

Proprioception and tension receptors in crab limbs: Student laboratory exercises.
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Question	Response

Proprioception and tension receptors in crab limbs: Student laboratory exercises.

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SHORT ABSTRACT:

Physiological and anatomical techniques are demonstrated to address function and structure for joint proprioceptors and muscle tension receptors in crustacean walking limbs.

LONG ABSTRACT:

The primary purpose of these procedures is to demonstrate for teaching and research purposes how to record the activity of living primary sensory neurons responsible for proprioception as they are detecting joint position and movement, and muscle tension. Electrical activity from crustacean proprioceptors and tension receptors is recorded by basic neurophysiological instrumentation, and a transducer is used to simultaneously measure force that is generated by stimulating a motor nerve. In addition, we demonstrate how to stain the neurons for a quick assessment of their anatomical arrangement or for permanent fixation. Staining reveals anatomical organization that is representative of chordotonal organs in most crustaceans. Comparing the tension nerve responses to the proprioceptive responses is an effective teaching tool in determining how these sensory neurons are defined functionally and how the anatomy is correlated to the function. Three staining techniques are presented allowing researchers and instructors to choose a method that is ideal for their laboratory.

INTRODUCTION:

Proprioception is the sensation of limb position and movement that enables coordinated motor behavior. Proprioceptors consist of position (static) and movement (kinesthetic) receptors. In insects and crustaceans, chordotonal organs are the structures that provide that information to the CNS.¹ Not all chordotonal organs span a joint but they can still monitor joint movements due to their attachment on the apodemes (tendon like structures) which span the joint and move in association with the skeletal muscle and joint articulation. Crab legs have six joints, each having one or two chordotonal organs.² Typically a chordotonal organ has 60-100 or more sensory neurons embedded within an

elastic strand, neurons that signal static joint position, direction and speed of movement.³⁻⁶ The input from chordotonal organs at each joint and leg is then centrally processed allowing coordinated movements by the animal.

The forces that leg muscles produce during isometric and isotonic contractions are detected by tension receptors associated with muscle fibers and their attachments to apodemes.⁷⁻⁹ In the crustacean walking leg protocols that follow we present methodology for recordings from primary sensory neurons that monitor proprioception and the neurons that respond to forces generated by muscle fibers. A technique for activating leg movements and quantifying force generation is also presented, as well as anatomical techniques that can be used to characterize the arrangement of these peripheral nervous system structures.

The procedures demonstrated below enable structural and functional analysis of the neurons that innervate both types of receptors relative to their location on a chordotonal elastic strand and apodeme. To illustrate, we use the propodite-dactylopodite (PD) chordotonal organ, the organ that spans the distal most segment of the crab leg.³ Though detailed electrophysiological studies began in the 1930's and are still being carried out today, some aspects have become known about the segmental connections of proprioceptors in the various joints and their roles in coordinated control of muscles.¹⁰⁻¹⁶ Establishing the structure-function relationship between the proprioceptive organs, muscles and the nervous system will further help define these roles. For instance, labeling the somata and distal endings of tension neurons inserted into the apodeme will reveal their location relative to muscle fibers.^{8,17-21}

We present three staining techniques for crustacean legs that can be used in research or academic laboratories. Methylene blue staining provides suitable contrast for muscles and nerves and is recommended as a simple technique for students to learn anatomy. Labs that have fluorescence microscopy setups can accomplish more selective neuronal staining by briefly exposing the nerves to the vital dye 4-di-2-ASP. The third alternative is CoCl_2 backfill, which stains and fixes the neurons, and does not require fluorescence imaging. Though it is labor and time intensive, this staining process gives high contrast and specificity for the nerves that are filled. Together these techniques can be used for comparing various chordotonal organs, not only within a limb or between limbs, but also among other crustacean and insect species.²⁰⁻²² Blue crabs (*C. sapidus*) used in physiological recordings and for anatomical staining are readily available all around the Southern and Southeastern border of the United States. This species serves as a representative of the chordotonal and tension nerve arrangements found in most crabs. Laboratories on the west coast will prefer to use the much larger Dungeness crab (*Cancer magister*) for these experiments.

PROCEDURE:

1) Dissection and recording electrical activity from the propodite-dactylopodite (PD) nerve

1.1) Hold the crab across the carapace from behind, and avoiding the claws, pinch the proximal

part of the meropodite with forceps. The leg will autotomize to prevent the animal from bleeding to death. Use caution when handling blue crabs as they are rather aggressive and very fast. [Place Figure 1 here]

1.2) Make a cut between the propodite and carpodite. Discard the carpopodite and the attached meropodite (Figure 1).

1.3) Cut a large window in the cuticle on the pigmented (lateral) side of the propodite with a scalpel with a #11 blade (Figure 2 and 3). **Note:** Do not cut deeply.

1.4) Remove the cuticle layer by sliding the scalpel blade beneath and parallel to the cuticle. This severs the muscle fibers attached to the cuticle.

1.5) Using the same technique cut a smaller window on the pigmentless (medial) side of the propodite, but leave the condyle (the socket joint or hinge between segments) attachment intact. [Place Figure 2 here]
[Place Figure 3 here]

1.6) Prepare a Sylgard-lined dish containing crab saline to pin the preparation down. **Note:** See table 1 for strain specific saline recipes.

1.7) Locate the PD organ by carefully probing with the fire-polished glass needles. The elastic strand spanning the joint has a silver appearance.

1.8) Remove muscle fibers that obscure your view of the organ from both sides of the tendon. Be very careful not to injure the PD organ or its nerve.

1.9) Once this has been accomplished, firmly re-attach the preparation to the dish with the pigment side (lateral) facing up (Figure 4).
[Place Figure 4 here]

1.10) Follow the PD organ nerve in the propodite as far proximally as possible in order to free-up a long length of nerve (1.5 cm) for recording purposes. This is best done while the PD nerve is still attached to the main leg nerve.

1.11) After separating the PD nerve from the main leg nerve with the aid of glass needles, sever the PD nerve proximally with the iris scissors. **Note:** Do not stretch or pull on the nerve during the dissection.

1.12) Move the dactylopodite to an extended and fully flexed position. Take note about where the extreme flexion and extension positions are and a half-way point for later use.

1.13) Place a ground wire inside the saline bath.

1.14) Turn on the electrophysiology recording hardware/software. **Note:** Our setup has been described previously.²³

1.15) Position the microscope so that it is overlooking the microscope stage. Once it is placed on the stage, you will need to adjust the position of the high intensity illuminator beam to best visualize the preparation.

1.16) Position the micromanipulator so the attached suction electrode assembly will have easy access to the saline bath and preparation. The suction electrode is constructed as shown in an online video.²⁴

1.17) To detect neural activity, draw the cut end of the PD nerve into the suction electrode.

1.18) Move the dactyl throughout extended and flexed positions for several cycles with the aid of a glass probe or wooden dowel.

1.19) Next observe activity when the dactyl is pinned in the extended, flexed and mid positions.

2) Recording electrical activity from the tension while monitoring force generation.

2.1) Determine the lateral and medial side of the leg. The medial side has a soft texture that can be felt by pinching gently in the meropodite region with a fingernail.

2.2) Face this soft cuticle side up in the dish. The excitor motor nerve that innervates the opener muscle also innervates the stretcher muscle in the carpopodite.

2.3) In order to stimulate the opener muscle the stretcher motor nerve in the carpodite region is isolated and stimulated with a suction electrode. The proximal part of the leg is removed by transecting the meropodite with scissors.

2.4) Remove a section of the cuticle in the carpus region on the inner side (medial side, **Figure 5A**).

2.5) Cut the apodeme of the bender muscle and remove the muscle carefully as not to pull the main leg nerve out of the leg cavity (**Figure 5B, C**- note the arrows where the bender apodeme is separated). The main leg nerve and a branch to the stretcher muscle can then be observed (**Figure 5D**).

2.6) Find the nerve branching from the main nerve bundle to the stretcher muscle (**Figure 5E, at arrow**). This can be cut close to the muscle and pulled into a suction electrode to stimulate (**Figure 5F&G, arrow depicts the branch**).

2.7) Tease out a section of the nerve that projects towards the opener without pinching the nerve. Transect the stretcher/opener motor nerve.

2.8) Pull the motor nerve into the suction electrode and then stimulate it.

2.9) To expose the opener muscle and tension nerve remove the closer muscle and the ventral region of the propodite. Cut off the closer muscle with a scalpel with a #11 blade in the propodite (Figure 6). **Note:** Do not cut deeply in the proximal region because it might damage the opener motor nerve.

