapt this protocol to monitor neuronal activity in conjunction with a variety of behavioral tasks in rats, mice, and other animals. Critically, the combination of these methods allows the experimenter to directly relate anatomically-identified neurophysiological signals to behavior.
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21 July 2013

Rachelle Baker
Associate Editor – Physical Sciences
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Dear Rachelle,

I submit the manuscript "Juxtacellular monitoring and localization of single neurons within sub-cortical brain structures of alert, head-restrained rats" by myself, Martin Deschênes, and David Kleinfeld, for consideration as a technical article and JoVE produced video.

Our manuscript demonstrates a particular method for monitoring and localizing neuronal activity in alert animals—a technique that achieves a common, but technically demanding, general goal for many systems neuroscience researchers. Our protocol contains specific technical instructions which can only be demonstrated via JoVE's unique video publication format. Additionally, it includes technical specifications for custom-made equipment that make such experiments more reliable and more convenient than previously possible. We suggest that our article will be beneficial to neuroscientists studying diverse experimental questions related to sensation and perception, motor control, and other sub-disciplines.

In addition to yourself, we have communicated with the following editors at JoVE:

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We request that the manuscript not be sent to Daniel Simons (U Pitt) in light of strong professional differences.

Author contributions: MD, DK, and JDM designed the protocol and the experiments. DK and JDM designed the mechanical and electrical components. MD and JDM performed the experiments. JDM wrote the paper with assistance from MD and DK.

Thank you so much for considering our work.

Most Sincerely,

Jeffrey D. Moore

TITLE:

Juxtacellular monitoring and localization of single neurons within sub-cortical brain structures of alert, head-restrained rats

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KEYWORDS:

electrophysiology; juxtacellular; iontophoresis; stereotaxic surgery; thalamus; vibrissa

SHORT ABSTRACT:

This protocol describes the design and surgical implantation of a head-restraining mechanism to monitor neuronal activity in sub-cortical brain structures in alert rats. It delineates procedures to isolate single neurons in the juxtacellular configuration and to efficiently identify their anatomical locations.

LONG ABSTRACT:

There are a variety of techniques to monitor extracellular activity of single neuronal units. However, monitoring this activity from deep brain structures in behaving animals remains a

technical challenge, especially if the structures must be targeted stereotactically. This protocol describes convenient surgical and electrophysiological techniques that maintain the animal's head in the stereotaxic plane and unambiguously isolate the spiking activity of single neurons. The protocol combines head restraint of alert rodents, juxtacellular monitoring with micropipette electrodes, and iontophoretic dye injection to identify the neuron location in post-hoc histology. While each of these techniques is in itself well-established, the protocol focuses on the specifics of their combined use in a single experiment. These neurophysiological and neuroanatomical techniques are combined with behavioral monitoring. In the present example, the combined techniques are used to determine how self-generated vibrissa movements are encoded in the activity of neurons within the somatosensory thalamus. More generally, it is straightforward to adapt this protocol to monitor neuronal activity in conjunction with a variety of behavioral tasks in rats, mice, and other animals. Critically, the combination of these methods allows the experimenter to directly relate anatomically-identified neurophysiological signals to behavior.

INTRODUCTION:

Monitoring neuronal activity in an alert animal actively engaged in a behavioral task is critical for understanding the function and organization of the nervous system. Extracellular recording of the electrical activity from single neuronal units has long been a staple tool of systems neuroscience and is still widely in use at present. A variety of electrode types and configurations are available depending on the scientific and technical demands of a particular experiment. Chronically implanted microdrives or electrode arrays are often used in freely moving animals, including birds, rodents, and non-human primates ¹⁻⁴. Alternatively, acute penetrations with metal or glass microelectrodes via an external micromanipulator are often used to record from anesthetized or head-restrained animals. Glass micropipette electrodes have the advantage that they can be used in the juxtacellular or "cell attached" configuration to unambiguously isolate the activity of single neurons without the complications of post-hoc spike sorting ⁵. These electrodes further permit recording from anatomically-identified cells or locations, as they can be used to inject small deposits of dye or neuroanatomical tracers, or even to fill the individual recorded cell. This configuration has been successfully applied in rats, mice and birds ⁶⁻¹⁰. The presently described technique focuses on juxtacellular monitoring and extracellular dye deposits in alert, head-restrained rats. Note that unlike single cell juxtacellular fills, these dye deposits do not provide information about cell morphology or axonal projections ¹¹, but they enable exact anatomical localization to approximately 50 μm and, critically, have a significantly higher yield in alert animals. Information regarding single-cell juxtacellular fills is nonetheless provided as an alternative strategy for anatomical labeling.

In brief, the protocol consists of three major phases. In the first phase, the rat is acclimated to body restraint in a cloth sock (**Fig 1**) over a period of 6 days. In the second phase, a head restraint apparatus (**Fig 2**) and recording chamber are surgically implanted such that the rat can be maintained in the stereotaxic plane during multiple subsequent recording sessions (**Fig 3**); this procedure enables the experimenter to target particular sub-cortical regions of the brain for electrophysiological study based on standard reference coordinates ¹². The third phase

involves placing the rat in an appropriate jig for conducting the behavioral and electrophysiological experiments (**Fig 4**), constructing the electrode from a quartz capillary tube (**Fig 5**), making juxtacellular neuronal recordings that unambiguously isolate single units⁶⁻⁹, and marking the anatomical location of the recording site with Chicago sky blue dye (**Figs 6 & 7**). The recordings are performed with simultaneous behavioral monitoring; however, the technical details of the behavior will depend on the scientific goals of each experiment and are thus beyond the scope of a single protocol. After completion of the experimental procedure, which can be repeated on multiple days, the animal is euthanized. The brain is extracted and processed according to standard neuroanatomical techniques using either bright field or fluorescence microscopy.

PROCEDURE:

Experimental protocols were carried out on female Long Evans rats (250-350 grams) in accordance with federally prescribed animal care and use guidelines and were approved by the Institutional Animal Care and Use Committee at the University of California San Diego.

1) Acclimating the rat to body restraint

Place the rat on a restricted diet. Feed the rat once per day immediately after each daily handling session to acclimate the rat to the restraint (described below). Provide enough feed to maintain the animal at 80% of its initial weight. This amount is approximately 6 grams of feed per day for a 250 gram female Long Evans rat.

1.1) **Acclimate the rat to being handled by humans.** Gently restrain the rat by hand for periods of approximately 5 seconds every 30 seconds. Do this for 15 minutes a day on two consecutive days. Monitor the rats daily for signs of stress during the training period. Signs include struggling in response to restraint, vocalization, and tooth-chattering.

1.2) On the third day, place the rat in a body-constraining cloth sock (**Fig 1**) for 15 minutes a day on two consecutive days.

1.3) On the fifth day, place the rat inside the sock, and place the sock inside a rigid tube on the experimental jig (**Fig 4**). Leave the rat in the tube for 15 minutes a day on two consecutive days.

1.4) Feed the rat immediately after each training session. At the end of the last session (6th day) give unrestricted access to food. Select for implantation only those rats which do not show signs of excessive stress on the last training day.

NOTE: Though selection of rats for implantation is subjective, in our hands over 90% of rats were found to be responsive to habituation and able to undergo implantation.

2) Implanting the recording chamber and head-restraint mechanism

- 2.1) Make a reference wire by soldering bare stainless steel wire to a pin connector. Cut the wire so that 3-5 mm remains uncovered by the pin. Autoclave the reference wire, along with all surgical tools.
- 2.2) Administer ketamine/xylazine anesthesia. Administer 95 mg/kg ketamine mixed with 5 mg/kg xylazine intraperitoneally. The initial dose lasts for 1½ to 2 hours. Supplement the anesthesia each 30-45 minutes thereafter, as needed.
- 2.3) Check the toe withdrawal reflex to determine the plane of anesthesia, and maintain anesthesia as necessary.
- 2.4) Place the rat in a stereotaxic holding frame with ear bars (**Fig 3A**). Shave the hair on the head and disinfect the wound site with povidone-iodine (10% solution). Take care to insert the ear bars appropriately so that they do not damage the ear drum. Ear bars with blunt tips are preferable.
- 2.5) Make an incision with a scalpel approximately along the midline of the cranium from the rostral-caudal level of the eyes to the back of the ears. Use scissors to cut and remove a 2 to 3 mm strip of skin on either side of the incision.
- 2.6) Scrape away the periosteum to expose the surface of the cranium out to the lateral ridges. Cover the exposed cranium with thin layer of superglue.
- 2.7) Drill a small hole, with a slightly smaller diameter than 0-80 screws, using a ½ mm diameter drill burr (see Equipment section). Drill the hole immediately posterior to where the bregma suture meets the lateral ridge, and at a 30-45 degree angle into the cranium, so that the screw can go in with the top more lateral than the bottom.
- 2.8) Screw in a 0-80 flat-bottom machine screw to the hole (approximately 3 turns), at 30-45 degree angle. Be careful not to screw in too deeply to avoid damaging the underlying brain tissue.
- 2.9) Repeat this procedure for 6 additional screws in the configuration shown (**Fig 3B**). Apply superglue to the base of all screws.
- 2.10) Place a syringe needle in the stereotaxic manipulator. Measure from the bregma suture, and make a dent in the cranium with the needle near, but outside of the desired craniotomy location, as a reference mark.

