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Preparation of Rhythmically-active In Vitro Neonatal Rodent Brainstem-spinal Cord and Thin Slice

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RE: JoVE manuscript 58870R1

Dear Dr. Myers,

Attached please find our revised manuscript entitled "Comprehensive dissection protocol for preparation of rhythmically-active in vitro neonatal rodent brainstem-spinal cord and thin slice" (JoVE 58870R1) which describes the methods we use to cut rhythmically-active *in vitro en bloc* brainstem-spinal cord and brainstem slices. We believe that the protocol as laid out within this manuscript provides extensive detail that is of value to our field and in general to understanding rhythmic motor networks in the nervous system. We thank the editorial team and the reviewers for their careful attention to the original submission and we have addressed their concerns to the best of our abilities. We feel that the manuscript is substantially improved by their input.

The authors are Samantha Palahnuk (email: palahns1@tcnj.edu), Vadim Gospodarev (email: vgospodarev@llu.edu), Jonathan Abdala (email: jabdala@llu.edu), and Christopher Wilson (email: cgwilson@llu.edu, phone: 909-651-5895). The manuscript is a detailed protocol and has not been published or submitted for consideration elsewhere.

Thank you for consideration of our manuscript.

Sincerely,

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TITLE:

Preparation of Rhythmically-active *In Vitro* Neonatal Rodent Brainstem-spinal Cord and Thin Slice

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KEYWORDS:

Brainstem-spinal cord dissection, vibratome sectioning, *en bloc* recording, *in vitro* slice, cranial rootlets, cervical rootlets, rhythmically-active slice, respiratory pattern generation

SUMMARY:

This protocol both visually communicates the brainstem-spinal cord preparation and clarifies the preparation of brainstem transverse slices in a comprehensive step-by-step manner. It was designed to increase reproducibility and enhance the likelihood of obtaining viable, long lasting, rhythmically-active slices for recording neural output from the respiratory regions of the brainstem.

ABSTRACT:

Mammalian inspiratory rhythm is generated from a neuronal network in a region of the medulla called the preBötzinger complex (pBC), which produces a signal driving the rhythmic contraction of inspiratory muscles. Rhythmic neural activity generated in the pBC and carried to other neuronal pools to drive the musculature of breathing may be studied using various approaches, including *en bloc* nerve recordings and transverse slice recordings. However, previously published methods have not extensively described the brainstem-spinal cord dissection process in a transparent and reproducible manner for future studies. Here, we present a comprehensive overview of a method used to reproducibly cut rhythmically-active brainstem slices containing the necessary and sufficient neuronal circuitry for generating and transmitting inspiratory drive. This work builds upon previous brainstem-spinal cord electrophysiology protocols to enhance the likelihood of reliably obtaining viable and rhythmically-active slices for recording neuronal output from the pBC, hypoglossal premotor neurons (XII pMN), and hypoglossal motor neurons (XII MN).

The work presented expands upon previous published methods by providing detailed, step-by-step illustrations of the dissection, from whole rat pup, to *in vitro* slice containing the XII rootlets.

INTRODUCTION:

The respiratory neural network of the brainstem provides a fertile domain for understanding the general characteristics of rhythmic neural networks. In particular, the interest is in the development of neonatal rodent breathing and understanding how the breathing rhythm develops. This may be done using a multi-level approach, including *in vivo* whole animal plethysmography, *in vitro en bloc* nerve recordings, and *in vitro* slice recordings that contain the breathing rhythm generator. Reductionist *in vitro en bloc* and slice recordings are an advantageous method to use when interrogating the mechanisms behind respiratory rhythmogenesis and neural circuitry in the brainstem-spinal cord region of developing rodents. The developing respiratory system includes approximately 40 cell types, characterized by firing pattern, including those of the central respiratory[1,2]. The central respiratory network includes a group of rhythmically active neurons located in the rostral ventrolateral medulla[1,3]. Mammalian respiratory rhythmogenesis is generated from an autorhythmic interneuron network dubbed the preBötzinger complex (pBC), which has been localized experimentally *via* both slice and *en bloc* preparations of neonatal mammalian brainstem-spinal cords[3-8]. This region serves a similar function to the sinoatrial node (SA) in the heart and generates an inspiratory timing system to drive respiration. From the pBC, the inspiratory rhythm is carried to other regions of the brainstem (including the hypoglossal motor nucleus) and spinal motor pools (such as the phrenic motor neurons that drive the diaphragm)[9].

Rhythmic activity may be obtained using brainstem spinal cord *en bloc* preparations or slices from a variety of cell populations, including C3-C5 nerve rootlets, XII nerve rootlets, hypoglossal motor nucleus (XII MN), hypoglossal premotor neurons (XII pMN), and the pBC[3,10-12]. While these methods of data collection have been successful across a handful of laboratories, many of the protocols are not presented in a way that is fully reproducible for new researchers entering the field. Obtaining viable and rhythmically active *en bloc* and slice preparations requires an acute attention to detail through all steps of the dissection and slice cutting protocol. Previous protocols extensively describe the various recording procedures and electrophysiology, yet lack detail in the most critical part of obtaining a viable tissue preparation: performing the brainstem-spinal cord dissection and slice procedure.

Efficiently obtaining a rhythmically-active and viable *en bloc* or slice preparation brainstem-spinal cord electrophysiology recordings requires that all steps be performed correctly, carefully, and swiftly (typically, the whole procedure related here can be performed in approximately 30 min). Critical points of the brainstem-spinal cord electrophysiology protocol that have not been previously well described include the dissection of nerve rootlets and the slicing procedure on the vibratome. This protocol is the first to stepwise visually communicate the brainstem-spinal cord dissection for both new researchers and experts in the field. This protocol also thoroughly explains surgical techniques, landmarks, and other procedures to assist future researchers in standardizing slices and *en bloc* preparations to contain the exact circuitry desired in each experiment. The procedures presented here can be used in both rat and mouse neonatal pups.

PROTOCOL:

The following protocol has been accepted and approved by the Institutional Animal Care and Use Committee (IACUC) of Loma Linda University. NIH guidelines for the ethical treatment of animals are followed in all animal experiments performed in the laboratory. All ethical standards were upheld by individuals performing this protocol.

1 Solutions

1.1 Prepare artificial cerebral spinal fluid (aCSF).

1.1.1 Prepare fresh aCSF the evening before an experiment in 1 L batches using the following recipe: 7.250 g NaCl (124 mM), 0.224 g KCl (3 mM), 2.100 g $NaHCO_3$ (25 mM), 0.069 g $NaH_2PO_4 \bullet H_2O$ (0.5 mM), 0.247 g $MgSO_4 \bullet 7H_2O$ (1.0 mM), and 5.410 g D-glucose (30 mM), 0.221 g $CaCl_2 \bullet 2H_2O$ (1.5 mM). Always add the $CaCl_2 \bullet 2H_2O$ last.

1.1.2 Dissolve the components into 1 L of deionized water. Stir for 20 - 30 min.

1.1.3 Measure the pH of the aCSF and adjust to 7.40 ± 0.02 using small volumes (typically < 0.5 mL) of dilute NaOH, KOH, or HCl.

NOTE: Prepared aCSF can be stored in the refrigerator (4 °C, pH 7.40 ± 0.02) up to three days before bacterial growth affects viability of the tissue during an experiment. Use 0.2 µm filters to reduce contamination by fungus or bacteria present in the laboratory since experiments can last up to 36 h. For efficiency, the aCSF recipe described may be scaled up to 4 L batches following the same protocol and this volume will last for up to three days of experiments.