[Place Figure 5 here]

[Place Figure 6 here]

2.10) Exchange the saline with fresh cooled saline throughout the dissection process to keep the neurons alive. For further dissection, place the preparation dish under a dissecting microscope and use fiber-optic illumination.

2.11) Carefully cut the closer tendon from its attachment to the dactyl using sharp-pointed medium-size scissors.

2.12) Be very careful not to disturb the branches to the opener muscle from the main leg nerve which should be clearly visible.

2.13) Remove and discard the closer muscle and tendon.

2.14) Make a hole in the dactyl with a dissection pin. This hole will be used later to hook the metal pin on the tension (force) transducer, but it is necessary to make it at this stage of the procedure.

2.15) Locate the nerve branch that projects to the opener muscle and apodeme in the distal region of the opener muscle. Carefully probe the nerve with the fire-polished glass tool.

2.16) Observe the tension nerve that arises from the distal end of the apodeme and proceeds to the motor nerve bundle.

2.17) To detect neural activity, fill a suction electrode with crab saline and draw the cut end of the opener tension nerve into the electrode. Ensure that the suction electrode fits tightly on the nerve.

2.18) Examine for the neural correlate of passive tension on the opener apodeme. While recording electrical activity from the tension nerve, rotate the dactyl rapidly into an extended joint (i.e. stretching the opener muscle).

2.19) Next, test active force development in relation to stimulation frequency.

2.20) Firmly attach a metal hook so that the tip of the hook goes through a small hole in the dactyl.

2.21) Attach the other end of the metal hook to the force transducer. **Note:** Make sure the transducer is at a 90 degree angle each time so that the pin is perpendicular to the transducer for maximal detection of the force generated.

2.22) Pull the hook and dactyl so that it is at about a 45 degree angle of the opener joint.

2.23) Now stimulate the motor nerve at 100 Hz for 250 msec and measure the force as well as the firing frequency of the tension nerve.

2.24) Place the joint into a fully extended position so that the opener muscle fibers are flaccid.

2.25) Stimulate the motor nerve at 100 Hz for 250 msec and measure the force as well as the firing frequency of the tension nerve.

2.26) Bend/flex the joint so that it is fully flexed (~90 degrees). In this position the muscle fibers of the opener are fully stretched. There may be some force measured by the transducer due to this passive stretch of the muscle.

2.27) Stimulate the motor nerve at 100 Hz for 250 msec and measure the force as well as the firing frequency of the tension nerve.

2.28) After measuring a force, proceed in a series of various frequencies: 20, 40, 60, 80 and 100 Hz for 8 to 10 seconds in each joint position.

2.29) Measure the response with a quick tension release. Arrange the preparation so that the hook on the force transducer can easily be pushed out of the hole in the dactyl.

2.30) Bend/flex the joint so that it is fully flexed (~90 degrees). Now stimulate the motor nerve at 100 Hz with a continuous stimulation of 5 seconds.

2.31) After the first second or less, as tension has built up on the opener muscle, push the pin out of the hole in the dactyl.

2.32) Examine the tension nerve recording before and after release of the pin holding the dactyl.

2.33) Modulatory effects of neuroactive substances such as octopamine, serotonin, and proctolin, which alter the output of the tension neurons, can be determined by adding these molecules to the bathing media over the exposed opener muscle and repeating the experiments.

2.34) Use a pipette and drip 1-2 ml over the preparation.

3) Staining peripheral nervous system structures in crustacean walking legs.

3.1) Methylene blue technique

3.1.1) Dilute one part methylene blue chloride stock solution (0.25%) with two parts of distilled water.

3.1.2) Add this mixture to five parts of buffered saline. Thoroughly irrigate the area of interest.

3.1.3) Dissect a leg according to the protocol in section 1. Incubate the preparation in the methylene blue solution at 12-13°C.

3.1.4) Examine the preparation every 10-15 minutes with the dissecting microscope using low intensity illumination to follow the progress of the staining. In some cases staining will be completed in an hour, in others it may take overnight.

3.2) 4-di-2-ASP technique

3.2.1) Fluorescent dyes can be used to back-fill the PD neuron or directly stain the preparation from the bath. However a microscope with abilities to view the fluorescent stain is required.

3.2.2) Dissect a leg according to a protocol in section 1.

3.2.3) Incubate the preparation in a 10 μ M concentration of 4-Di-2-ASP solution and leave the preparation in the refrigerator for 15 minutes. Use just enough solution to cover the preparation.

3.2.4) Photograph the preparations quickly and avoid over exposure to the mercury light. This fluorescent dye fades away relatively quickly.

3.3) Cobalt chloride technique

3.3.1) Expose the PD nerve according to the protocol in section 1 and keep it immersed in cold saline.

3.3.2) Make a petroleum jelly well to hold the CoCl_2 . If any CoCl_2 spills into the saline bath the entire preparation will stain black, and the preparation should be discarded.

3.3.3) Slip a small cut of a polystyrene sheet, to make a plastic platform, and pin it in such a manner that it will not float away or become immersed in the saline bath (Figure 7).
[Place Figure 7 here]

3.3.4) Eject petroleum jelly from a fine hypodermic needle fastened to a disposable syringe, then make a barrier (a circle might work well), which should be about 1-1.5 mm high except for a shallow "V" at midpoint where the nerve will be draped across, on top of the slip of polystyrene.

3.3.5) Make a small puddle of saline on either side of the barrier near the "V". Taking care not to stretch or pinch the nerve. Lift the nerve carefully from the dish and place it in the saline puddle in the petroleum jelly well.

3.3.6) Work quickly so that the section of nerve does not dry out, carefully eject petroleum jelly to cover the exposed nerve. Now test the barrier with saline and make sure it is not leaking.

3.3.7) Blot away saline on the inside of the barrier and see if it fills up when the bath saline is high around the wall of petroleum jelly to be sure that the two sides are isolated from one another (**Figure 8**).
[Place Figure 8 here]

3.3.8) Wick away the saline in the well using a piece of tissue paper. Avoid letting the paper wrap up the nerve.

3.3.9) Make a new puddle using a few small drops of distilled water, and then cut the nerve end. Be very careful not to pull the nerve cord through the barrier when making the cut. The osmotic shock of the distilled water will "balloon" the axons of the connectives.

3.3.10) Within 30 seconds add a small drop of CoCl_2 to the water, soak up this solution, and then add enough CoCl_2 to form a puddle over this shortened section of nerve (**Figure 9**). Preparations are best kept refrigerated at 13°C for 12-24 hrs.
[Place Figure 9 here]

3.3.11) Remove the humidifying puddles. Blot away the cobalt solution using a tissue and wash away the remnants of cobalt with several changes of saline.

3.3.12) Transfer the isolated preparation to a small glass Petri dish containing about 10 ml of crab saline. The neurons are washed and the following steps are performed *in situ*. Good metal tools are not to be used to handle the preparation after this step (you should use specific tools that are not to be used at a later time for physiology).

3.3.13) Add 1-2 drops of ammonium sulfide $(\text{NH}_4)_2\text{S}$ to the saline. Cap the $(\text{NH}_4)_2\text{S}$ bottle tightly and place back into the hood. Observe the reaction in the preparation under a dissecting microscope

3.3.14) Within a minute or two, cobalt-filled neurons and their processes should begin to appear because they will stain black.

3.3.15) After 5-10 min, replace the development solution with fresh saline.

3.3.16) Make certain that the development solution you have poured into the sink drain is followed by running tap water for a few minutes or in a waste bottle with a lid.

3.3.17) Pour out the saline and fix the nerve preparation for about 15 minutes with two changes of Bouin's solution fixative. For larger tissues like the opener muscle (to stain tension neurons), increase the duration of fixation to 30 minutes.

3.3.18) Dehydrate in a series of ascending order of ethanol concentration beginning at 70% (i.e. 70%, 80%, 90%, 100%). About 10 minutes at each concentration is sufficient for small tissues.

3.3.19) After about 10-15 minutes in two changes of 100% ethanol, clear the tissue by replacing ethanol with 100% methyl salicylate. The preparation will stay in this solution permanently for repeated viewing. With time the filled cells will become more apparent because the surrounding tissue will become clearer. Intensification methods can be used to help prevent fading over time.

25

3.3.20) Use the same process to fill the tension nerve with CoCl_2 . However, change ethanol solution multiple times in each ethanol dehydration step and incubate the preparation inside alcohol for about 20 minutes for each step to thoroughly dehydrate the muscles and allow them to clear well.