NOTE: In this demonstration, the stereotaxic coordinates of the mark are 3 mm posterior and 1 mm lateral to the bregma suture.

2.11) Mark the dent with a permanent marker. Note the stereotaxic coordinates of the mark, as it will serve as a stereotaxic reference point (**Fig 3B**).

2.12) Make a craniotomy centered on the desired coordinates (3 mm posterior and 3 mm lateral to bregma in this example). Leave the dura mater intact. Cover the craniotomy with modified artificial cerebral spinal fluid (125 mM NaCl, 10 mM glucose, 10 mM HEPES, 3.1 mM CaCl_2 , 1.3 mM MgCl_2 , pH 7.4)¹³ (**Fig 3C**).

2.13) Cut a 0.2 mL centrifuge tube to 4 to 5mm in length, and cut off the cap. Place the tube on the cranium and center it over the craniotomy.

2.14) Apply dental cement around the bottom of the tube to seal the base of the tube to the cranium. Be careful not to leak cement into the exposed craniotomy (**Fig 3D**).

2.15) Drill another small hole (about ½ mm diameter) in the contralateral cranial plate and carefully insert the reference wire. Construct the reference wire by soldering a pin that interfaces with the recording amplifier to the end of the wire (see Equipment Section). Do not move the reference wire once it is in the brain as this may cause damage.

2.16) Apply superglue to the hole in which the wire was inserted. This will seal the pin and wire in place temporarily.

2.17) Mix the two parts of the silicone gel kit (see Equipment section) in approximately equal portions. Wait two minutes for mixing and fill the centrifuge tube approximately 1/3 full with gel.

2.18) Attach a right angle post clamp (see Equipment section) to the head restraint bar. Attach the head plate (**Fig 2A**) to the holding bar (**Fig 2B**) at a 45 degree pitch angle so that the bottom of the plate is toward the nose of the animal. It is helpful to use an inclinometer to set the pitch angle to match that of the experimental jig (**Fig 4**).

2.19) Attach the holding bar to the stereotax manipulator arm so that the bar is parallel to the ear bars. Lower the bar and plate so that the plate is posterior of the lambda suture and anterior to the caudal-most screw.

2.20) Grasp the front head-bolt (an 8-32 stud or screw, see Equipment section) at approximately 45 degree angle with a helping hands arm and clamp, so that the head of the screw faces down and towards the tail of the animal. Lower the screw so that it touches the anterior 0-80 screws (**Fig 3 F,G**).

2.21) Secure the bolt and plate in place with dental acrylic. Apply dental acrylic around the bone screw heads, reference pin, and around the sides of the centrifuge tube. Wait approximately 10 minutes for the dental acrylic to dry (**Fig 3H**).

2.22) Apply a layer of dental cement around the edges of the dental acrylic, cementing the skin to the implant. Wait for the cement to dry.

2.23) Poke several holes in the cap of the centrifuge tube, and place the cap on the tube.

2.24) Remove the helping hands clamp. Then carefully remove the head-holding bar from the stereotax, and then remove head plate from the bar (**Fig 3I**).

2.25) Remove the animal from the stereotax and administer post-operative care and monitoring in accordance with all applicable rules, regulations, and laws. If the animal shows signs of distress, or if required, administer an appropriate analgesic (e.g. Buprenorphine 0.02 mg/kg, or a thin layer of lidocaine gel applied topically to wound sites), every 8-12 hours until the problem is resolved. If inflammation develops around the edges of the implant, apply a thin layer of antibiotic ointment to the affected site daily until resolved.

3) Juxtacellular monitoring of neuronal units

3.1) Pull quartz capillary tubing on a carbon dioxide laser micropipette puller (see Equipment section) with tip diameters of less than 1 μm (**Fig 5A**).

NOTE: the heating parameters will vary according to the particular instrument, and that the required neck diameter will vary depending on the brain structure of interest. For recordings in rat thalamus, micropipettes have necks ranging from 5 to 7 mm.

3.2) Secure the pipette in place under a differential interference contrast (DIC) microscope equipped with long working distance objectives. Use modeling clay to hold the pipette in place on the microscope's stage.

3.3) Slowly move a glass block (0.5 to 1 cm thick; a piece of glass used for making ultra-microtome knives is convenient) into the field of view with the pipette tip. Using the stage micromanipulator, gently touch the pipette tip to the glass, causing it to break. Repeat as necessary until the pipette tip outer diameter is between 1 and 3 μm (**Fig 5 B,C**). Ensure that these micropipettes have impedances between approximately 5 and 15 M-Ohm.

3.4) Prepare extracellular saline (135 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2 , 5 mM HEPES, pH 7.2 with NaOH) ⁸ and add 2% (w/v) Chicago sky blue. Using a syringe with a 30-gauge needle or smaller, fill the back end of the pipette with the solution. Alternatively, for single cell juxtacellular labeling add 2% (w/v) Neurobiotin or biotinylated dextran amine (BDA-3000 or BDA-10000) to the saline solution in place of Chicago Sky Blue.

3.5) Place the rat inside the cloth sock inside the rigid tube on an appropriate experimental jig (**Fig 4**). Wait a minimum of 72 hours after surgery before placing the animal on the head-

restraint jig. Tighten the drawstring around the upper sternum, rather than the neck, to avoid obstruction of the airway. If breathing appears to be labored or obstructed, loosen the drawstring or move it posteriorly. Carry out this procedure while the rat is alert.

3.6) **Attach the head-restraining plate on the rat to the corresponding piece on the jig.** To do this, first pin the head-restraint plate in place, and then secure it using a 4-40 screw. Be sure to wait until the animal is calm in order to avoid applying excessive torque to the head.

3.6.1) **Next, place an 8-32 nut on the head-restraining bolt that is implanted on the rat. Then screw in a threaded stainless-steel rod to the head-bolt.** Affix the rod to the experimental jig in such a way that it can be tightened in place **(Fig 4)**.

NOTE: Securing the animal with head-bolt as well as the head-restraint plate minimizes bending of the apparatus and improves recording stability. In most cases recording can commence on the first day the animal is head-restrained. However, if the animal fidgets excessively or vocalizes, provide one day of head-restraint habituation training before commencing any recordings. In this case, leave the animal on the jig for 15 minutes and then place it back in its cage. Repeat this step the following day and continue with the protocol.

3.7) **Open the recording chamber and remove the silicone gel.** Clean the craniotomy using fine forceps if tissue has re-grown in the craniotomy.

3.8) **Attach the pipette to the motorized micromanipulator, and plug in the headstage pre-amplifier.**

NOTE: In the present demonstration, a small relay circuit on the headstage is used to switch between the amplifier lead wires and an external current source with high compliance **(Fig 6A-C)**. Note that some amplifiers have an appropriate built-in high compliance source (see Discussion and Equipment sections).

3.9) Use the motorized micromanipulator to move the tip of the pipette to the stereotaxically identified mark in the recording chamber. Note the coordinates of this location. Be careful not to break the tip of the pipette on the cranium.

3.10) **Move the pipette to the desired recording location in the anterior-posterior and medio-lateral axes.** Then advance the pipette ventrally through the dura until it is approximately 500 μm dorsal to the intended recording location.

3.11) **Slowly advance the pipette while listening for spiking events on an audio monitor of the amplified voltage recorded between the pipette tip and the reference wire. Once spiking events are identified, continue to move the pipette 0-100 μm until positive going voltage deflections greater than approximately 500 μV are observed.**

NOTE: The electrode resistance will increase by a factor of approximately 1.5 to 10 when this occurs.