2 Preparation of Dissection and Vibratome Rig

2.1 Set up blade.

2.1.1 Use a fresh double-edged razor blades for cutting tissue on a vibratome. Wash the blade with 100% ethanol and rinse with deionized water before cutting or snapping the blade in half and inserting the blade into the mounting clamp on the vibratome.

2.1.2 Change the blade every time a new tissue preparation is mounted on the vibratome for slicing or if it cuts into the paraffin slab while slicing. Any paraffin residue will make it nearly impossible to cut cleanly through neonatal neural tissue.

2.2 Set up paraffin wax slab.

2.2.1 Use any embedding-style paraffin wax. Place 10 g of embedding media in a heat-tolerant glass beaker and use low heat to melt the paraffin wax beads to liquid.

2.2.2 Add approximately 0.5 g of graphite powder and mix solution thoroughly and uniformly into the liquid paraffin.

2.2.3 Use a small plastic slab as the substrate for the paraffin. Cut a small (0.5 - 1 cm thick and approximately 2 x 2 cm wide) piece of plastic (polycarbonate or aluminum can also be used). Use a machinist's file to scratch grooves into the plastic as this will ensure that the paraffin adheres to the plastic.

NOTE: Alternatively, use a heated 18 G hypodermic needle to place angled holes into the sheet to ensure that the paraffin-graphite mixture adheres to the plastic.

2.2.4 Use the back of a cotton tip applicator to drip the paraffin-graphite mixture onto the plastic by repeatedly dipping the applicator into the molten paraffin-graphite mixture, dripping the slurry onto the plastic block, and building the paraffin-graphite to approximately 1.5 cm in thickness on top of the plastic.

2.2.5 Once a sufficiently thick layer of the paraffin-graphite mix is deposited upon the block, set the block aside to cool and then shape to accommodate the brainstem-spinal cord.

3 Dissection and Isolation of the Neuraxis

3.1 Perform initial dissection and anesthetization.

NOTE: Animals used in this study may range in size from embryonic day 18 (E18) to postnatal day 10 - 20 in mice, or rats ranging from E18 to postnatal day 5/6. Rats or mice of any strain, treatment, or gender may be used, depending on the experimental design. Perform anesthesia and preliminary dissection under a fume hood since isoflurane is used (0.25 mL in a 25 mL chamber).

3.1.1 Weigh the pup and then place it in an anesthesia chamber containing 0.25 mL of isoflurane placed on a 2 x 2 inch piece of gauze. After the animal reaches a surgical plane of anesthesia (verified by toe pinch with no withdrawal reflex), pin the animal on a petri dish filled with paraffin or silicone elastomer.

NOTE: Neonatal rodents may also be anesthetized *via* cryoanesthesia[13,14], isoflurane vaporization[15], or injection[16] balanced with oxygen.

3.1.2 Place the animal ventral side down and make a midline incision from just behind the eyes to the mid-lumbar region of the spinal column with a number 10 or 11 scalpel blade or surgical scissors. Reflect the skin and decerebrate the animal at the level of the parietal/occipital suture (see **Figure 1**) and remove the skin transecting the animal below the diaphragm. Then remove the arms by cutting at the shoulder joint with scissors or a scalpel.

3.1.3 Once the skin is removed, transfer the animal to a perfusion chamber with silicone elastomer in the bottom for further dissection and preparation of the brainstem-spinal cord.

3.1.4 Bubble an aCSF reservoir (500 mL side-port bottle) continuously with chilled (4 °C, pH 7.40 ± 0.02) aCSF and oxygenate with a mixture of 95% O_2 and 5% CO_2 (also called "carbogen"). During the dissection, use chilled aCSF to periodically flush the chamber, providing fresh oxygenated aCSF throughout isolation of the brainstem-spinal cord.

NOTE: Red blood cells can be seen in the remaining arteries and veins and, when sufficiently oxygenated, these are bright red.

3.1.5 Use gravity-flow perfusion *via* tubing and a stopcock, to intermittently or continuously perfuse the tissue with oxygenated aCSF (bolus volume or *via* slow drip using a stopcock, approximately 0.5 - 1.0 mL per minute). This oxygenates the tissue and maintains a clear surgical field.

3.1.6 Remove excess fluids as needed by vacuum suction filtration system.

3.1.7 Perform the dissection using a dissecting microscope. A continuous zoom model is preferred to allow fine adjustment of magnification and allow optimal visualization of the region of interest during each step of the dissection.

NOTE: Microsurgery tools recommended include a range of toothed tissue forceps, blunt forceps, #5 forceps, angled tissue forceps, a range of micro-dissecting scissors (spring scissors), and regular insect and micro-dissecting pins.

3.1.8 Expose the brain *via* mid-line section of the skull along the sagittal suture then pin down the reflected flaps with micro-dissecting pins and pin the spinal column at the caudal end to the silicone-covered bottom of the dissection chamber using a 27 G needle.

NOTE: The rest of the dissection requires a dissection microscope to provide the clearest view of the tissue and landmarks used to ensure the maximum likelihood of obtaining rhythmic fictive output on the cranial rootlets of the brainstem and spinal rootlets. Perform these steps of the dissection using medium spring scissors or iris scissors and fine forceps.

3.2 Promptly transfer the isolated trunk of the animal to an aerated dissection chamber. Place the tissue dorsal side up with the rostral end (brain) facing the front of the chamber. Pin the tissue at the shoulders and most caudal end of the spinal cord.

3.2.1 Make a mid-sagittal incision through the skull following the parietal suture to avoid damaging the cortex and brainstem underlying the skull.

NOTE: Neonatal bone tissue is not fully calcified and is very brittle. The bone/tissue will be flexible to a degree, but tougher than the surrounding connective and muscle tissue.

3.2.2 Use medium spring or iris scissors to snip the occipital sutures of the skull, beginning at the sagittal suture and working laterally. This will create “flaps” of skull that may be reflected and pinned to anchor the rostral part of the skull and provide some stability to the tissue (see **Figure 2A**).

3.2.3 After reflecting the skull flaps, cut off the rest of the cerebral cortex, leaving the caudal portion of the cerebellum (vermis) relatively intact (**Figure 2B**).

3.3 Perform dorsal laminectomy.

3.3.1 Remove the musculature surrounding the skull and vertebral column using micro spring scissors and forceps. Remove tissue along the dorsal side of the rib cage, leaving the rib cage intact, as this will be pinned later to anchor the tissue during the ventral laminectomy, minimizing the chance of damage to the spinal cord.

3.3.2 Carefully snip away the lateral processes of the vertebral laminae using medium spring scissors or fine iris scissors. Cut away any tissue overlying the pons and medulla. The vermis, cerebellum, pons, and beginning of the spinal cord will now be clearly visible (**Figure 2C**).

3.4 Perform ventral laminectomy.

3.4.1 Turn the tissue dorsal side down and pin at the rib cage and most caudal end of the spine. Pin the rostral side of the tissue down using the skull flaps (**Figure 3A**).

3.4.2 Remove the ventral half of the rib cage, including the sternum and all abdominal organs using same scissors and forceps. Dissect away soft tissue attached to the ribcage exposing the ribs and spinal cord (**Figure 3B**).

3.4.3 Remove the tongue, esophagus, trachea, larynx, and all other soft tissue and musculature overlying the base of the skull and the spinal column (as seen in **Figure 3C**).

3.4.4 Dissect tissue overlying the hard pallet. Identify the hard palate by locating a rectangular plate of bone at the base of the skull with a V-shape indentation on it (**Figure 3C**). Cut along the midline of the palate, carefully lift it upwards, and perform a transverse cut to remove it (**Figure 4A**).