REPRESENTATIVE RESULTS

When the PD organ is stretched by fully extending the joint, activity in the PD nerve is robust during the movement as shown for the first second in **Figure 10**. Some activity remains while it is held still in the open position. This activity is from the static position sensitive neurons (second half of the recording shown in Figure 10). Movement evokes a response during displacement, and the firing is mostly present during the stretching of the chordotonal strand (**Figure 11**).

Further analysis of the spikes can be readily approached by sorting the relative amplitudes. This is an approach to demonstrate different populations of sensory neurons being recruited for positions or types of movements.⁵ Typical amplitudes range from 0.25-1.5mV, but these values are dependent on the resistance (i.e., tightness) of the suction electrode seal. Frequency of spikes in the various size ranges can also be graphically represented for analysis.

Forces generated by the opener muscle with respect to the stimulation frequency can be compared by superimposing the respective voltage-time traces on top of each other (**Figure 13A,B**). This can also be performed for each joint position at each given stimulation frequency. Activity of the tension nerve can then be correlated with the amount of relative force generated at each stimulation frequency, and for each joint position. As in the PD nerve, a variety of spike amplitudes are seen in response to contraction of the opener muscle (**Figure 13C**).

Anatomical arrangement of neurons in the walking leg is clearly observed with methylene blue staining (**Figure 14**). Note the elastic chordotonal strand and tension neuron that is close to the apodeme. Several somata with different diameters and specific locations are also visible in this figure. The entire course of the tension nerve and stretcher motor nerve are shown in **Figure 15**. Individual neurons of the PD nerve are shown with higher contrast using 4-Di-2-ASP (**Figure 16**) and CoCl_2 (**Figure 17**) backfill techniques. At high magnification the sensory endings can be seen inside of the supportive scolopales (**Figures 16B**).^{21,22,26}

Figure legends

Figure 1: First walking leg of a crab. The anatomical location of chordotonal organs (hatched regions) are superimposed on this schematic. The double arrow head indicates where to transect the leg for the PD nerve experiments.

Figure 2: Cut along the dotted line on the propodite

Figure 3: Expose the nerve in the window (the arrows outline the nerve bundle)

Figure 4: Exposed PD organ and nerve.

Figure 5: Dissection steps for exposing the motor neuron for stimulating the opener muscle.

Figure 6: Cut the cuticle on the ventral half of the propodite to expose the closer muscle so it can be removed.

Figure 7: Polystyrene sheet with pins to hold in place in dish

Figure 8: Petroleum jelly well with saline and PD nerve spanning the well. The PD nerve has not been cut yet.

Figure 9: The cut PD nerve being exposed to CoCl_2 . The cut nerve swells up in the presence of water that facilitates and accelerates the movement of dye molecules through the axons into the neuron cell bodies.

Figure 10: Move and hold at 0 degrees. The dynamic neurons are robust in firing during the movement and spikes from static sensitive neurons are present while the joint is held open.

Figure 11: Rapid open and closing from fully flexed to extended (90 to 0 degrees) position.

Figure 12: Extracellular spikes recorded from the PD nerve. The joint is fully extended and then quickly moved to a $\frac{1}{2}$ flexed position and then held still. Notice the activity during the movement and decreased activity when static.

Figure 13: The relative forces that are developed with the joint fully flexed and stimulated at the various frequencies. (A) Voltage-time traces from the force transducer are shown with each stimulation frequency. (B) The traces in panel A are superimposed in different colors for ease in comparison. (C) Voltage-time traces of electrical activity recorded from the tension nerve when the motor nerve was stimulated at 80 Hz. Note the regular pattern of the stimulus artifacts as compared to the neural activity. Also, note the various amplitudes of the neural responses.

Figure 14: Methylene blue stain of the walking leg preparation. Individual somas are shown with their sensory endings projecting into the elastic strand. Close to the apodeme a tension neuron is shown.

Figure 15: The tension nerve arising from distal end (red arrows) and joining the motor nerve (green arrow).

Figure 16: (A) A back-fill of the PD nerve in *Cancer magister* with 4-Di-2-ASP. (B) Higher magnification of sensory endings. The neurons are slightly stained with methylene blue from a blue crab. The PD nerve in *Cancer magister* was filled with 4-Di-2-ASP.

Figure 17: Neurons that were filled with CoCl_2 and processed (A). Traced outline of the stained preparation shown (B).

Table 1: Recipes for crab saline

DISCUSSION

The purpose of this set of experiments is 1) to teach and exhibit the fundamental principles of extracellular recordings from an identifiable proprioceptive organ and tension nerve and 2) to stress the importance of anatomical mapping in relation to physiological function of particular sensory systems. This experimental approach and the animal models utilized are inexpensive and relatively easy to conduct in neurophysiology teaching laboratories.

The neurons of chordotonal organs are of two specific functional types, those that respond to movement and those responding to static positions. Single cell recordings from a variety of chordotonal organs, no matter which joint is examined, have shown this to be the case.^{3,5} Indeed, chordotonal organs associated with the antennal joints of lobsters reveal the same two sensory types and basic anatomy.²⁷ In addition to there being two neuron types (movement and position), the neurons share the same anatomical arrangement on their respective elastic strands. The large somata located proximally on the strand tend to belong to the dynamic movement sensitive neurons. Neurons that signal static positions have small somata and are located distally. These cells are tonically active. The PD joint only contains a single chordotonal organ while there are two chordotonal organs in the carpus-propodus (CP) and merus-carpus (MC) joints.

The dissection to expose proprioceptive structures in blue crabs (*C. sapidus*) for electrophysiological recording requires a strategy that allows joint movements to take place in the natural positions while recording from sensory neurons. The tension nerve for the opener muscle in the walking leg is a very fine nerve made up of several neurons. Unless precaution is taken the tension nerve, as well as the motor nerve innervating the muscle to be stimulated, can readily be damaged during this dissection. For optimal recordings the suction electrodes need to be tailored to the size of the nerve. Recordings are readily accessible in a student laboratory using a 30-40x magnification dissecting microscope and low-end micromanipulators.

Future types of experimentation that would be interesting to pursue with the joint chordotonal organs would be to examine the structural and physiological profiles during leg regeneration in various species at different stages in the life cycle as a follow up to an initial study that used *Cancer magister*.¹⁹ Questions remaining to be addressed are 1) does the distribution and organization of regenerated neurons depend on the age of the animal when regenerating a limb, 2) are the axonal projections to the CNS (ventral nerve cord) in a regenerating limb functional or

does it take time and joint use to establish functional connections, and 3) what happens to the severed axons proximal to the autotomy plane when the limb is autotomized?²⁸

Crustaceans conform to environmental conditions and their surrounding temperature, but it is unclear how they maintain coordination within a neural circuit as neurons alter their activity in response to temperature changes. A slow rate of change might allow the animal some time for acclimatization whereas a rapid change may not.^{29,30} Physiological changes in pH or osmolarity due to metabolism, behavior,³¹ or environmental impact may present similar challenges to neural circuits involved in proprioception. These crustacean preparations are ideal for addressing these types of problems because their function is well characterized at a single cell level.

In this protocol we have demonstrated the physiological importance of tension neurons in monitoring force generated by the opener muscle. These tension receptors can be traced to their location within the apodeme by using staining procedures. These neurons, as in mammals, detect force at various levels and recruit additional neurons as the force increases. The frequency in activity is related to the stimulation frequency of the motor neuron until saturation in reception is reached. Using a quick release protocol with the flexed dactylus joint, tension activity quickly disappears but then returns upon regaining tension in a fully extended joint. This is a classic experimental procedure to illustrate the force measured by tension receptors. Various neuromodulators can be applied to the preparation to see how it effects the development of force and neuronal response. One of the important aspects is how the neural responses are processed and integrated in the central nervous system and their impact on activity of motor neurons. The techniques we have shown allow one to start to address more information about the tension (sensory) nerve-motor neuron circuit function, i.e., signal in an intact leg to the ganglion and back to the muscle.

The staining procedures demonstrated are key to understanding the physiology of sensory neurons that innervate proprioceptive organs. Anatomical arrangement of the neurons based on function and the size of the soma are similar in the various chordotonal organs within the crab legs. It is not known if similar neuronal arrangements are also similar in other crustacean species or insects. Combining physiological recordings from single cells and mapping the location allows direct structure function relationships. The long term preservation of the anatomical arrangement with CoCl_2 staining and fixation allows one to repetitively make measures and assess the structural arrangement.