3.12) Begin recording once a unit has been isolated as according to step 3.11, along with all other behavioral and physiological measures of interest. In the present demonstration, self-generated vibrissa movements are monitored with high speed videography (see Equipment section).

3.13) To label the recording site, switch the electrode leads to connect to the current source. There are several ways to do this, using either a relay circuit (**Fig 6A-C**), a built-in current source on the amplifier, or manually (**Fig 6D**, see step 3.8 and Discussion). Pass -4 μ A with 2 s pulses at 50% duty cycle for 4 minutes to iontophoretically inject Chicago sky blue through the pipette.

3.14) Kill and perfuse the animal after several labeling procedures according to standard practice. Section the brain and counterstain as necessary to identify the anatomical location of the recording site. Counterstain the tissue for cytochrome oxidase reactivity as per ¹⁴. Alternatively, identify the Chicago sky blue deposits using fluorescence microscopy.

NOTE: To accurately differentiate between different labeled units it is important that all labels are made with the same pipette on the same day, without unclamping the pipette between labels. In this case recording sites can be differentiated by their relative locations in post-hoc histology along with the noted manipulator coordinates of each site (see step 3.9). For unambiguous identification, mark the labels at least 200 μ m apart from one another, and no more than 3-5 labels per brain region.

REPRESENTATIVE RESULTS:

Neuronal units in ventral posterior medial (VPM) thalamus encode the phase of vibrissa movement during self-generated whisking ^{15,16}. **Figure 7A** shows sample spiking activity of a VPM thalamic unit as a rat is actively whisking. **Figure 7B** shows a histogram of spike times aligned to the instantaneous phase of vibrissa motion ¹⁷. There are more spikes during the retraction phase of whisking. After the recording, the location of the unit was labeled via iontophoresis of Chicago sky blue dye, as shown in **Figure 7C**. The tissue is counterstained for cytochrome oxidase activity to reveal the neuroanatomical borders of VPM thalamus. A similar version of this protocol was recently applied to monitor whisking-related multiunit neuronal activity (10-20 μ m diameter pipette tips) in specific brainstem nuclei ¹⁸.

FIGURE LEGENDS:

FIGURE 1. CLOTH RESTRAINING SOCK

(a) Photograph of a rat sock with drawstrings and drawstring clasps. The small end is placed snugly around the sternum, and the large end around the tail. (b) Design pattern for the sock in **panel 1A**. Dimensions are in inches.

FIGURE 2. MECHANICAL DESIGN OF HEAD RESTRAINT APPARATUS

(a) Mechanical design of a precision head restraint plate for rats. Dimensions are in inches. (b) Mechanical design of a head restraint bar for use with the plate in **panel 2A**. The protocol requires two of these parts, mounted using right-angle post clamps at the same pitch angle.

FIGURE 3. SURGICAL PROCEDURE

(a) Rat in a stereotaxic holding frame (protocol step 2.4). (b) Surgical site with implanted screws and cranial markings (protocol step 2.10). (c) Surgical site with a craniotomy (protocol step 2.12). (d) Surgical site with the recording chamber (protocol step 2.14). (e) Surgical site with the reference wire and silicone gel (protocol step 2.17). (f-g) Surgical site with head restraint plate and bar held in place (protocol step 2.20). (h) Surgical site in with plate and bar cemented in place (protocol step 2.21). (i) Final head-restraint and recording chamber implant (protocol step 2.24).

FIG 4. EXPERIMENTAL JIG

Diagram of rat in the mechanical jig for electrophysiological and behavioral monitoring experiments (protocol steps 3.5 - 3.6). The jig utilizes the bar and plate in **Figure 2**.

FIG 5. MICROPIPETTE ELECTRODE CONSTRUCTION

(a) Micrograph of an unbroken micropipette (protocol step 3.1). Scale bar is as in **panel C**. (b) Micrograph of the unbroken micropipette and glass block under microscope (protocol step 3.2). The reflection of the tip can be seen in the glass. Scale bar is as in **panel C**. (c) Micrograph of the broken micropipette (protocol step 3.3). (d) Micrograph of the tip of the unbroken micropipette in the boxed region of **panel A**. Scale bare is as in **panel E**. (e) Micrograph of the tip of the broken micropipette in the boxed region of **panel C**.

FIG 6. EQUIPMENT FOR SWITCHING BETWEEN THE AMPLIFIER AND CURRENT SOURCE

(a) Setup to switch the leads from the amplifier to the current source via a magnetic relay (optional). A printed circuit board containing the voltage-controlled relay switch is attached to the amplifier head stage. (b) Layout for the printed circuit board in **panel 6A**. The board connects the amplifier leads and current source leads to the input pins of the relay, and the electrode leads to the output pins. The electrode is connected to either the current source or the amplifier by applying either zero or five volts to the relay control pins. Bottom view. Dimensions are in inches. (c) Top view of the board layout in **panel 6B**. (d) Setup to switch the leads from the amplifier to the current source manually (optional) requires an open pipette holder and flexible lead wire like the ones shown (see also: Equipment section). This approach is an alternative to the relay circuit in **panels 6A-C**.

FIG 7. EXPERIMENTAL SETUP AND REPRESENTATIVE RESULTS

(a) Spikes and vibrissa movement from one unit (protocol step 3.12). (b1) Normalized vibrissa position versus phase in the whisk cycle. Each whisk is normalized such that the most caudal position during the whisk is defined as zero and the most rostral position defined as one. The trajectory represents the normalized position versus instantaneous phase in the whisk

cycle¹⁷. All identified whiskers are superimposed. (b2) Raster of the phase in the whisker cycle at which spikes occur. Each trial on the vertical axis represents a single whisker. (b3) Histogram of spike rates with respect to whisker cycle phase for the same unit. (c1) Location of Chicago sky blue dye with cytochrome oxidase counterstaining (protocol steps 3.13 - 3.14). (c2) High resolution view of the outlined region in **panel C1**. The arrowhead points to the dye label.

DISCUSSION

Construction of the experimental jig

The description of the mechanical parts used to build the experimental jig (**Fig 4**) is omitted from the protocol, as it can be constructed in a variety of ways. In this demonstration standard opto-mechanical parts and support clamps are used to mount the head restraint bar and the body restraint tube (see Equipment section). Similar opto-mechanical parts can be used to mount the electrode holder to the motorized micromanipulator. It is important that the head restraint bar on the jig is mounted at the same pitch angle as the bar used to implant the head-restraint plate during surgery. If the electrode is to be moved along the standard stereotaxic axes, then the x-axis of the electrode should move parallel to the head-restraint bar, and the z-axis perpendicular to plane containing lambda and bregma and parallel to the ground (**Fig 4**).

Types of appropriate amplifiers and current sources

There are a variety of commercial amplifiers and current sources that can be used for this experiment. Most extracellular and intracellular electrophysiology amplifiers with a current clamp mode that achieve a total gain of 1000x are appropriate. For iontophoresis of Chicago sky blue with 1 to 3 μm pipettes, a current source that can deliver up to 10 μA with a resolution of at least 1 μA and a compliance of at least 100 V is recommended. Note that some commercial amplifiers have appropriate built-in current sources (see alternate parts in Equipment section). However, to circumvent the requirement of an appropriate combined amplifier/current source, a magnetic relay circuit can be attached to the front end of the amplifier headstage (**Fig 6A-C**). Such a circuit is optional but it allows the experimenter to switch between the amplifier and a separate current source to deliver iontophoresis pulses without physically perturbing the electrode. An alternative approach is to manually replace the electrode lead wires with ones that connect to the current source. In this case, it is best to use a pipette holder which does not enclose the top (unbroken end) of the pipette, and to use flexible lead wire (**Fig 6D**).

Steps that may require repeated practice

There are several steps that may require practice to master. The step that requires the most dexterity is breaking the tip of the pipette. It is helpful to paint the edge of the glass block to visualize the reflection of the tip in the glass. Then one can slowly advance the block and the pipette until the tip barely meets its reflection. Advancing pipette or block too fast can shatter the micropipette and make the tip diameter too large or uneven. We recommend using micropipettes made of quartz, as described in the protocol. Quartz micropipettes effectively penetrate intact rat dura in chronically prepared craniotomies without bending or breaking. This eliminates the need for dura resection, which can cause additional complications. Borosilicate micropipettes with short tapers (<5 mm) also penetrate the dura, but their use is

limited to recording from superficial structures like the cerebral cortex or striatum. Recording from deeper structures, particularly in thalamus or brainstem, requires long tapered, preferably quartz micropipettes. A second step that requires practice is positioning the pipette in the juxtacellular configuration. Moving the pipette too quickly when close to a cell can rupture the membrane. Therefore, it is helpful to monitor the resistance of the pipette to know when the tip is close to a putative cell. Changes in membrane resistance of at least two-fold indicate that the electrode may be near or touching a cell membrane.