3.4.5 Begin a ventral laminectomy by removing laminae exposing the ventral surface of the brainstem and the spinal cord from the first cervical vertebra to approximately thoracic vertebra 7 (T7) as seen in **Figure 4B**.

3.4.6 At this stage, the ventral rootlets will be visible. Using small micro-dissection or spring scissors, take care to make cuts as close to the laminae and as far from roots’ origin at the spinal cord as possible; avoid stretching the roots.

3.4.7 Snip 5 - 10 mm along both sides of the spinal column at the laminae. Do not cut too close to the spinal cord or into the vertebrae. This step allows removal of the cervical vertebrae to expose the spinal cord. Avoid cutting the rootlets.

3.4.8 Snip rootlets approximately 20 - 25 mm (bilaterally) along the spinal column (approximately T7). Throughout this procedure avoid cutting into the spinal cord and manipulate the edges of the vertebrae as seen in **Figure 4C**.

3.4.9 Remove C1, C2, and C3 by carefully lifting the rostral edge of each of the vertebrae and snipping closely underneath the bone. The closer to the bone that the cut is made, the longer the rootlet. Use hooked or bent forceps to aid achieving a firm grip on each cervical vertebra while making cuts (**Figure 4C**).

3.4.10 Once the desired length of spinal cord is isolated and dissected from the vertebral column, make a transverse cut to remove the spinal cord (**Figure 5A and Figure 5B**).

3.5 Remove dura mater.

NOTE: The isolated brainstem-spinal cord can be used for *en bloc in vitro* recording as has been previously described[3,7]. With the brainstem-spinal cord removed from the vertebral column, the dura must be removed to provide optimal access for suction electrodes to perform recordings from the cut cranial and spinal rootles. Additionally, removal of the dura makes it possible to cleanly slice the brainstem and obtain rhythmically active thin slices for in vitro recording.

3.5.1 Carefully pin the isolated brainstem-spinal cord tissue dorsal side up to allow access to the dura surrounding the cranial rootles (**Figure 5B**). Pins should be applied through the lateral margin of the brainstem, rostral to the XII rootlets.

3.5.2 Remove the dura from the dorsal surface of the brainstem by lifting the dura with fine forceps (#5) at the dorsal margin of the dura and cut from lateral-to-medial across the length of the brainstem with fine spring scissors. Be careful to avoid cutting into the brainstem itself. Gently lift blood vessels from the brainstem surface and cut to avoid impediment of the vibratome blade when sectioning the brainstem.

3.5.3 Carefully dissect dura from the medial and lateral aspects of the brainstem, as the cranial nerve rootlets pass through the dura and are easily ripped away when the dura is lifted. Minimize the likelihood of the rootlets being pulled off by gently cutting around them with small spring scissors.

3.5.4 Flip the brainstem-spinal cord so that the ventral surface faces up and the cranial rootlets are clearly visible. Dissect the dura from the dorsal side of the brainstem by gently lifting the dura directly over the *area postrema*, cutting from rostral to caudal. The *area postrema* will appear slightly pink due to the large number of microcapillaries perfusing this region.

3.5.5 Use a fine insect pin to tease away any remaining dura and remove remaining blood vessels in close proximity to cranial and cervical rootlets.

4 Slice Protocol

4.1 After removing the dura, place the brainstem in the center of the paraffin platform on the plastic block (hereafter referred to as the "cutting block"). Pin the caudal end of the brainstem through the distal spinal cord using fine insect pins that have been trimmed to no more than 1 cm in length as shown in **Figure 5C**.

4.2 Align the paraffin-covered cutting block with the pinned brainstem in the vibratome block holder so that the blade cuts perpendicular to the rostral face of the brainstem.

4.3 Make an initial slice to remove the uneven, extraneous tissue on the rostral-most end. This initial cut can be 200 - 300 µm typically but carefully avoid removal of IX, X, and XII cranial rootlets. This step will typically reveal the caudal extent of the facial nucleus (VII) and the glossopharyngeal rootlets and makes it possible to align the brainstem so that its face is par-planar to the vibratome blade. Make small adjustments as necessary to ensure par-planarity and make small cuts to remove tissue that is uneven.

NOTE: Glossopharyngeal (IX) rootlets will be visible at the lateral edge of the brainstem as one cuts each successive slice, and these provide a landmark for the rostro-caudal distance to the brainstem neural circuitry necessary for rhythm-generation. On the ventral side of the brainstem, the hypoglossal rootlets (XII) should also be visible slightly caudal to the cut face showing the IX rootlets. A final landmark will be the obex (the point in the human brain at which the fourth ventricle narrows to become the central canal of the spinal cord) on the dorsal face of the brainstem. With these three points of reference visible, the correct cutting plane is established (see **Figure 6A**) and a rhythmically-active slice will be reliably obtained.

4.4 Continue cutting rostral-to-caudal until the IX rootlets are near the surface of the cut face of the brainstem.

4.5 Refer to **Figure 6** for landmarks and correct orientation. Adjust the paraffin-slab in the vibratome clamp so that the next angle of transection will create a very slight "wedge" shape to the cut slice and cut slices of approximately 100 - 200 µm until the landmarks described can be clearly seen (**Figure 6A**).

NOTE: Cutting this slight wedge increases the number of XII motor neurons captured in the slice and maximizes the likelihood of obtaining rhythmic activity during recording. Using the landmarks described (rostral rootlets from the glossopharyngeal nerve bundle, rootlets from the hypoglossal nerve bundle, the obex) will ensure that the slice reproducibly contains at least a large portion of the pBC (**Figure 6B**).

4.6 Cut a 300 - 500 µm slice of brainstem from these rostral neuroanatomical markers to capture the pBC and associated transmission circuitry.

NOTE: An "ideal" slice will contain the most rostral rootlet of the hypoglossal rootlet bundle to reliably obtain inspiratory activity without resorting to surface recordings using a suction electrode over the rhythm-generating/transmitting loci within the brainstem.

5 Recording Procedures

5.1 Place the slice in a recording chamber, perfuse continuously with aCSF (0.5 - 1.0 mL per minute) and use suction or extracellular electrodes to record population activity from the XII rootlets or from the pBC, XII PMNs, or XII motoneurons. For detailed recording procedures, see previous publications on brainstem-spinal cord electrophysiology and patch clamping[10,11].

REPRESENTATIVE RESULTS:

The method presented here allows a researcher interested in obtaining rhythmically active slices of brainstem to reproducibly and reliably cut a viable, robust slice that will allow recording of fictive motor output for many hours. All of the minimally necessary neural circuit elements for generating and transmitting inspiratory rhythm can be captured in a thin slice using this method. These elements include: the preBötzinger Complex, premotor neurons projecting to the hypoglossal motor neurons (pXII MNs) and the hypoglossal motor neurons (XII MNs), and hypoglossal nerve rootlets. The pBC, XIIn, and C4 nerve rootlets are commonly used for inspiratory rhythm recordings, as illustrated in **Figure 7**.