Proprioception and tension reception of skeletal muscles are sensory modalities that enable coordinated behaviors and responses to external and internal environment for articulated animals in a variety of skeletal muscle configurations. The muscle receptor organ in the abdomen of the crayfish is another well-documented preparation (see the Crawdad Project; <http://www.crawdad.cornell.edu/>) for teaching purposes of proprioception with only two neurons per abdominal hemi-segment.²³ Being able to record from single neurons to sensory nerve bundles provides further details that aid in understanding the basic principles of sensory reception. These relatively simple crustacean preparations allow one to address fundamental aspects of proprioception and tension monitoring, with the potential to dissect the neural circuits that enable central integration of proprioceptive and other sensory inputs.^{9-12, 32, 33}

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DISCLOSURES

The authors declare that they have no competing financial interests.

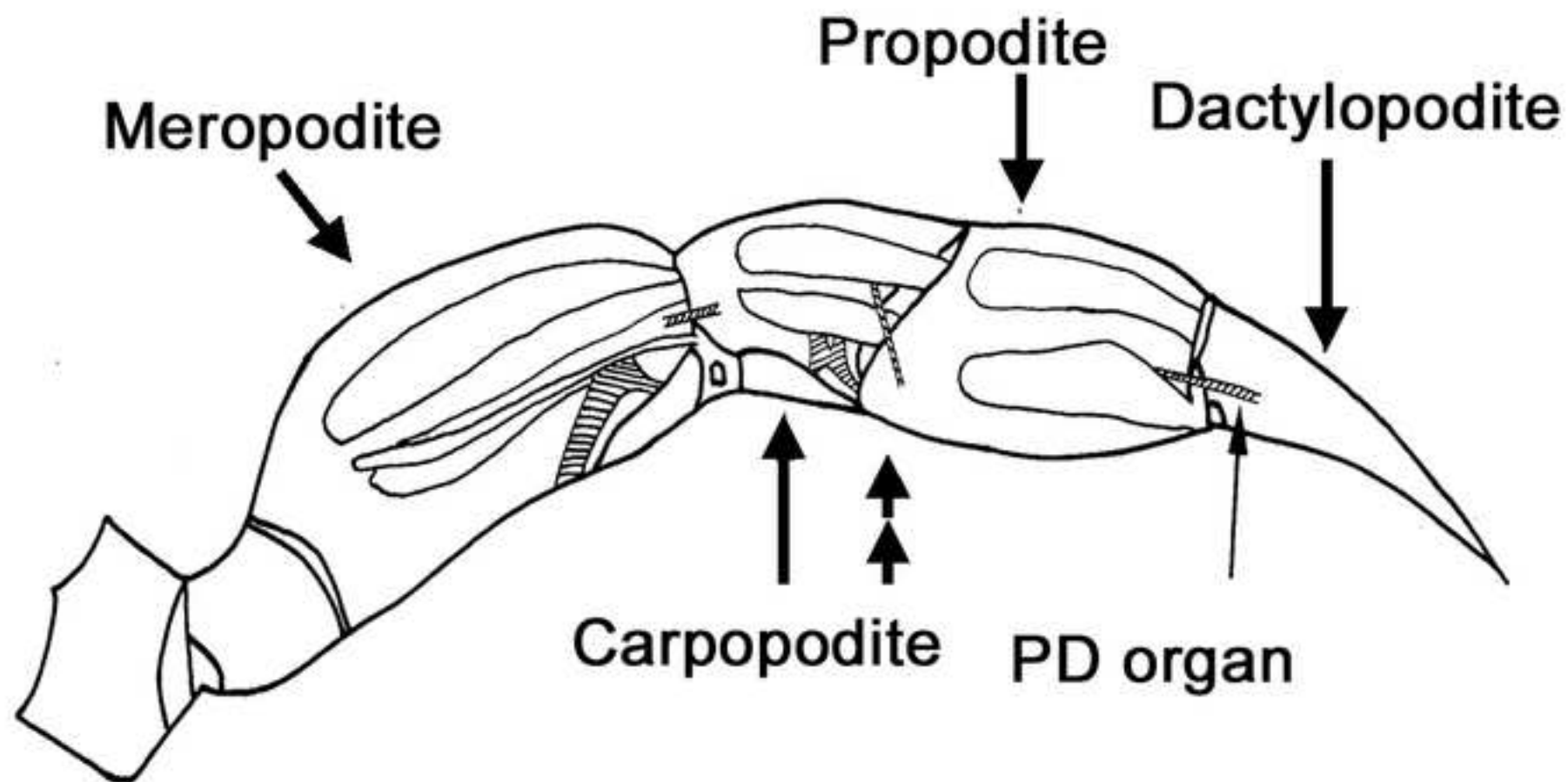
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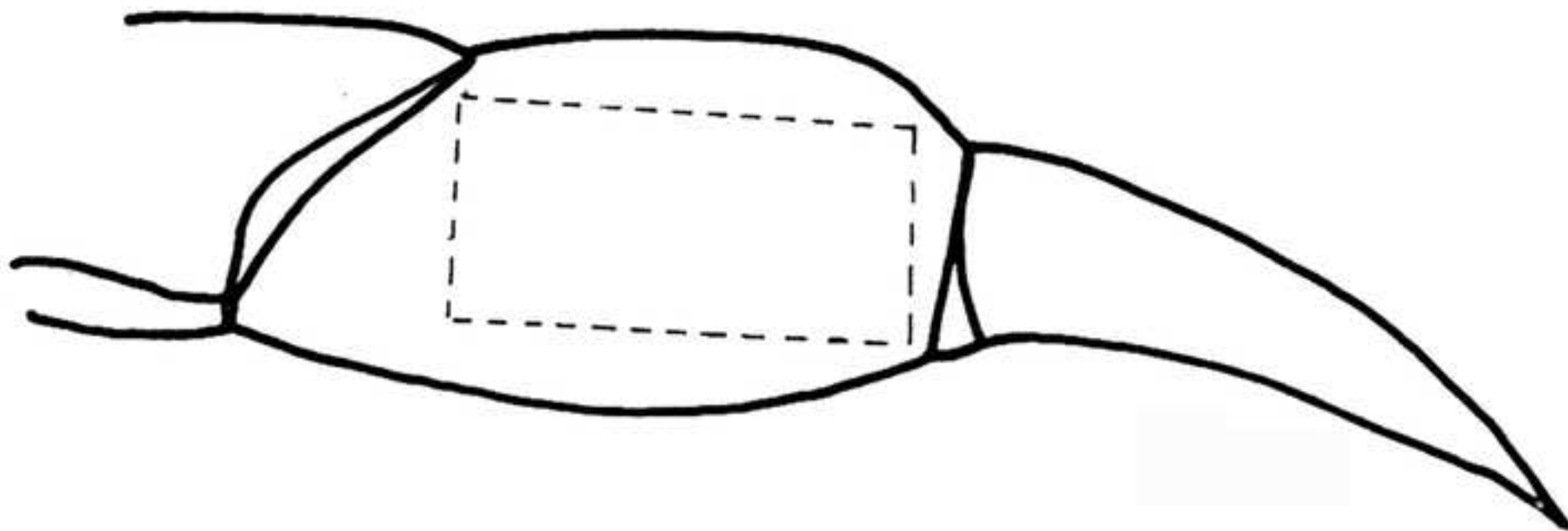
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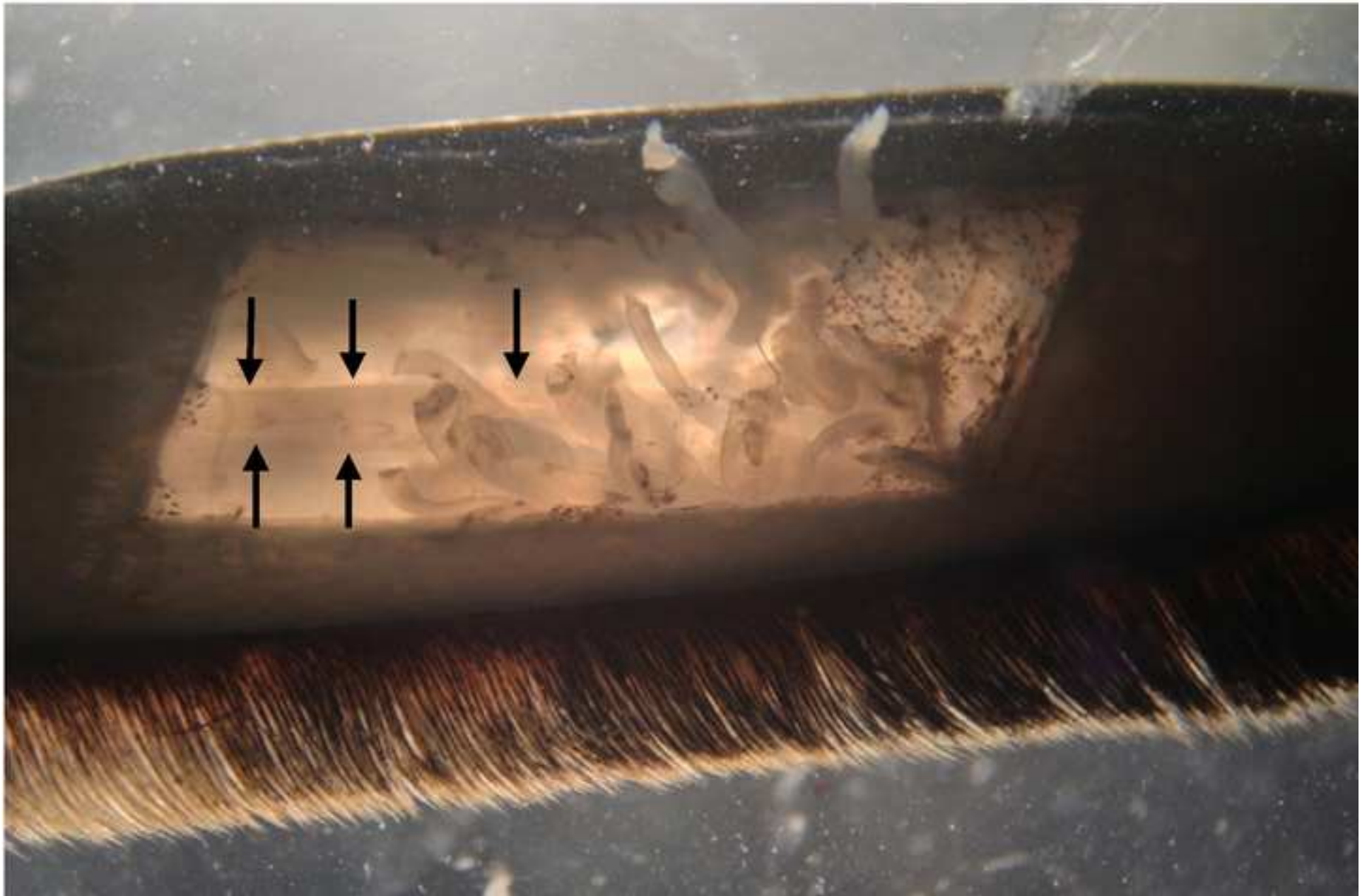
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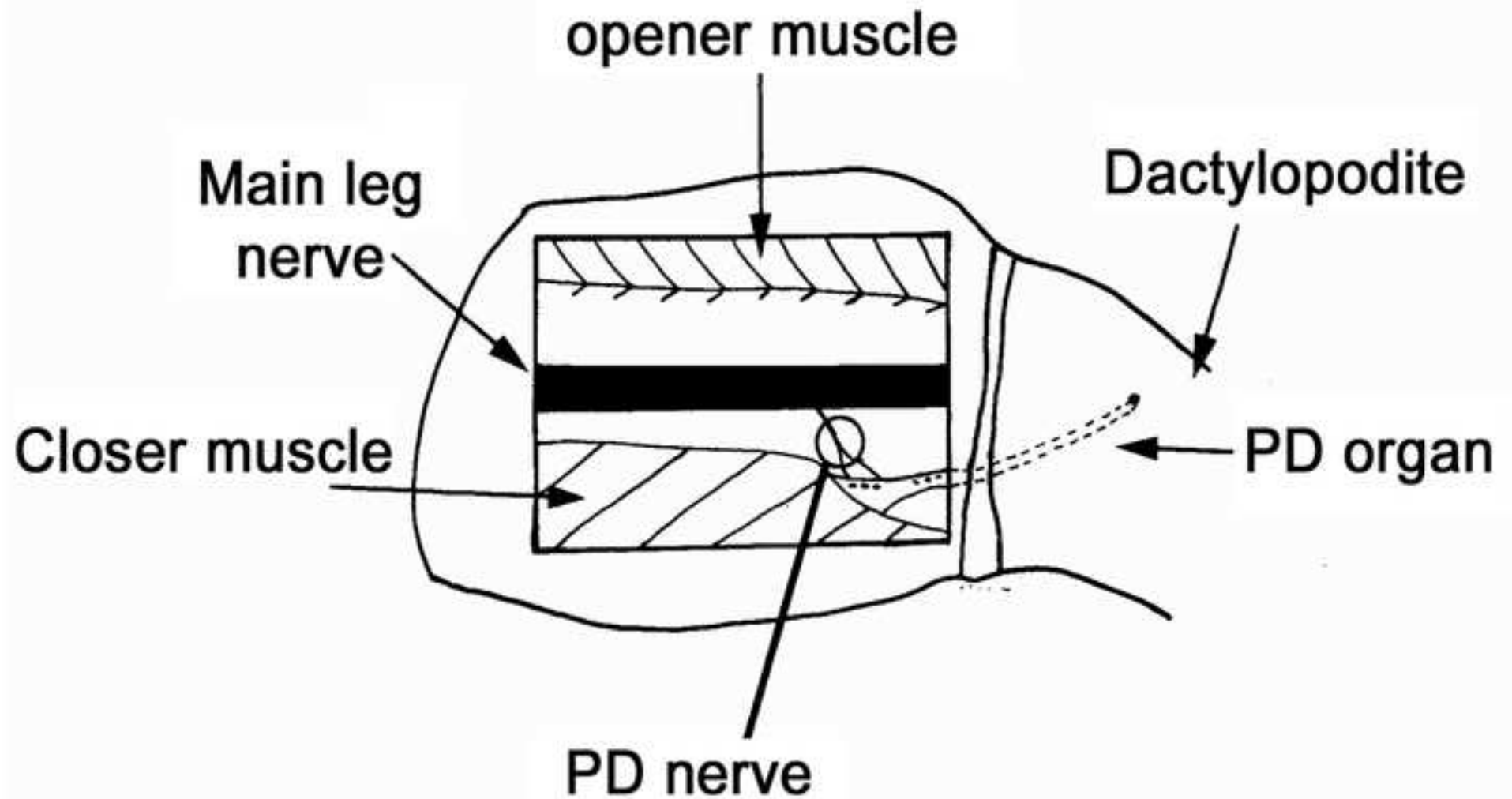
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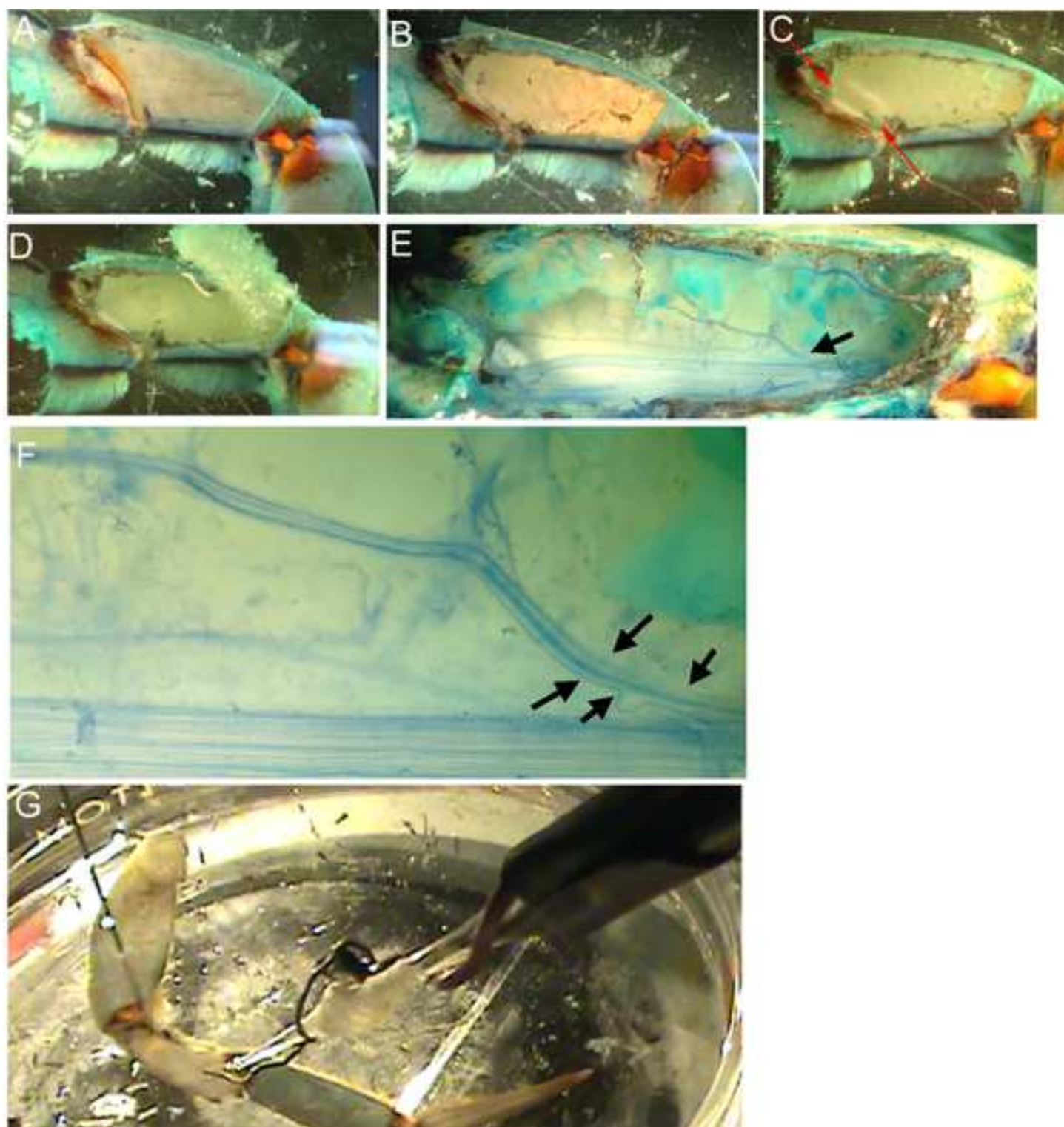