Common pitfalls

There are several common pitfalls to bear in mind. First, because the pipette tip is necessarily very close to the cell, it is possible to “lose” the cell if the pipette drifts away from the membrane. It is also possible for a cell to become agitated or rupture midway through the recording. If it does, the spike width and firing rate may increase and the spike amplitude may decrease. These are indications that the cell may be unhealthy. Animal movement will inevitably cause these events to happen on some proportion of the recordings, but can be minimized by having the animal well habituated and by avoiding making unnecessary, sudden loud noises or causing vibrations that startle the animal.

Potential modifications to the protocol

It is possible to use this recording and labeling technique in contexts in which the animal is performing a variety of behavioral tasks. Training for a particular behavioral task can be carried out following surgery but before beginning to record. Neuronal activity can be recorded at least 1 to 2 weeks after preparing the craniotomy, and iontophoretic spots can last for at least two to three days. This time frame permits a variety of experimental manipulations, including recording from multiple brain regions during separate recording sessions.

The technique may also be modified to fill the individual neurons from which the recordings are made. This alternative strategy of juxtacellular labeling of single neurons requires mechanical stability that is only achieved when the animal is still or anesthetized. For this labeling strategy, the use of BDA is recommended over Neurobiotin. BDA is not degraded by proteases, which significantly increases the rate of success over Neurobiotin. Labeling should be attempted during the last recording session before perfusing the animal. Note that detailed descriptions of the juxtacellular protocol have been described previously ^{6,19}. While the technique has been employed successfully in alert rats ⁸, in our experience iontophoretic injection of Chicago sky blue has a substantially higher yield. Note that both Chicago sky blue and Neurobiotin or BDA labeling can be done in the same animal.

This technique is likely to be appropriate for a variety of species. Note that juxtacellular recordings have been made in cortical regions of alert mice ⁹, for example. While training, handling, and head-restraint are species specific, the particular techniques for monitoring and labeling described in this protocol should remain the same. Finally, simply by changing the size of the pipette tip and parameters of the current pulses this technique may be used for iontophoretic injection of a variety of molecules, including pharmacological agents ^{20,21}. Thus,

the technique could also be useful for neuropharmacological studies which involve behaving animals.

Conclusions

It is often the case that brain regions which are extremely close together can have markedly different properties and/or functions²². In such cases, relating the activity of single neurons from anatomically defined regions to organismal behavior is critical for understanding neural circuits and computation. The present article demonstrates a protocol to accomplish this using a combination of standard techniques and describes sensory encoding in VPM thalamic neurons as an example. The technique is likely to be relevant and feasible in a variety of brain regions and behavioral paradigms in different animal models. It has a substantially higher success rate than single cell juxtacellular labeling in alert animals^{6,8}, and has the advantage of better anatomical resolution over techniques that utilize multi-electrode arrays or microdrives.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors declare that they have no competing financial interests.

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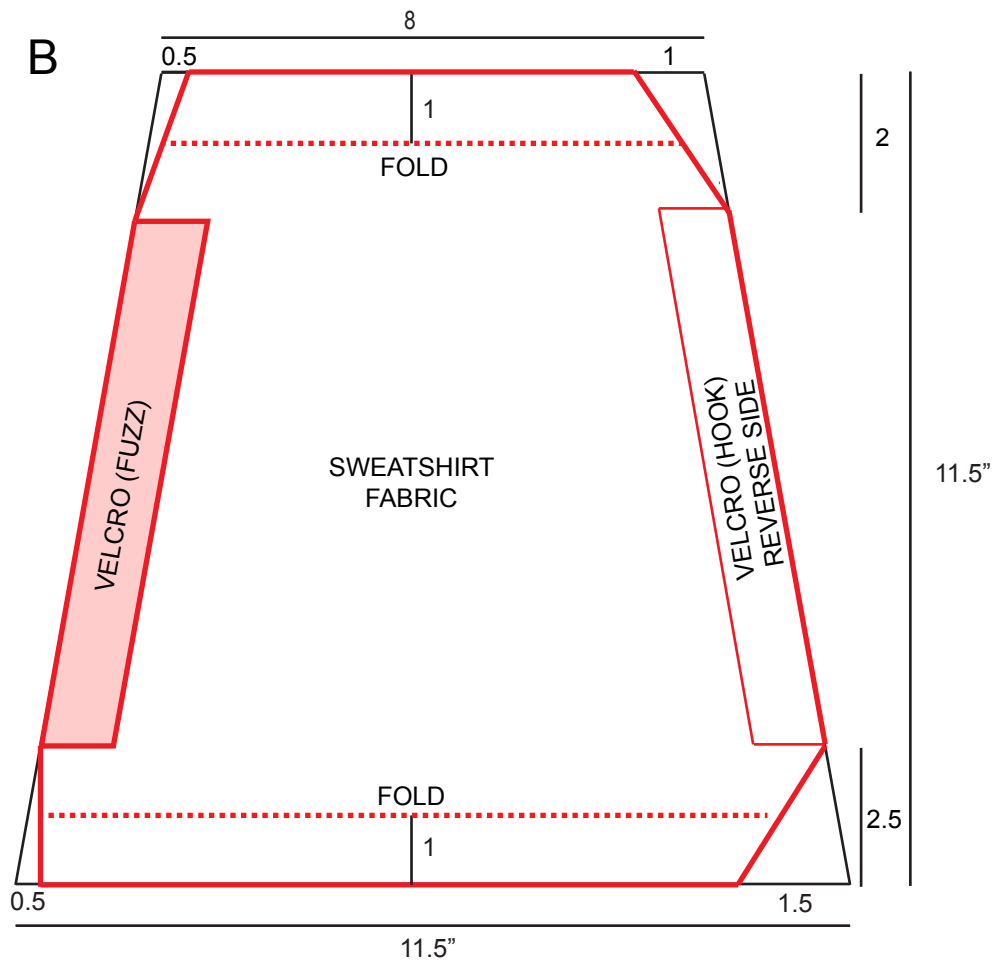
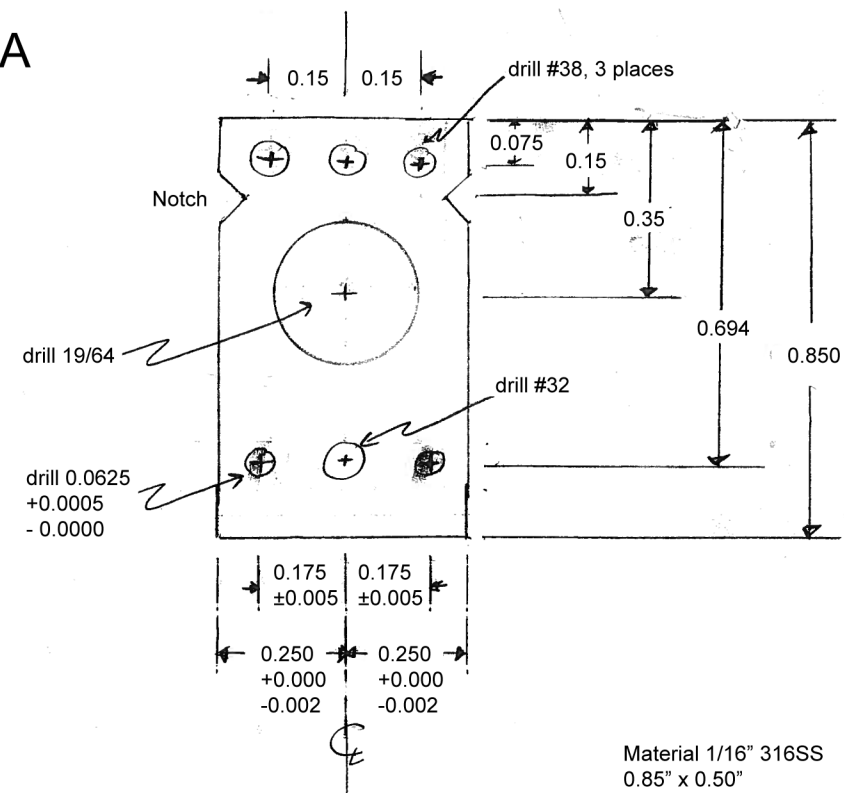