Successful use of this procedure will produce a viable and rhythmically active *en bloc* preparation in 10 - 15 minutes, or a rhythmically-active slice in < 30 minutes. After isolation of the *en bloc* brainstem-spinal cord or a thin slice, 15 minutes of equilibration in the recording chamber is sufficient for the production of fictive motor output. In the case of the slice, increasing the extracellular [K+] to approximately 8 - 9 mM will produce robust neural drive that can last for 24 to 36 hours in this laboratory's experience. The preparation should be continuously superfused with carbogenated (95% O_2/5% CO_2) and heated aCSF (~ 27 °C). Successful dissections are performed quickly and avoid pinching, stretching, or damaging nerve rootlets used for recordings. For optimal results, all steps in the procedure are performed swiftly and the tissue must be bathed or continuously perfused with carbogenated aCSF when performing the dissection and tissue isolation (as described above). For extensive details concerning the neuroanatomy and precise atlases of the brainstem, please see the work of Ruangkittisakul *et al.*[13-14], Ballanyi[15] and colleagues. The protocol presented here is one method that has proven reliable in the senior author's laboratory and this report provides a graphical, step-by-step method for generating these slices relying only upon surface landmarks visible on the brainstem or within the brainstem as detailed here.

FIGURE LEGENDS:

Figure 1: Initial dissection for the brainstem-spinal cord extraction. (**A**) Anesthetized pup pinned for dissection. Dashed lines indicate incision guidelines for sagittal incision into skin and transverse cuts caudal to eyes and below the diaphragm. (**B**) Guide for removing skin and forelimbs from animal. (**C**) Dorsal aspect of skinned rodent. Dashed lines indicate incision guidelines for skull-flap "clamshell" exposure.

Figure 2: Stages of the dorsal laminectomy. (**A**) Dashed lines indicate incision guidelines for removing the cerebrum. (**B**) Result after removing tissue. Dashed lines indicate incision guidelines for dorsal laminectomy. (**C**) Exposed brainstem-spinal cord following dorsal laminectomy.

Figure 3: Stages of initial ventral dissection. (**A**) Ventral side of a skinned rodent with severed forebrain and abdomen. Dashed lines indicate incision guidelines for removing the xyphoid process and opening the rib cage to subsequently remove abdominal and thoracic cavity organs. (**B**) Abdominal and thoracic cavity organs removed. (**C**) Oropharyngeal structures removed. Dashed lines indicate incision guidelines for hard palate removal.

Figure 4: Stages of the ventral laminectomy. (**A**) Hard palate and remaining skull parts removed. Incision guidelines indicate how to remove remaining tissue surrounding the brainstem-spinal cord. (**B**) First cervical vertebra (C1) exposed. (**C**) Demonstration on lifting C1 and making a transverse cut to the vertebral body to remove it. Nerves are carefully cut flush to the dorsal side of the vertebral body before removing it. This is the most critical step in the dissection.

Figure 5: Preparation of brainstem-spinal cord for slicing. (**A**) C1-C3 removed from spinal cord. Incision guidelines indicate where to sever the brainstem-spinal cord from the caudal spinal cord. (**B**) Ventral side of brainstem-spinal cord with labeled nerve rootlets. Incision guidelines indicate recommended transection or remaining rostral structures at the ponto-medullary before recording from the brainstem-spinal cord region. (**C**) Paraffin slab setup for slicing on the vibratome. The slicing region includes tissue between cranial nerves IX and XII. Do not place micro-pins into the slicing region.

Figure 6: Landmarks used for obtaining slices with an intact hypoglossal motor nucleus and PreBötzinger Complex. (**A**) Transverse view of brainstem-spinal cord after initial cut has been made rostral to the glossopharyngeal (IX) rootlets. Incision guideline indicates transection level just caudal to the hypoglossal (XII) rootlets. (**B**) Orientation of paraffin block to blade before cutting a slice containing the pBC and the hypoglossal motor nucleus. Creating a trapezoidal "wedge" shape cut maximizes the likelihood of capturing enough inspiratory neurons in the slice to obtain rhythmic activity during recording. The wedge will include the pBC, the hypoglossal premotor neurons, and the hypoglossal motor neurons, which collectively provide the necessary circuitry to transmit rhythmic drive, which is an index of respiratory rhythmogenesis.

Figure 7: Representative recordings from *en bloc* or slice preparations. (**A**) Integrated trace from XII rootlet. (**B**) Integrated trace from the pBC. Recording from the pBC using an *en bloc* preparation will require a blind patch clamp. (**C**) Integrated trace from the C4 nerve rootlet.

DISCUSSION:

Adapting the protocol presented here into an *en bloc* or slice workflow is advantageous for laboratories and studies that would like to utilize either *en bloc* brainstem-spinal cord and/or thin slice preparations for electrophysiology recordings. The dissection and slice method presented, combined with methods previously reported by others[17-19], will allow reproducible preparation of robust and viable tissue that is widely adaptable to a range of experiments using the rodent hindbrain or spinal cord. The dissection presented is highly detailed and includes dorsal and ventral laminectomy as well as extensive detail regarding orientation of the brainstem-spinal cord when preparing to cut slices. The detailed dissection and slice protocol presented allows the researcher to include all circuitry necessary for the generation and transmission of inspiratory rhythm. The main neural populations/structures that can be captured using this method include: the hypoglossal premotor neurons, the hypoglossal motor nucleus, and the preBötzinger complex. Note that regions of the brainstem that contain central CO_2 sensors or expiratory rhythm-generating circuits (RTN/pFRG) are not included in the preparation detailed here[20,21]. Once the *en bloc* preparation or slice has been obtained, rhythmic activity may be obtained using suction electrodes, surface electrodes, or single-cell recording methods such as extracellular unit or patch-clamping methods[3,10,11].

The goal of this protocol is to provide a clear, easy-to-follow method for generating either a brainstem-spinal cord preparation or a thin, rhythmically-active slice. This protocol should be valuable to both experienced and new researchers interested in incorporating rhythmic brainstem/slice preparations in their armamentarium, as there are several critical steps detailed within the procedures that facilitate capture of robust, reproducible, and long-lasting rhythmically active slices.

Once the researcher has become comfortable with identification of the anatomical landmarks and the skills necessary for dissection, the speed of the dissection will increase, and the procedures can be optimized for each individual investigator. The protocol detailed here was taught to the senior author (CGW) during his post-doctoral work with Jeffrey Smith, the originator of the rhythmic brainstem slice preparation[3]. To ensure rhythmic activity and viability in both the slices and *en bloc* preparations, all procedures to generate a slice should be finished within approximately 30 minutes from beginning of the procedure to a slice in the recording chamber. With continuous perfusion, there is very little impact on tissue viability but minimizing the time for dissection allows for longer recording and data acquisition. For respiratory studies, a slice that is as thin as approximately 280 µm thick[3] will have rhythmic, robust inspiratory activity, but slices may range up to 550 µm[17-19,22]. Careful practice is required to develop the necessary skills to perform this procedure and minimize the variability in thickness and rostral-caudal position of each slice. Including or excluding certain cell populations will influence the quality and robustness of rhythmic activity obtained in later recording as both excitatory and inhibitory nuclei in the brainstem can impact the "quality" of the rhythm recorded from the slice[3].

Finally, it is also critical that aCSF is prepared exactly according to protocol, and is at a standardized pH[20], temperature, and oxygenation level. Anoxic preparations will not be rhythmically active and will affect data collection[21].