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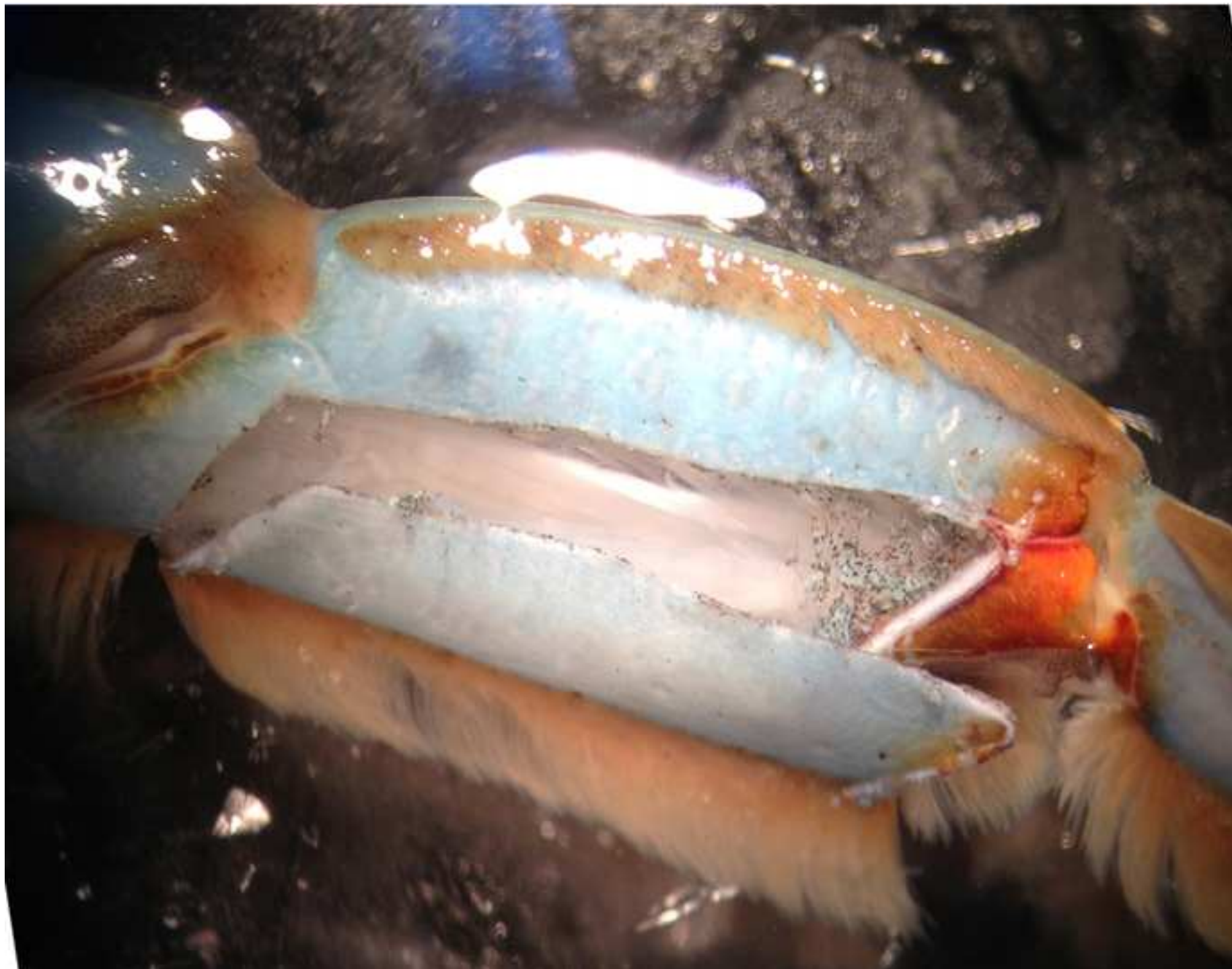
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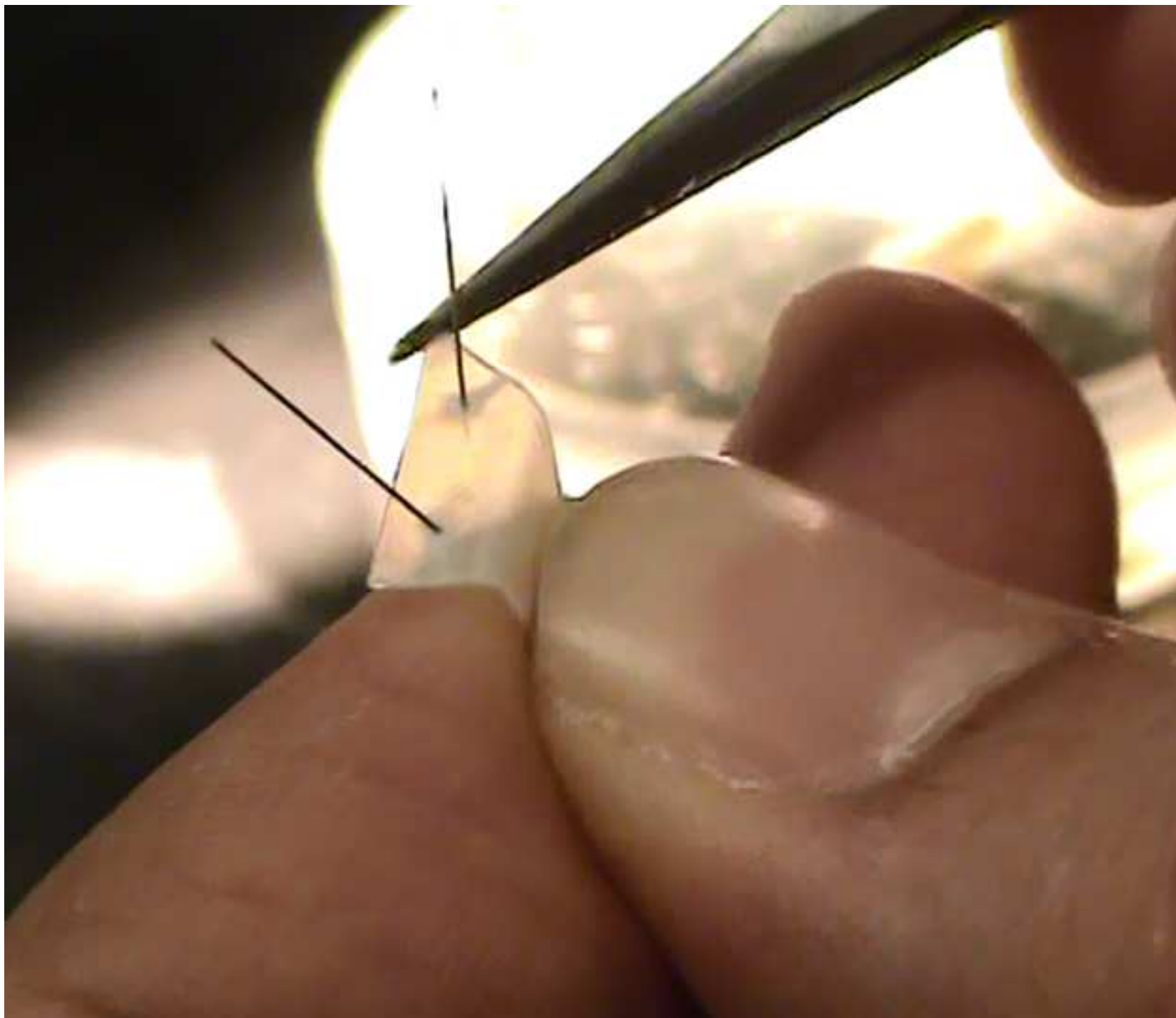
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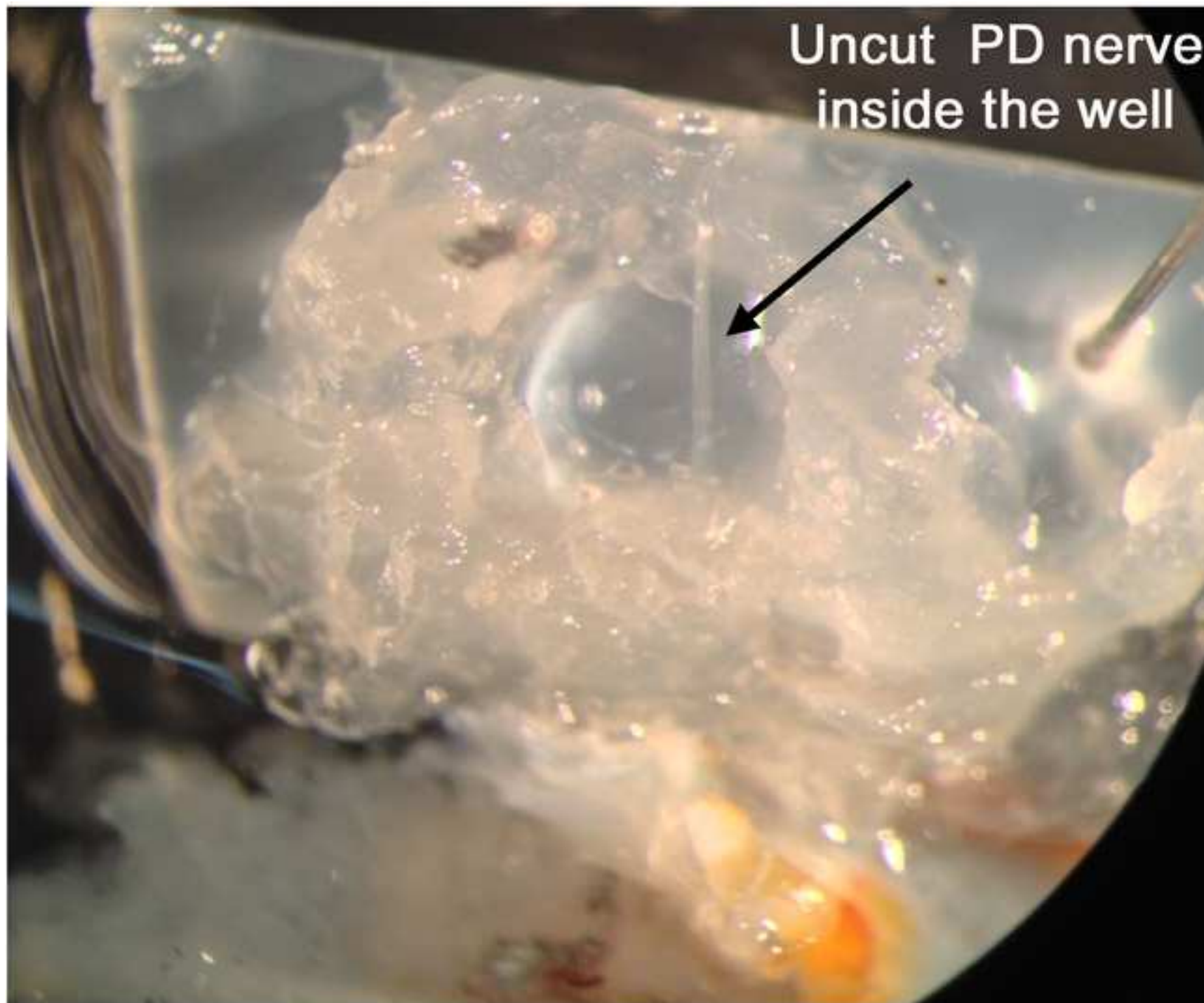
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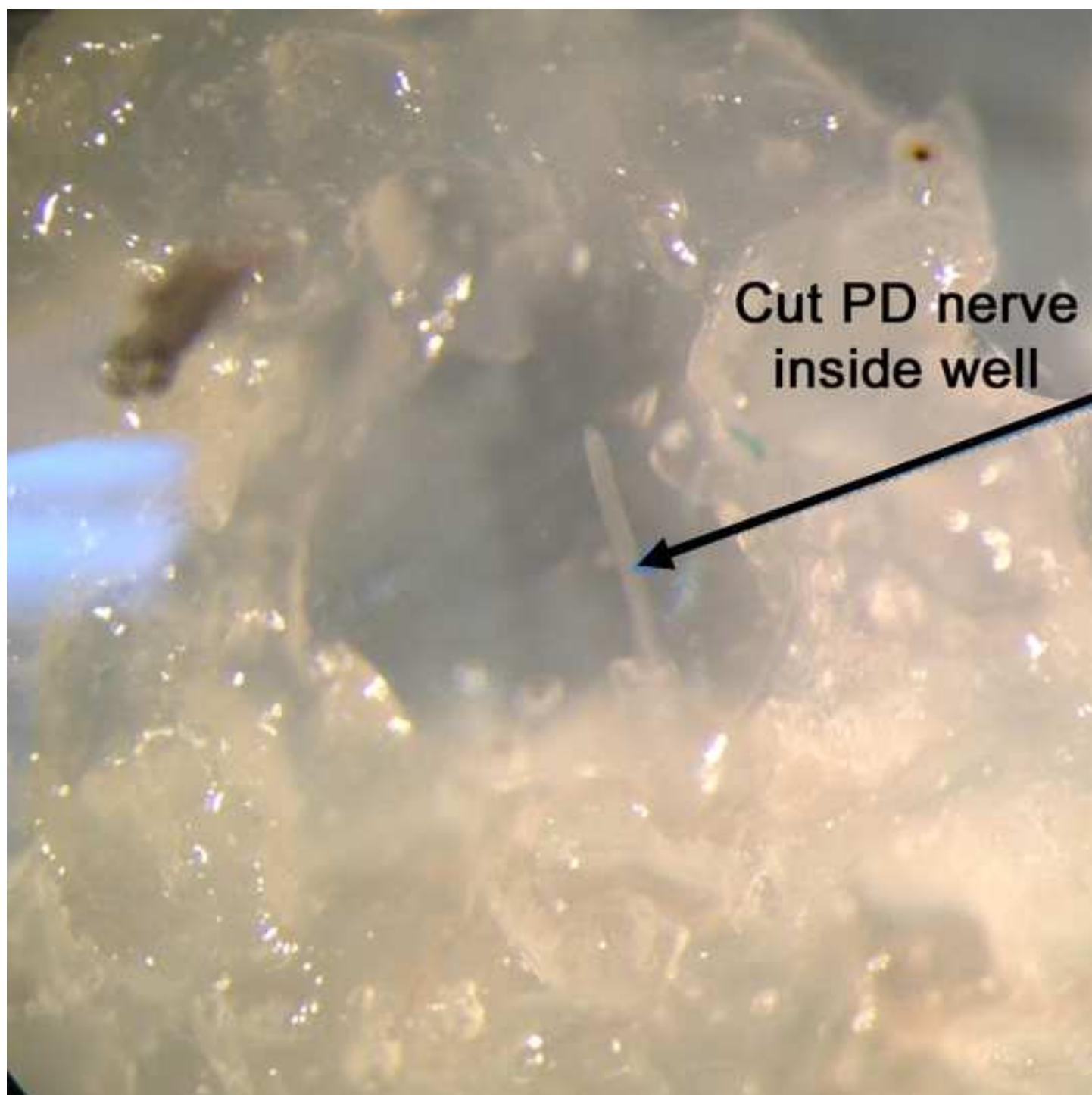
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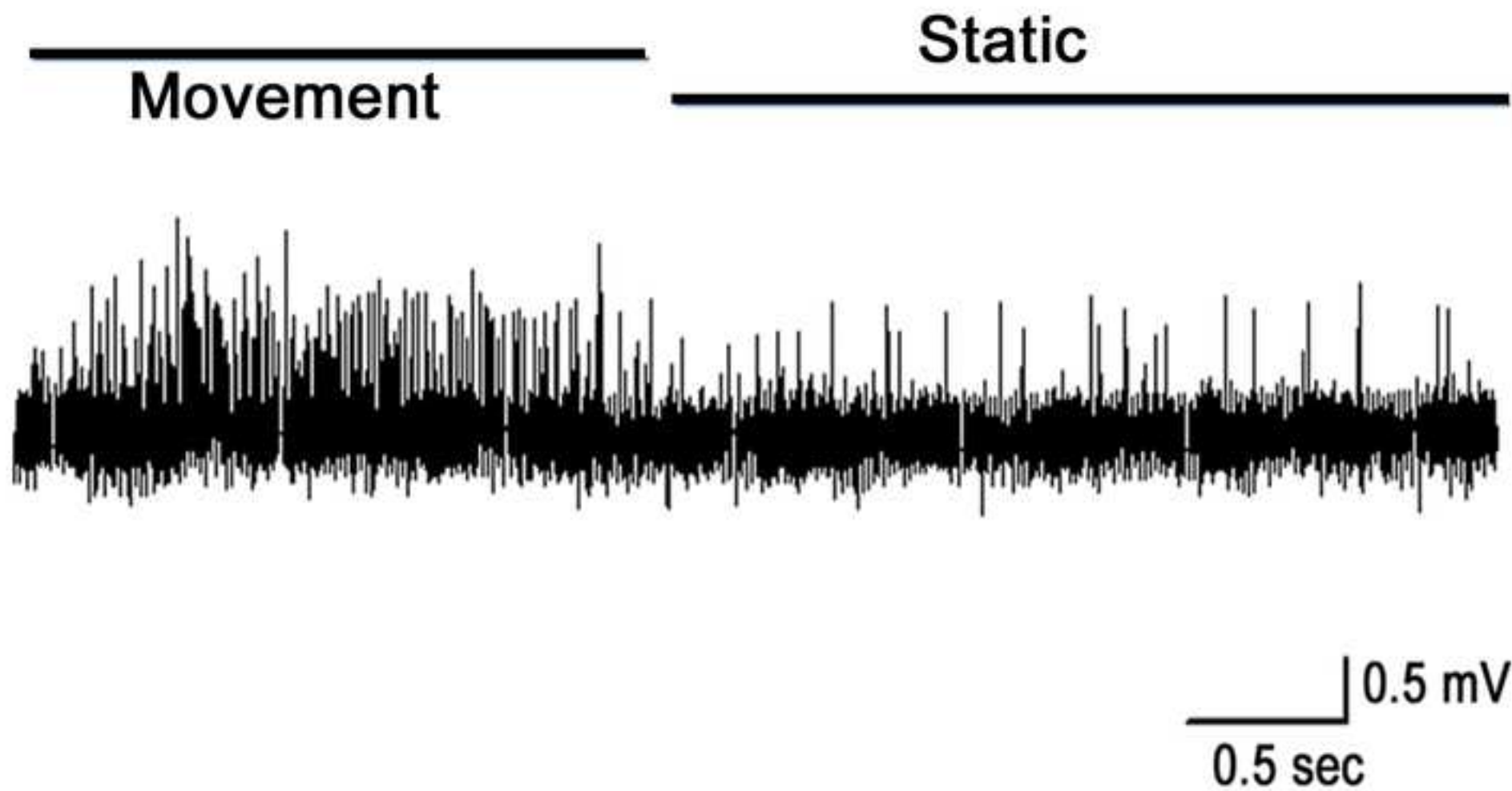