Figure 2
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A



B

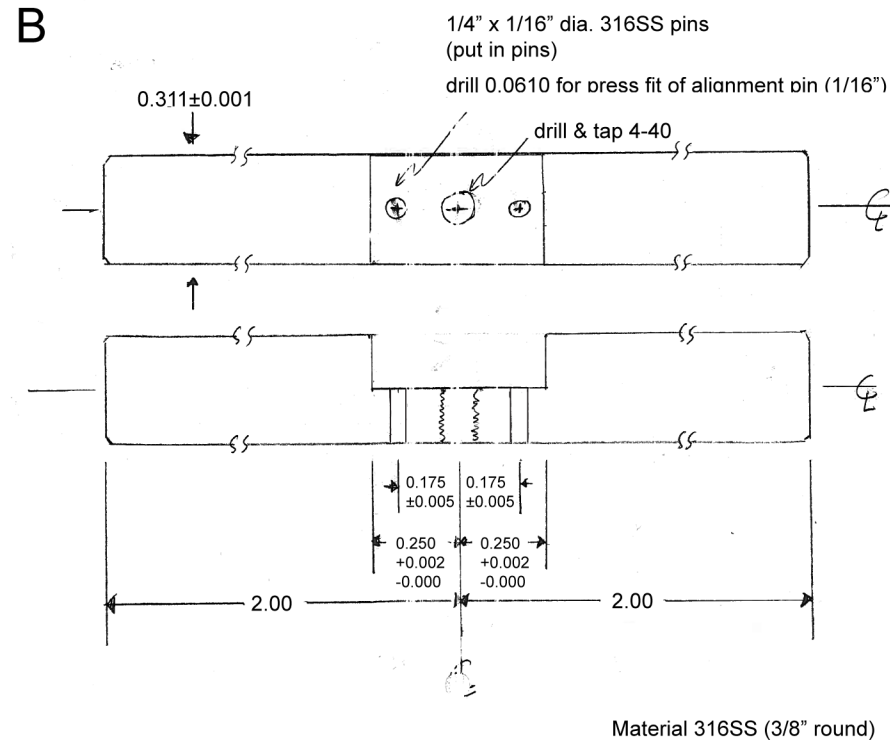
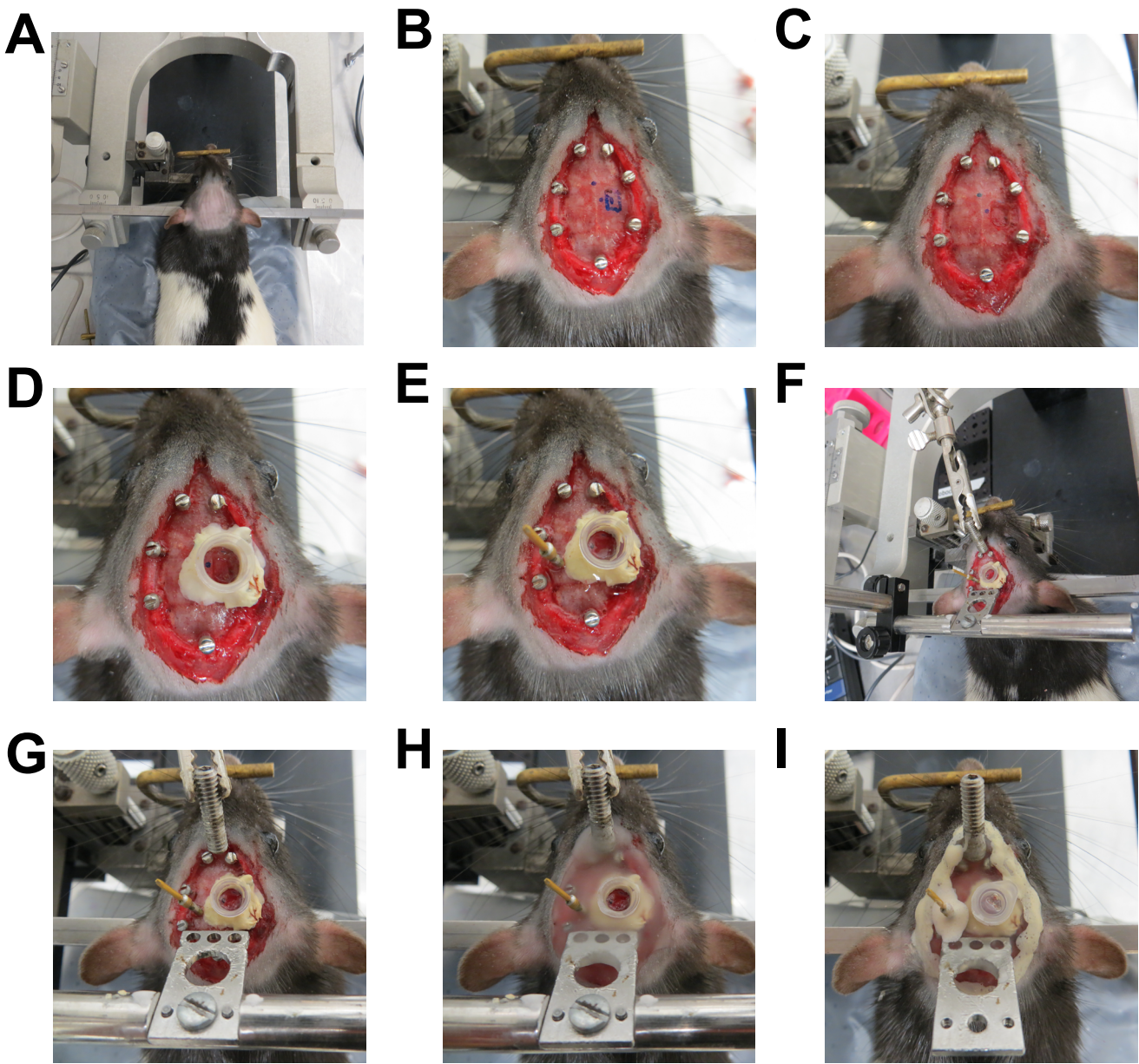


Figure 3
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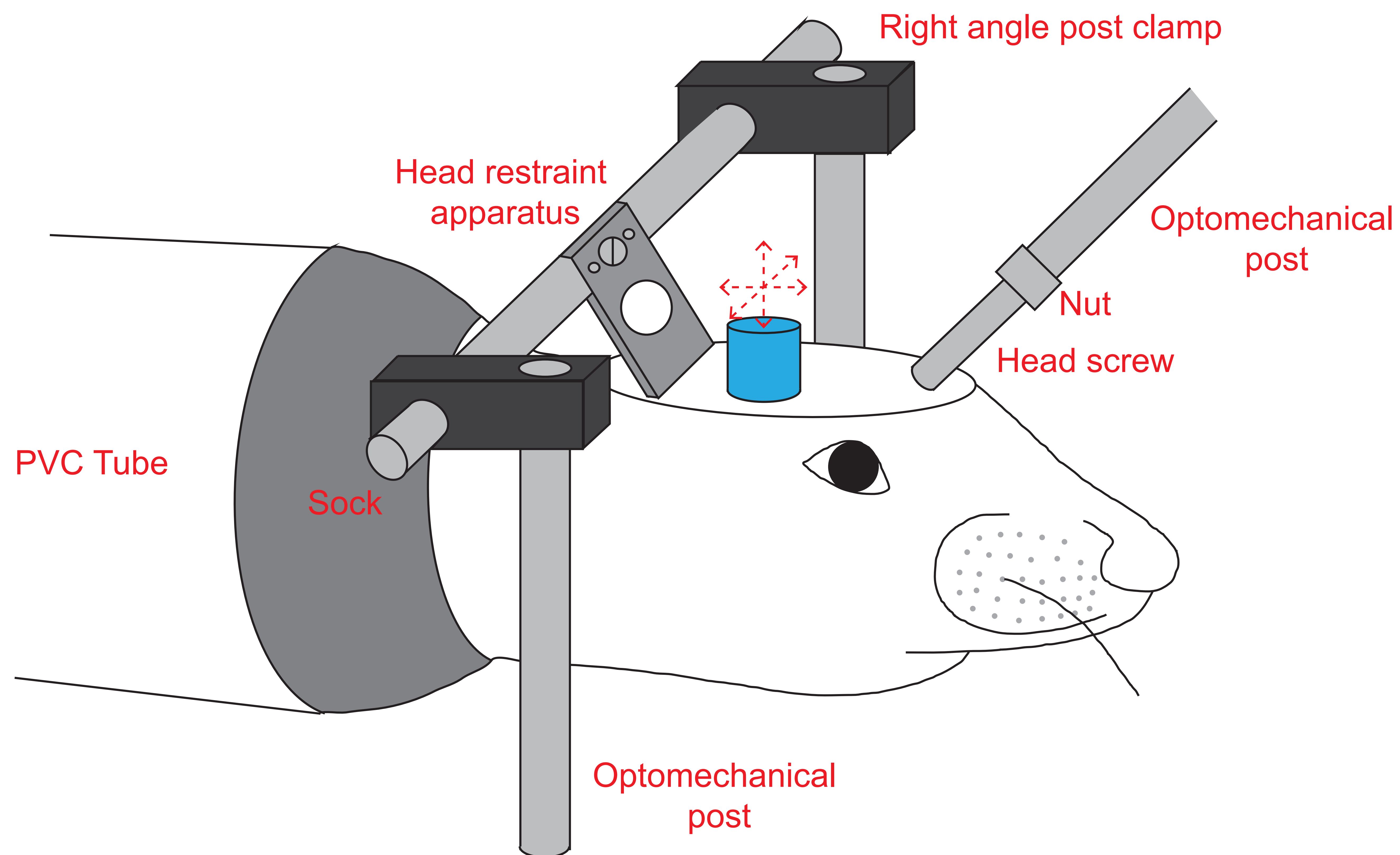