There are several key steps in the procedure that facilitate an investigator capture of viable and rhythmically active preparations. During the ventral laminectomy, keeping the dorsal rib cage intact allows extra leverage and provides more tissue for securing the preparation while performing the relatively delicate isolation of the brainstem-spinal cord from the vertebral column. The dissection steps detailed here allow the investigator to easily produce *en bloc* preparations or thin slices so, by necessity, extra steps are detailed that can be omitted to obtain just a thin slice. Other useful tips include, while performing the ventral laminectomy, it is important to gain a firm grip on the vertebral body to lift it upwards while severing the nerve rootlets. Once the brainstem-spinal cord has been dissected and extracted, the dura mater and remaining vasculature must be dissected off of the surface of the neural tissue. Blood vessels and dura are tough and can prevent the vibratome blade from cutting a clean slice. Dissection of the dura is not vital for the *en bloc* preparation, but is recommended to optimize nerve-suction electrode coupling. Finally, careful and methodical practice in the use of the vibratome is necessary for cutting a clean, reproducible slice. This protocol provides a very comprehensive list of detailed steps for the dissection and cutting procedures. The emphasis here is to provide a step-by-step detailed list for an investigator to use as a reliable foundation and training tool from which they can modify their laboratory's procedures as needed.

As a whole, this methodology represents a thirty-year evolution of methods of *in vitro* electrophysiology. The scope of this paper focuses on the standardization of *en bloc* and slice brainstem-spinal cord recordings that are typically used for studying inspiratory activity in P0-P5 rats and mice[22]. However, this dissection process may be utilized across a range of animal sizes, ages, and species for a wide variety of studies, including E18 rats/mice, neonatal rats and mice (postnatal days 0 to 6), and adult mice. Future studies may use the methods presented to streamline protocols across species and ages, allowing studies to interrogate mechanisms in rhythm generation or other physiological process across both species and ages. Ultimately, changing the orientation, thickness, or location of the slice or *en bloc* preparation will influence rhythmic activity[23]. Extensive literature has been published in the past concerning the neural populations captured by slice procedures and there are stereotaxic rat and mouse atlases available to provide more detail concerning the neural circuitry discussed here[24-26]. The procedures presented here provide extensive detail with clear illustrations that allow a new investigator to learn to cut rhythmic slices from landmarks readily visible under a laboratory dissecting scope.

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The authors have nothing to disclose.

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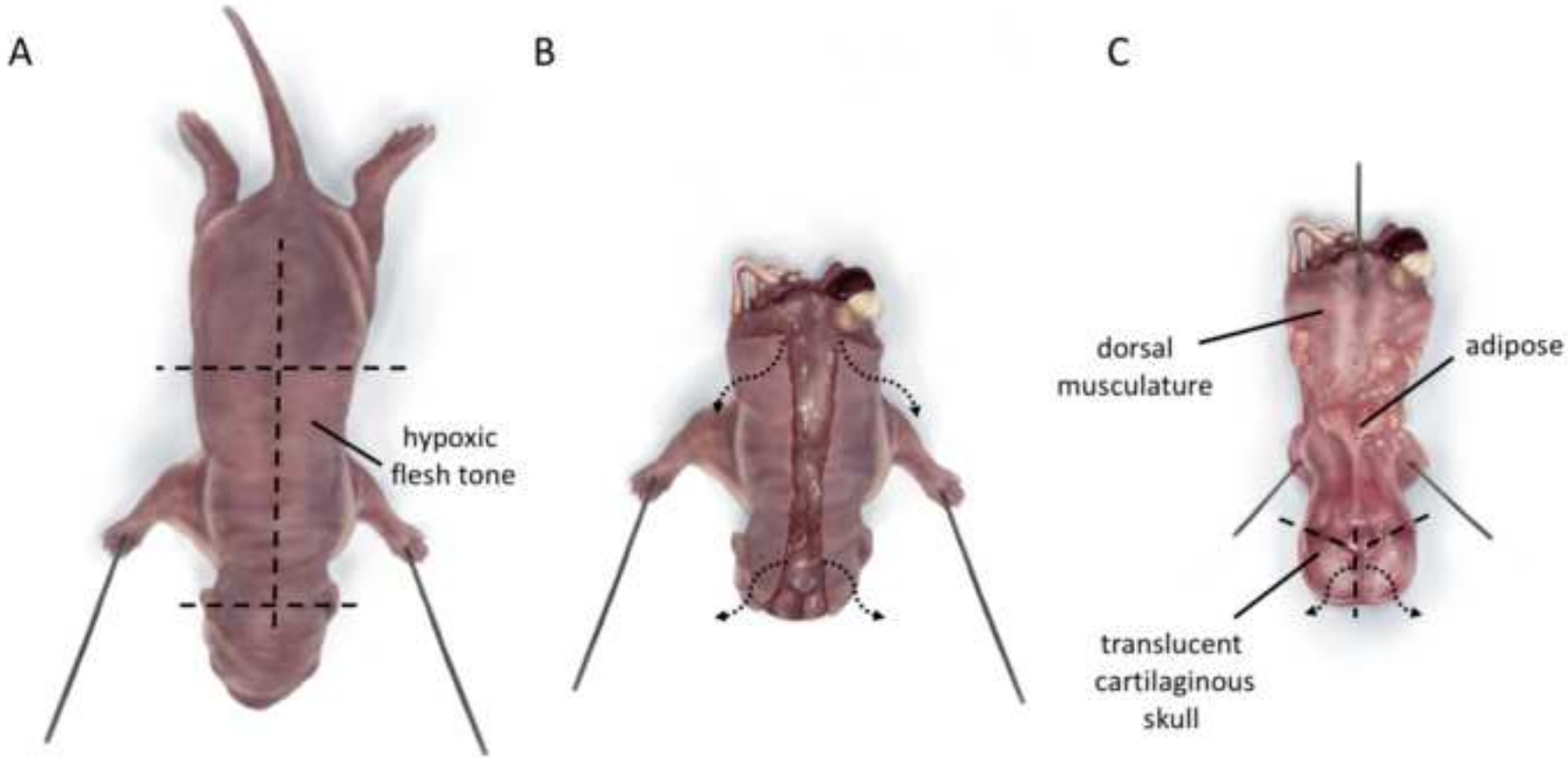