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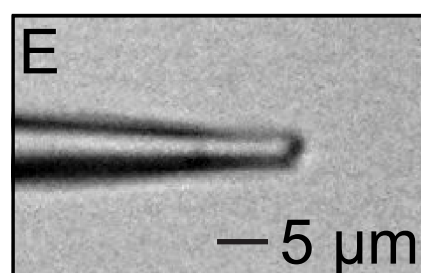
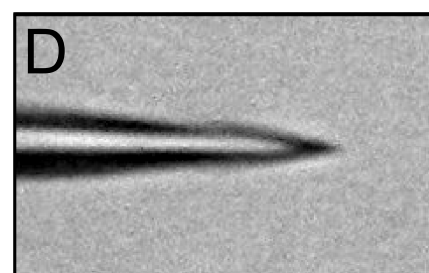
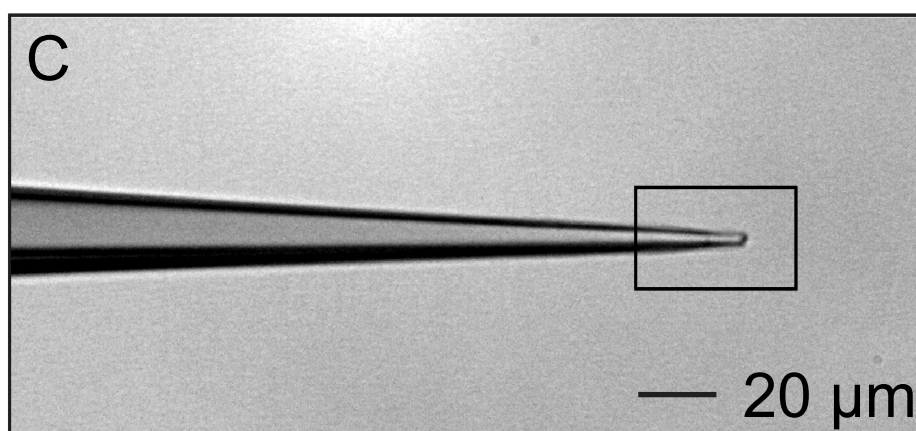
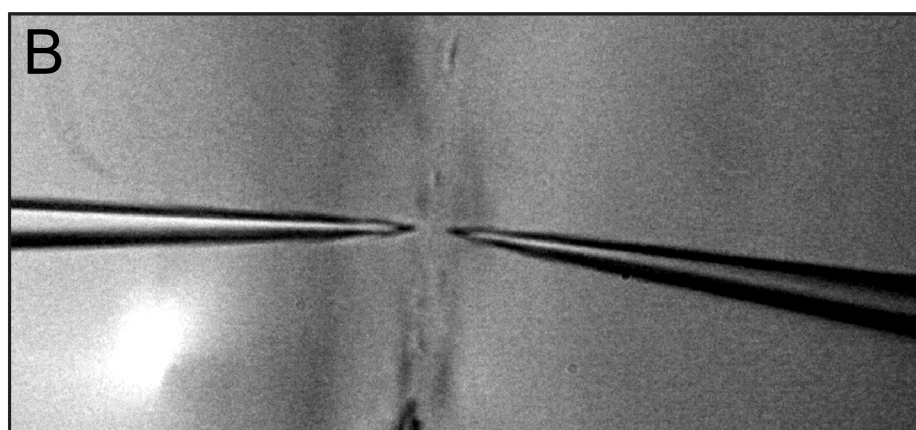
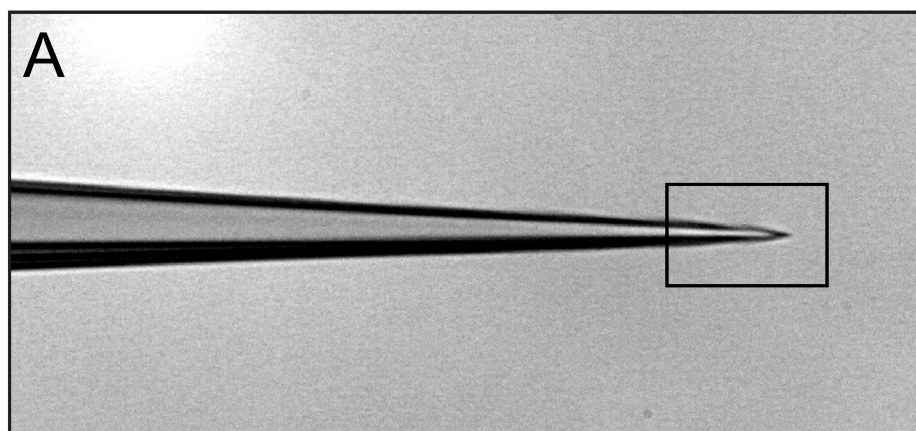


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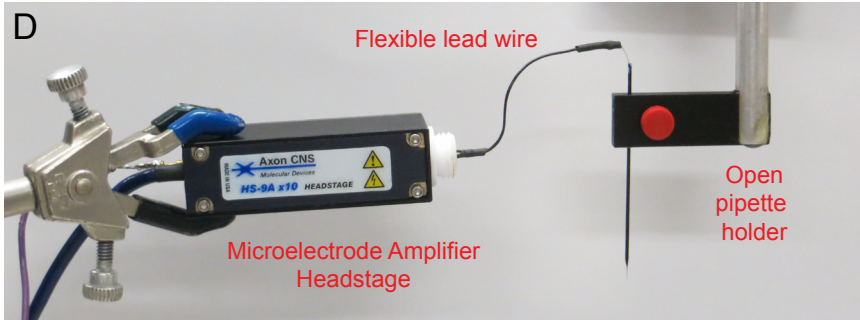
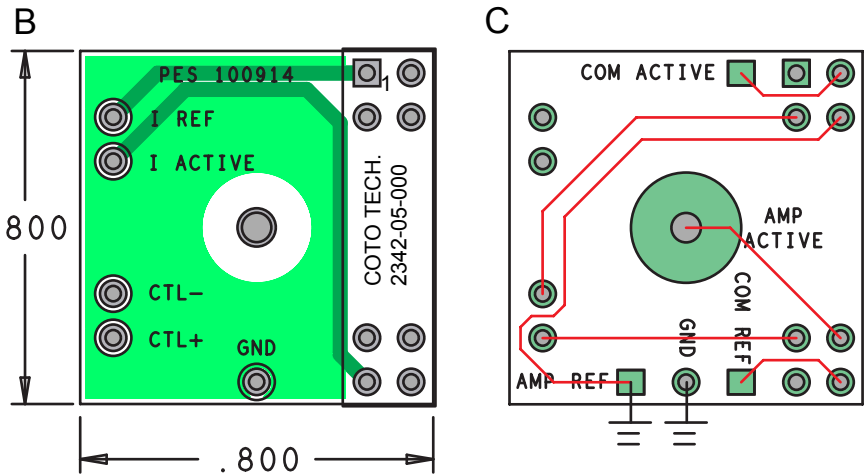
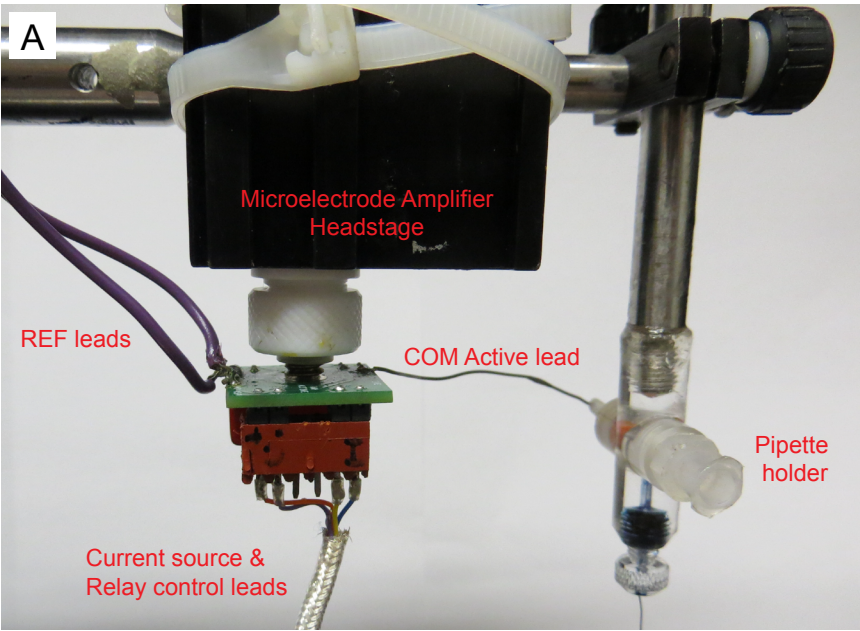
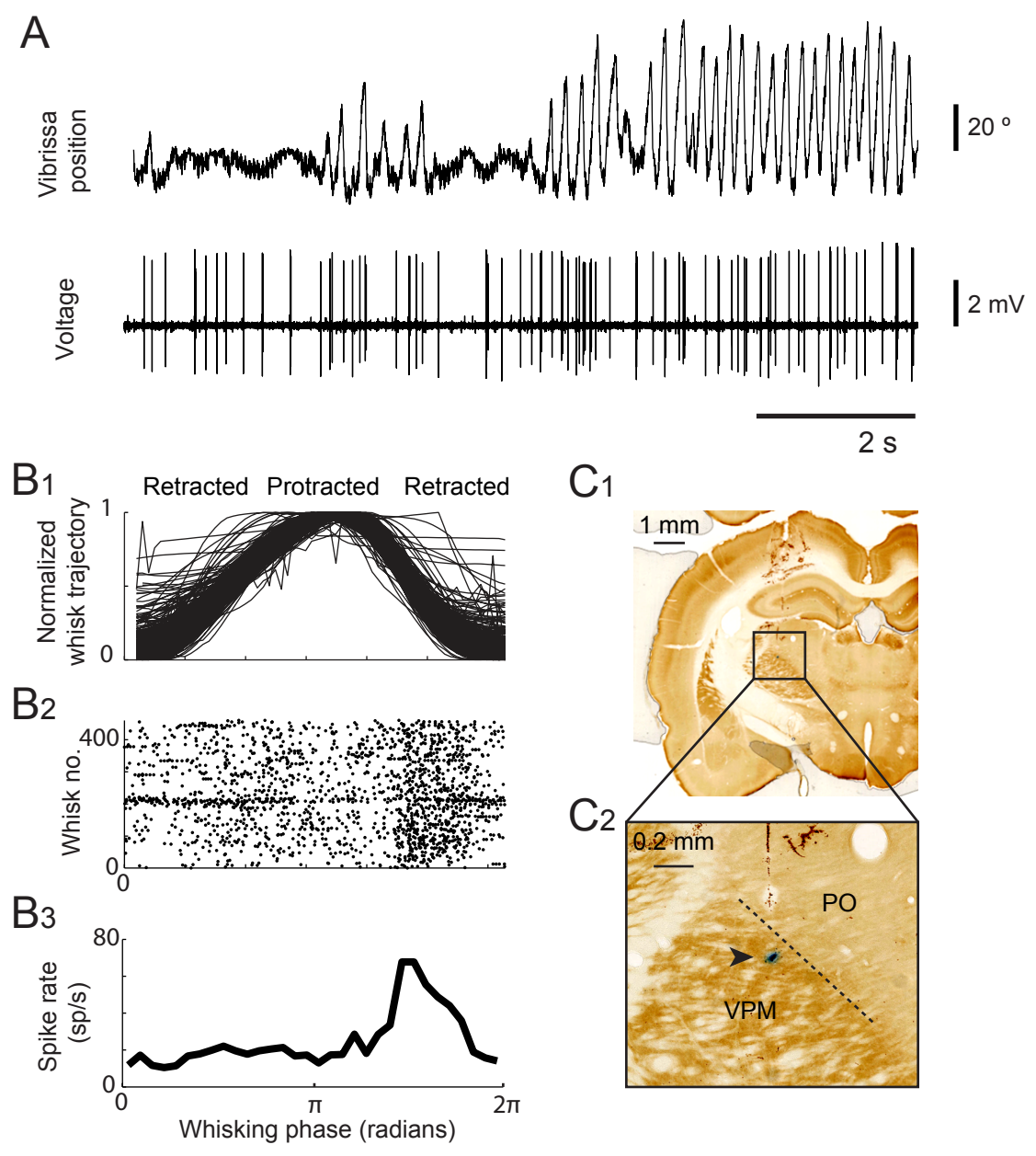


Figure 7
[Click here to download Figure: JOVE_fig7.pdf](#)



Name of the Reagent	Company	Catalogue Number
Ketaset (Ketamine HCl)	Fort Dodge	N/A
Anased (Xylazine solution)	Lloyd Laboratories	N/A
Betadyne (Povidone-Iodine)	CVS Pharmacy	269281
Loctite 495	Grainger Industrial Supply	4KL86
Vetbond	3M	1469SB
Grip cement powder	Dentsply Intl	675571
Grip cement liquid	Dentsply Intl	675572
Silicone Gel	Dow Corning	Mar-80
Jet denture repair acrylic powder	Lang Dental Manufacturing Co.	N/A
Ortho-Jet Fast curing orthodontic acrylic resin liquid	Lang Dental Manufacturing Co.	N/A
Chicago sky blue	Sigma	C8679
Paraformaldehyde	Sigma	158127
Phosphate-buffered saline	Sigma	P3813
Cytochrome C	Sigma	C2506
Diaminobenzidine	Sigma	D5905

Material Name	Company	Catalogue Number
Rat sock	Sew Elegant (San Diego, CA)	N/A
PVC tube 2 ½"	U.S. Plastic Co.	34108
Subminiature D pins & sockets	TE Connectivity	205089-1
Stainless steel music wire 0.010" diameter	Precision Brand Products, Inc.	21010
Stereotaxic holding frame	Kopf Instruments	Model 900
Stereotaxic ear bars	Kopf Instruments	Model 957
Stereotaxic manipulator	Kopf Instruments	Model 960
½ mm drill burr	Henry Schein	100-3995
Quiet-Air dental drill	Midwest Dental	393-1600
Stainless steel 0-80 1/8" screw	Fastener superstore	247438
0.2mL centrifuge tube	Fisher Scientific	05-407-8A
Custom head-holding bar	UCSD SIO Machine Shop	N/A
Custom head-holding plate	UCSD SIO Machine Shop	N/A
Right angle post-clamp	Newport	MCA-1
8-32 3/4" screw	Fastener Superstore	240181

4-40 ¼" screw	Fastener Superstore	239958
Quartz capillary tubing	Sutter Instruments	QF-100-60-10
Carbon dioxide laser puller	Sutter instruments	P-2000
Motorized micromanipulator	Sutter Instruments	MP-285
Microelectrode amplifier	Molecular Devices	Multiclamp 700B
Microelectrode amplifier head stage	Molecular Devices	CV-7B
Isolated pulse stimulator	A-M Systems	Model 2100
Audio monitor	Radio Shack	32-2040
Pipette holder	Warner Instruments	#MEW-F10T
Electrode lead wire	Cooner wire	NEF34-1646
Relay for amplifier head-stage	COTO Technology	#2342-05-000
Digital video camera	Basler	A602fm