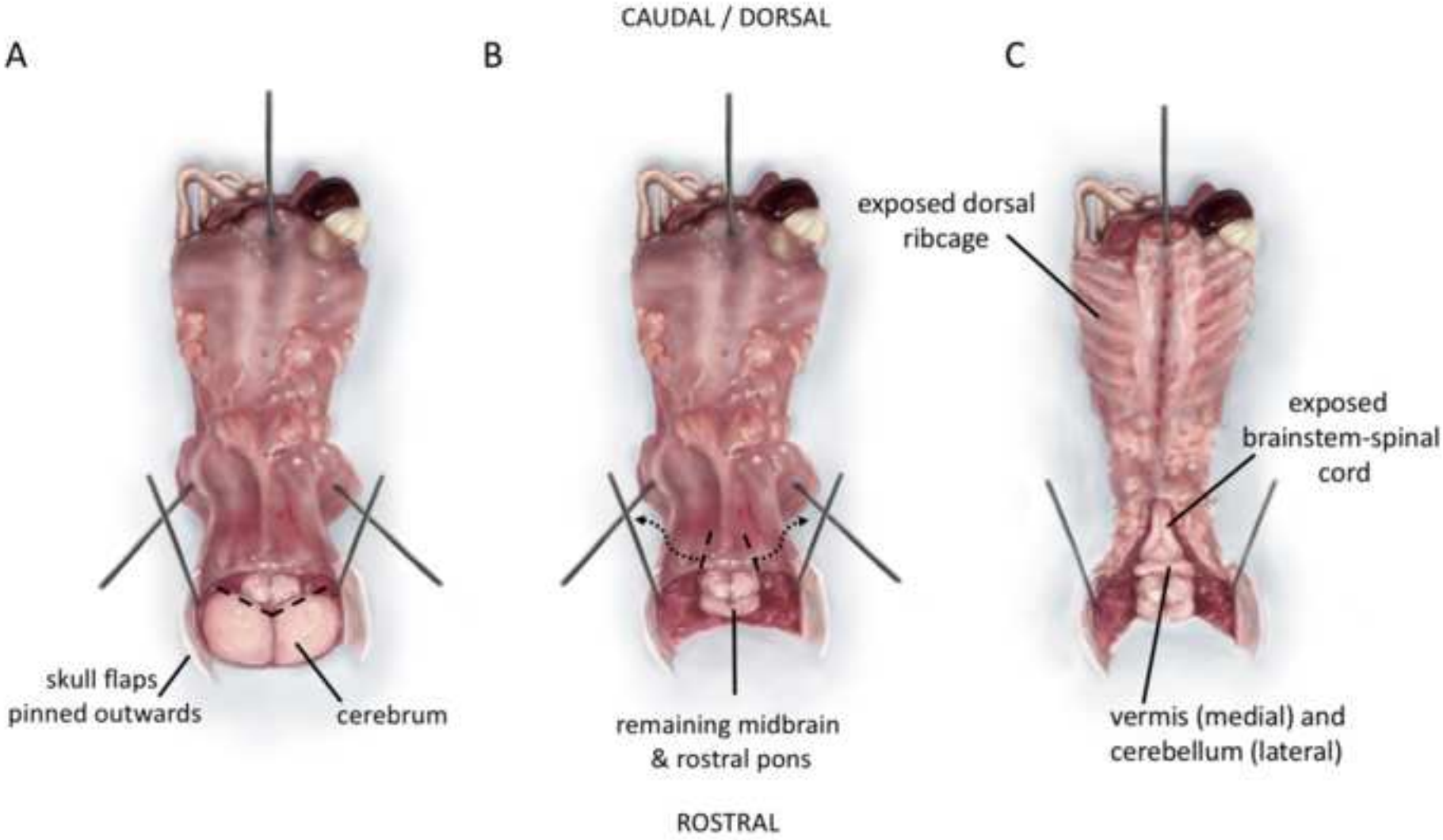

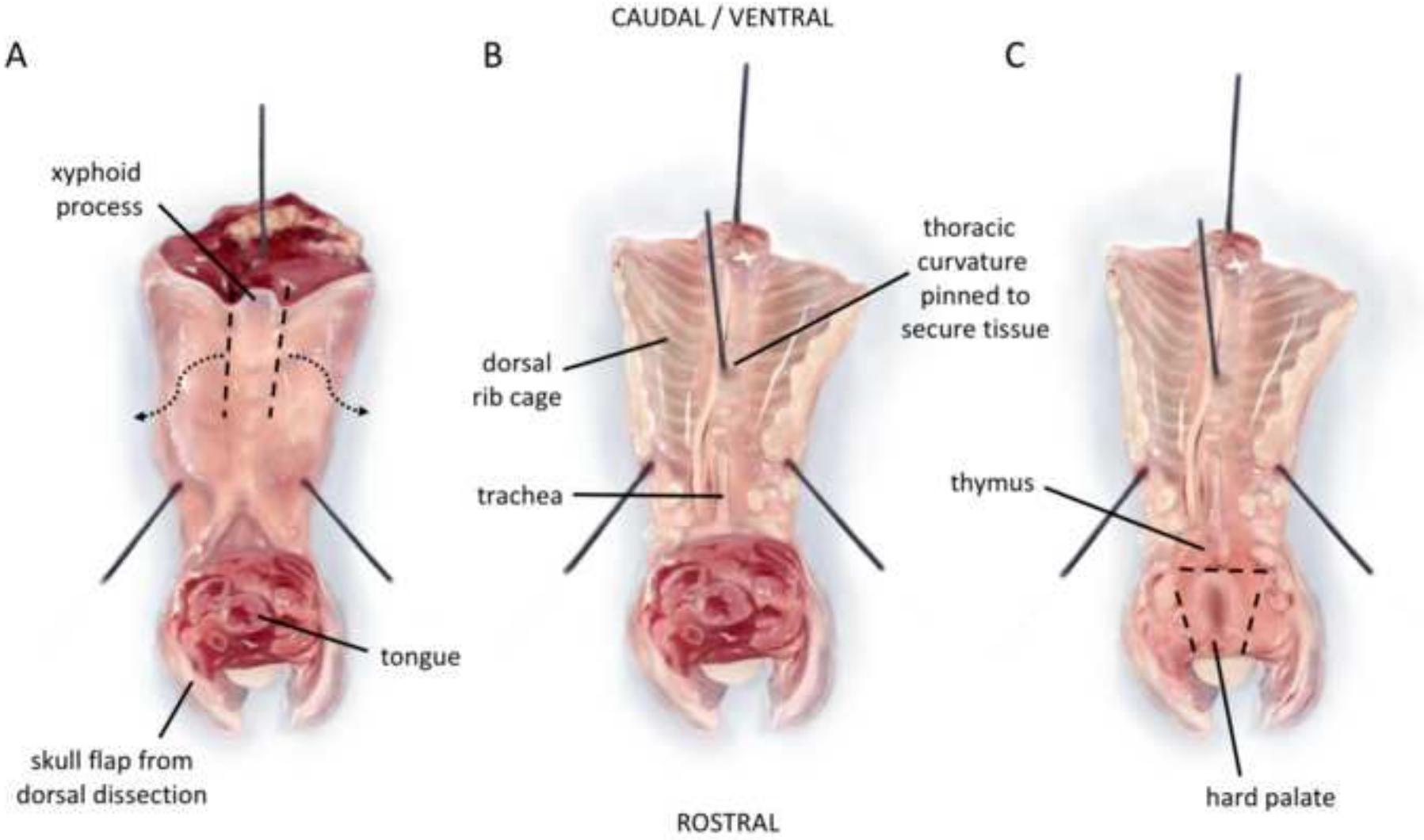

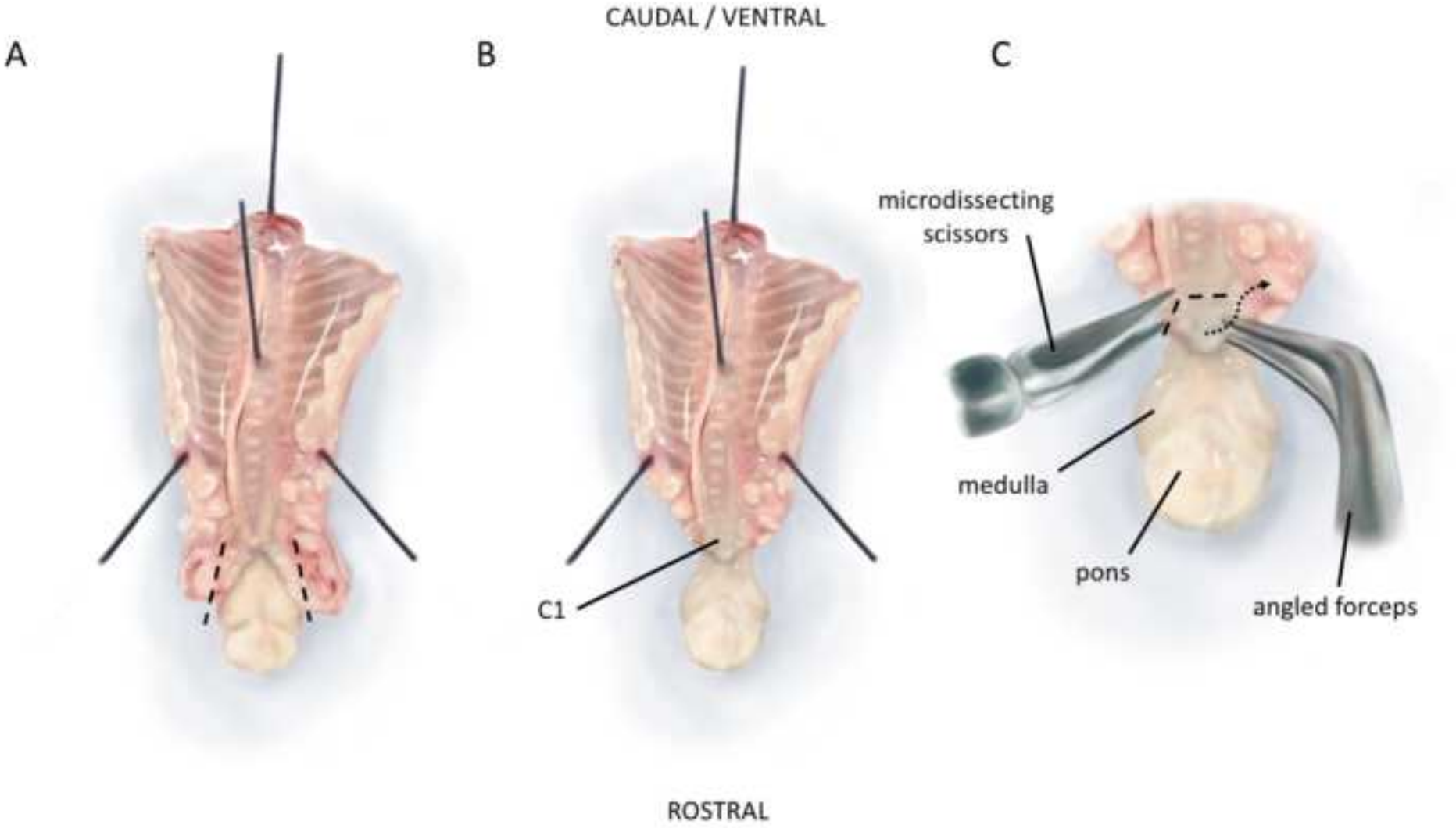

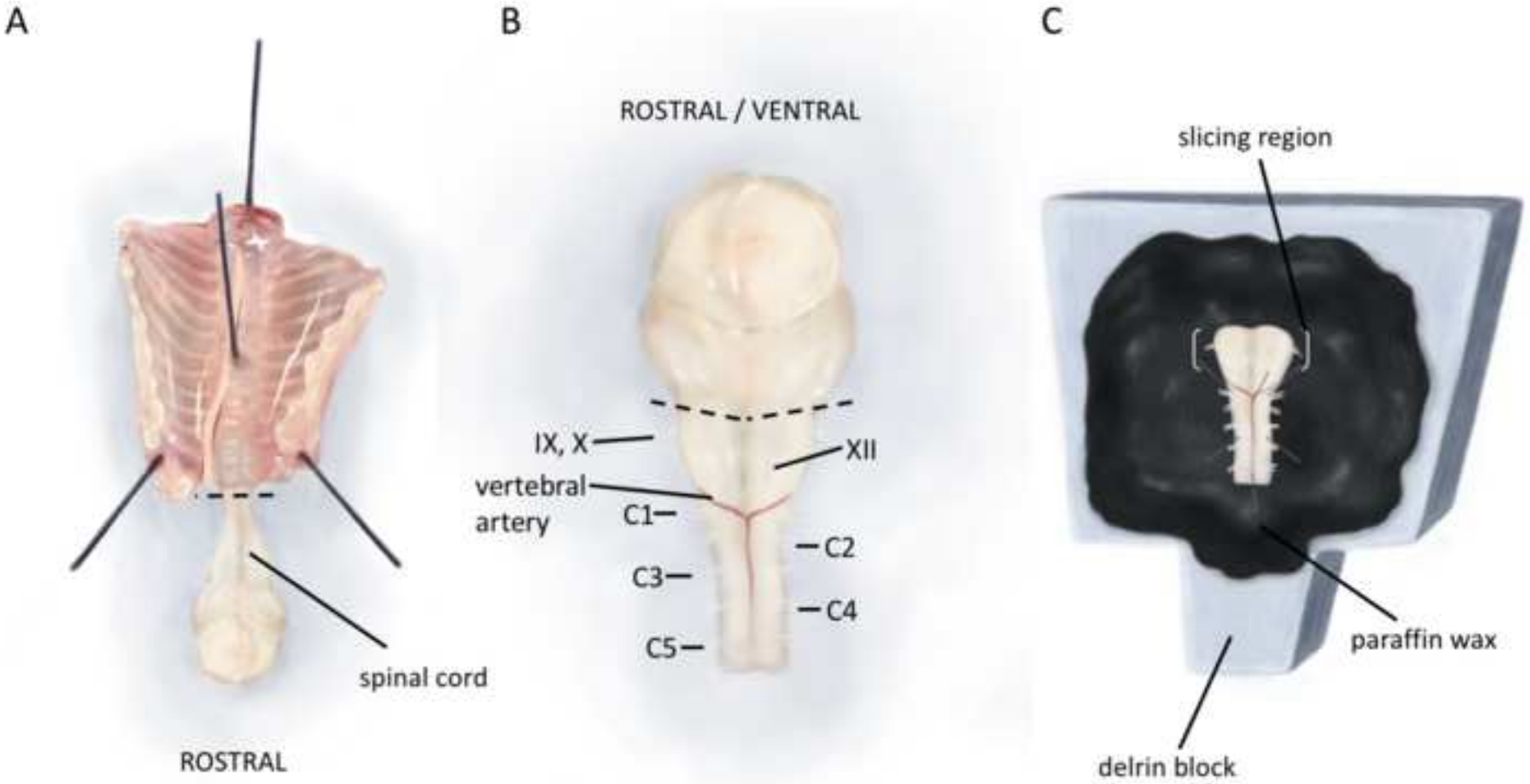

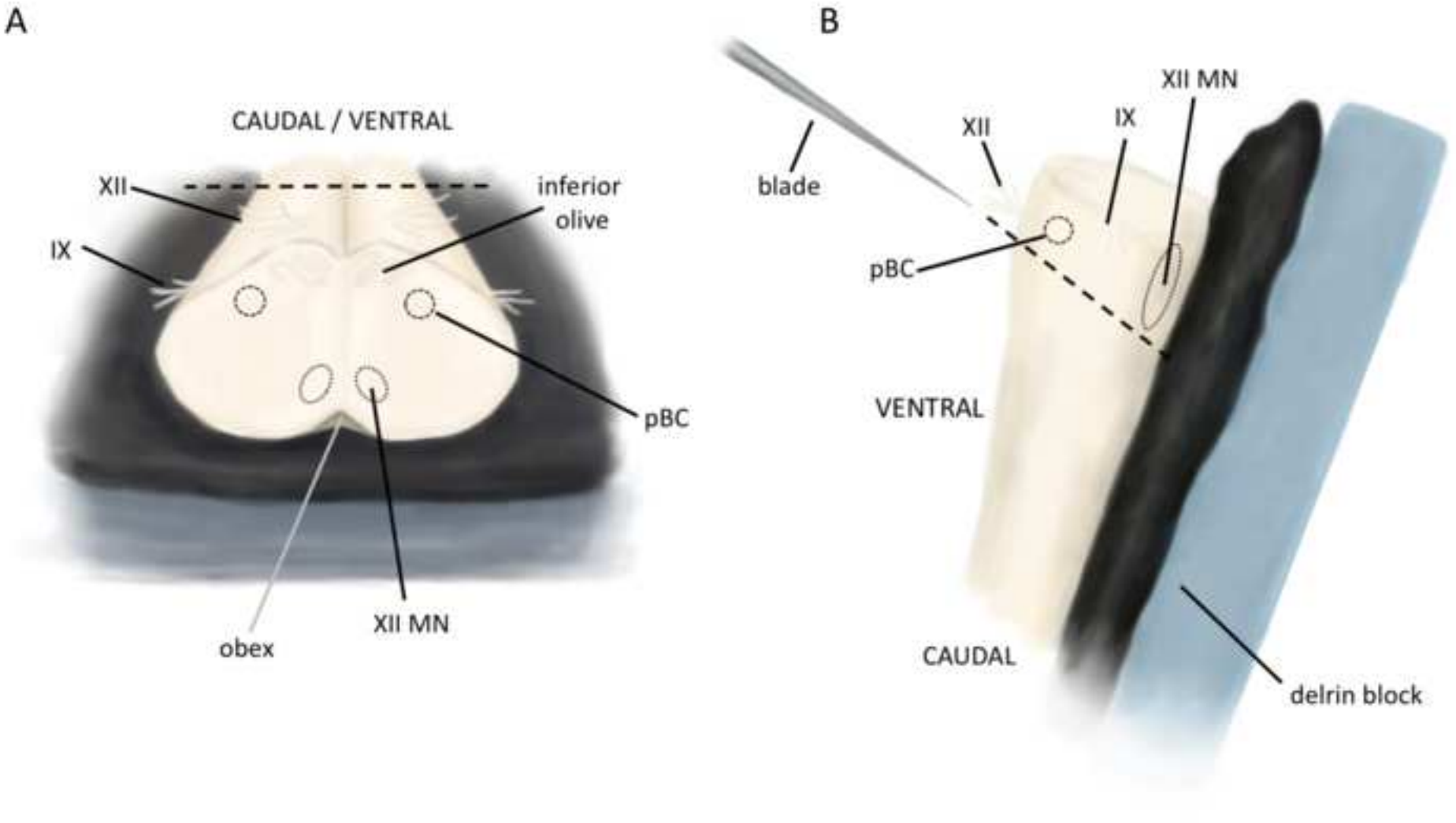

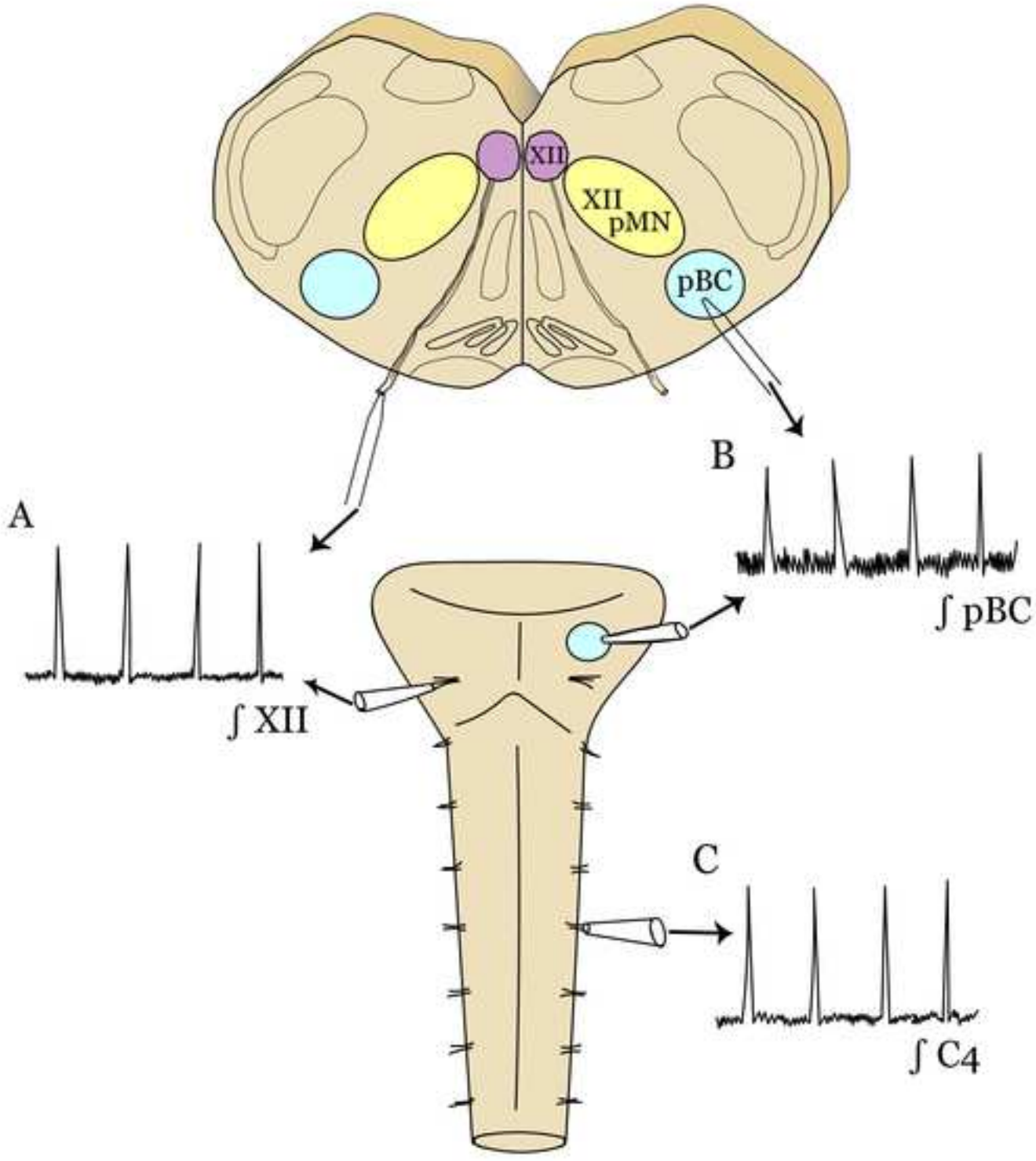

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Editorial Revisions (Sept 24, 2018)

Line 112. We have added more specific language regarding sources of contamination.

Line 149. Rephrased in imperative tense. More directed language will help guide the procedure more efficiently. We have changed the text to make it imperative and re-labeled “slides” to “Figures”.

Line 150. Clarified how the block with paraffin is formed.

Line 172-174. Rewrote in imperative tense.

Line 176-177. Removed duplicate note to ensure proper flow of protocol.

Line 191-194. Rewrote in imperative tense.

Line 196. Added the flow rate of aCSF

Line 204-206. Clarified that these tools are used differently in the initial euthanasia than the dissection protocol.

Line 208. Accepted format and word changes in text and made some other text changes.

Line 224. The symbol is a typo and removed it from text

Line 229,232,236,242, 247, 249. Accepted format and word changes.

Line 236 and 249. Specified which tool was used (medium spring scissors or fine iris scissors).

Line 286-291. Converted the step into a note for useful information prior to dissection.

Line 299- 301. Changed text to imperative text.

Line 303-306. Changed suggestive text into a note and the imperative text into a step.

Line 316. Accepted format changes

Line 335- 342. Created two notes that are critical to the process and kept the imperative tense as a step in the protocol.

Line 344-348. Rephrased the text to an imperative and informative step in the protocol. Removed the use of personal pronouns.

Line 359. We have added clarifying text.

Line 379. Change molecular formula to subscript.