Comments
20-40 cp cyanoacrylate
For the base of the recording chamber
For the base of the recording chamber
For securing the head restraint apparatus to the cranium
For securing the head restraint apparatus to the cranium
For perfusion and tissue fixation
For perfusion and tissue fixation
For cytochrome-oxidase staining (Figure 7)
For cytochrome-oxidase staining (Figure 7)

Comments
Custom made, Figures 1, 4
Figure 4
Figure 3
Figure 3
Figure 3
Figure 3
Figure 3
Figure 3
Figure 3
Figure 3
Custom made, Figures 2, 3, 4
Custom made, Figure 2, 3, 4
Figure 3,4; standard opto-mechanical parts for the experimental jig (Figure 4) are also from Newport Corp.
For head-restraint, Figure 3

For head restraint, Figures 3, 4
Figure 5
Alternate part: Molecular Devices Axoclamp 900A
Alternate part: HS-9Ax10 with Molecular Devices Axoclamp 900A
Alternate part: HS-9Ax10 with Molecular Devices Axoclamp 900A
Alternate parts: see Discussion
Figure 6A
(optional), Figure 6D
(optional) Used with a custom-made printed circuit board (UCSD Physics Electronics Shop), Figure 6A-C
(optional) For behavioral monitoring, Figure 7

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MS # (internal use):

Summary of changes to the manuscript

1. We include information on single cell juxtacellular labeling as an alternative procedure for labeling cells, as requested by Reviewers #1 and #2.
2. We add specific experimental details and commentary as requested by all reviewers.
3. We include higher magnification views of pipette tips in **Figure 5**.

Reply to Reviewer #1:

Manuscript Summary:

This is a very nice compilation of a complex protocol, which is otherwise difficult to obtain. In most cases the details are worked out by time consuming trials and errors thus the paper is very timely.

We thank the reviewer for his/her careful read of the manuscript and recommendation to report our protocol.

I have the following notes:

Please indicate that blunt ear bars should be used to protect the ear drum during the operation.

We include a statement warning of this potential problem in protocol step 2.3, and suggest the use of blunt ear bars.

Please indicate to what extent the method can be used for mice or what modifications are needed.

This is presently covered in the "Modifications" subsection of the discussion section, however it is admittedly vague. Therefore, we now further clarify which parts of the protocol are likely to be applicable to other species, and which are species specific. Additionally, we state specifically in the introduction that juxtacellular monitoring has been successfully applied in previous studies in rats, mice, and birds.

Please give more details about the postoperative care, common problems (inflammations, loose implants) and solutions

We apologize for this omission. Post-operative care, including analgesics, antibiotics, monitoring, and reporting, is heavily regulated by law and local guidelines. We note this but include recommendations for alleviating potential post-operative complications in protocol step 2.18.

Indicate the optimal resistance of the quartz capillary.

We agree. The resistance is now indicated in protocol step 3.2.

Please indicate that Neurobiotin can also be added to the pipette solution, to label individual neurons.

A similar request was made by reviewer #2. As such, we state in the introduction and discussion that single cell juxtacellular labeling is an alternative strategy. This procedure is then described in protocol steps 3.3 and 3.11.

Please indicate, to what extent there is individual variability among the rats to be accustomed to the head restrain apparatus. What is the success rate of the handling, as described?

We thank the reviewer for bringing up this important point which we neglected. In fact the rats' behavior was closely monitored during training. Each rat was given a subjective score between 1-5 by the trainer based on signs of stress in response to training. The scoring system is as follows:

1 - quiet, calm, responsive to training

3 - quiet, but struggling against restraint

5 - struggling against restraint, tooth chattering, vocalization

Dropout – rat struggles too much that it cannot be placed in the sock for training

Score	1	2	3	4	5	Dropout
Day 1 (handling)	39%	33%	27%	0%	1%	N/A
Day 6 (body restraint in tube)	55%	36%	6%	0%	1%	2%

Although selection for surgery is subjective by the experimenter, in practice we implanted rats with final scores of 1 or 2. This corresponds to a success rate of >90% for Long Evans rats from Charles River labs (N=122 rats). We now state that rats should be monitored for these signs of stress in protocol step 1.1. We quote this expected success rate of “well-behaved” rats in protocol step 1.3. We further note the strain, sex, weight, and vendor of our rats at the beginning of the procedure.

Please indicate if the rats needs to placed to the apparatus AFTER the head-mount is made, if yes how many times? If no, how many days should pass between the surgery and recording?

We agree that the procedure is vague in this regard. This information, along with a more detailed description of how to initially head-restrain the animal, is now included in protocol steps 3.4 – 3.6

Editorial comment:

[Please keep JoVE's protocol guidelines and length requirements in mind while addressing reviewer comments(use short steps, imperative tense, proper spacing, etc).]

Reply to Reviewer #2:

In their paper 'Juxtacellular monitoring and localization of single neurons within sub-cortical brain structures of alert, head-restrained rats' Moore et al. describe procedures to recording and identify neurons in awake head-fixed animals. As the authors acknowledge there is nothing ground-breakingly new in the ms, but the special compilation of techniques used to identify cells in awake behaving animals has not yet been published.

I agree with the authors that such a publication makes sense, because there is a need to identify neurons in behaving animals and hence a need for this very powerful combination of techniques.

The paper is fairly didactical and I think it will meet its goal, i.e. it will make neuron identification more accessible. Prior to publication a few improvements are indicated.

We thank the reviewer for his/her careful read of the manuscript and recommendation to report our protocol.

1. The micrographs of the electrodes should include higher magnification views.

We agree. We include higher magnification views in **Figure 5**.

2. I am not sure that screws are still the gold standard for securing implants in rodents. Very stable implants can be achieved with various gluing procedures. More often than not skull screws lead to inadvertent damage of the underlying brain tissue.

We agree that for mice, the implants can be secured with dental acrylic alone. For rats, who can apply substantially more torque to the restraint mechanism, we prefer the use of screws for several reasons: (1) arranging the screws in a circular fashion at an oblique angle to the skull, as shown in Figure 3, ensures that if the rat resists against restraint the resulting forces will be distributed across the entire skull; (2) the screw heads provide a substrate to anchor the head restraint plate and bolt with acrylic. To address concerns of damage, we include additional details on the type of screw and the surgical procedure in protocol step 2.5.

3. I am not sure if the suggested laser puller and the use of quartz pipettes really make the technique more accessible for outsider. Conventional borosilicate pipettes can also be introduced through the dura and will be much more easily available in most labs. Perhaps the author can comment on this issue from their own experience.

We agree that the use of borosilicate would make the procedure more accessible. Therefore in protocol step 3.1 we specify, based on our experience, the experimental

conditions in borosilicate pipettes can be used as an alternative, as well as those in which quartz is preferable.

4. More experimental detail on labeling procedures would be desirable. A few lines from Deschenes could make a big difference for the investigator that actually tries out these procedures.

A similar request was made by reviewer #1. As such, we state in the introduction and discussion that single cell juxtacellular labeling is an alternative strategy. This procedure is then described in protocol steps 3.3 and 3.11.

5. I did not exactly get the application of Chicago Sky Blue. Can it also be used for intracellular labeling?

No, and this is stated explicitly in the introduction. However, as discussed above we now include information on juxtacellular labeling with neurobiotin or biotinylated dextran amine as an alternative strategy, as well as the limitations of such an approach. The reason we prefer Chicago sky blue is because of its high success rate, which is further explained in response below to comment 6.

6. I was surprised to see that the authors suggest several labeling procedures. What about the disambiguation of multiple labeled neurons? Can Chicago Sky Blue help here? Again a few words from a labeling expert could be very illuminating here.

We agree that it is important the protocol include an explicit description of how to differentiate between different units labeled with Chicago sky blue. This is now included in protocol step 3.12. Importantly, note that assigning recording sites to labels requires that 100% of labels are recovered. Thus, the high success rate of labeling with Chicago sky blue is the basis for our recommending it in this protocol.

Reply to Reviewer #3:

Manuscript Summary:

This is a excellent description of the methods used to implant a headholding device on rats used for recording neuronal activity in awake rats.

Major Concerns:

No concerns

Minor Concerns:

There is an internal inconsistency on lines 151 and 156. The coordinates should be 3 mm posterior and 3 mm lateral to bregma, not 1 mm lateral to bregma.

We apologize for the confusion. The mark must be made near the craniotomy but not on the craniotomy, because the mark must remain on the skull after the bone is removed.

In this case a mark is made 2 mm medial to the center of the craniotomy. The text in protocol step 2.7 is revised to clarify this point.

Additional Comments to Authors:

N/A