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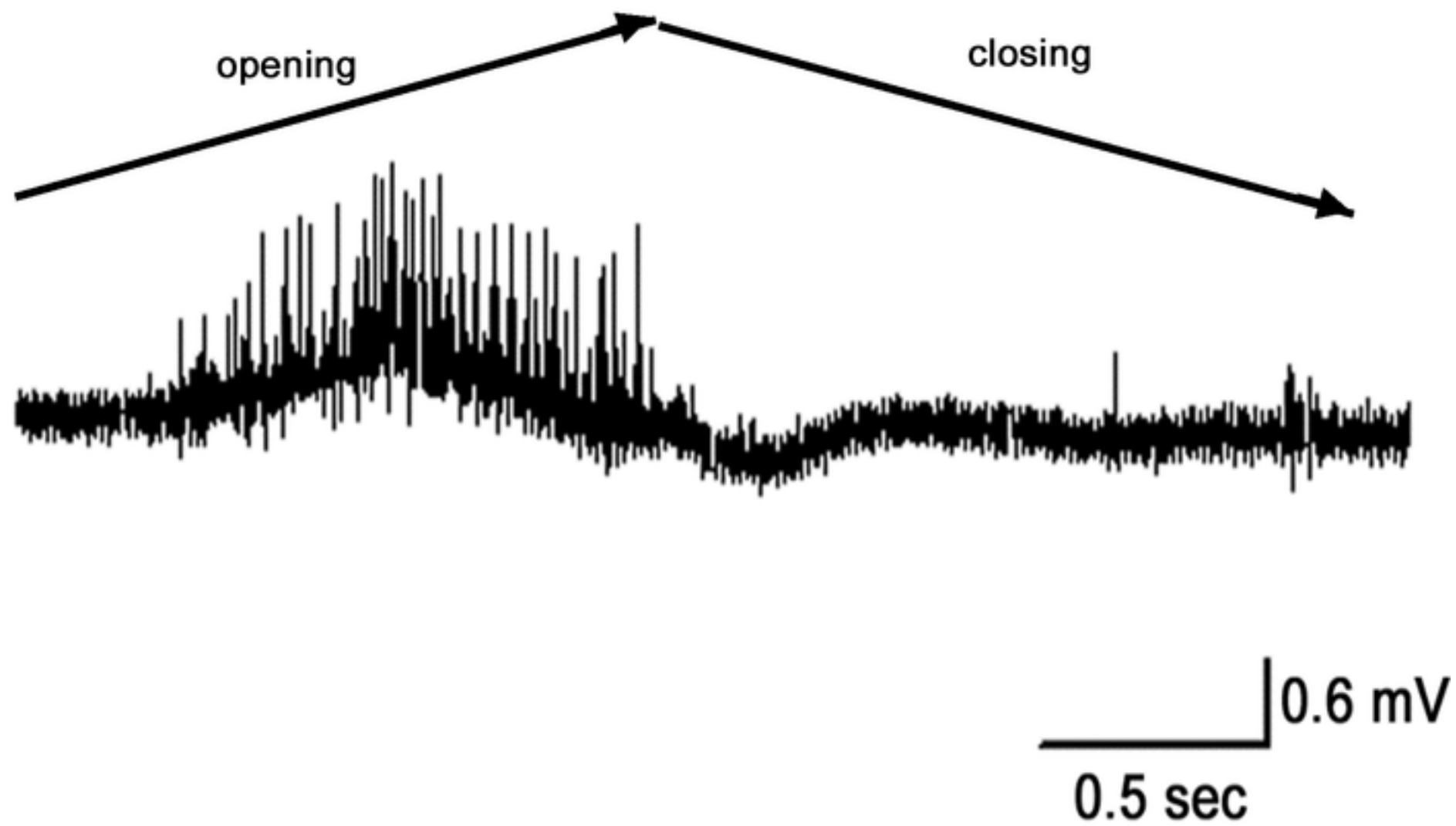


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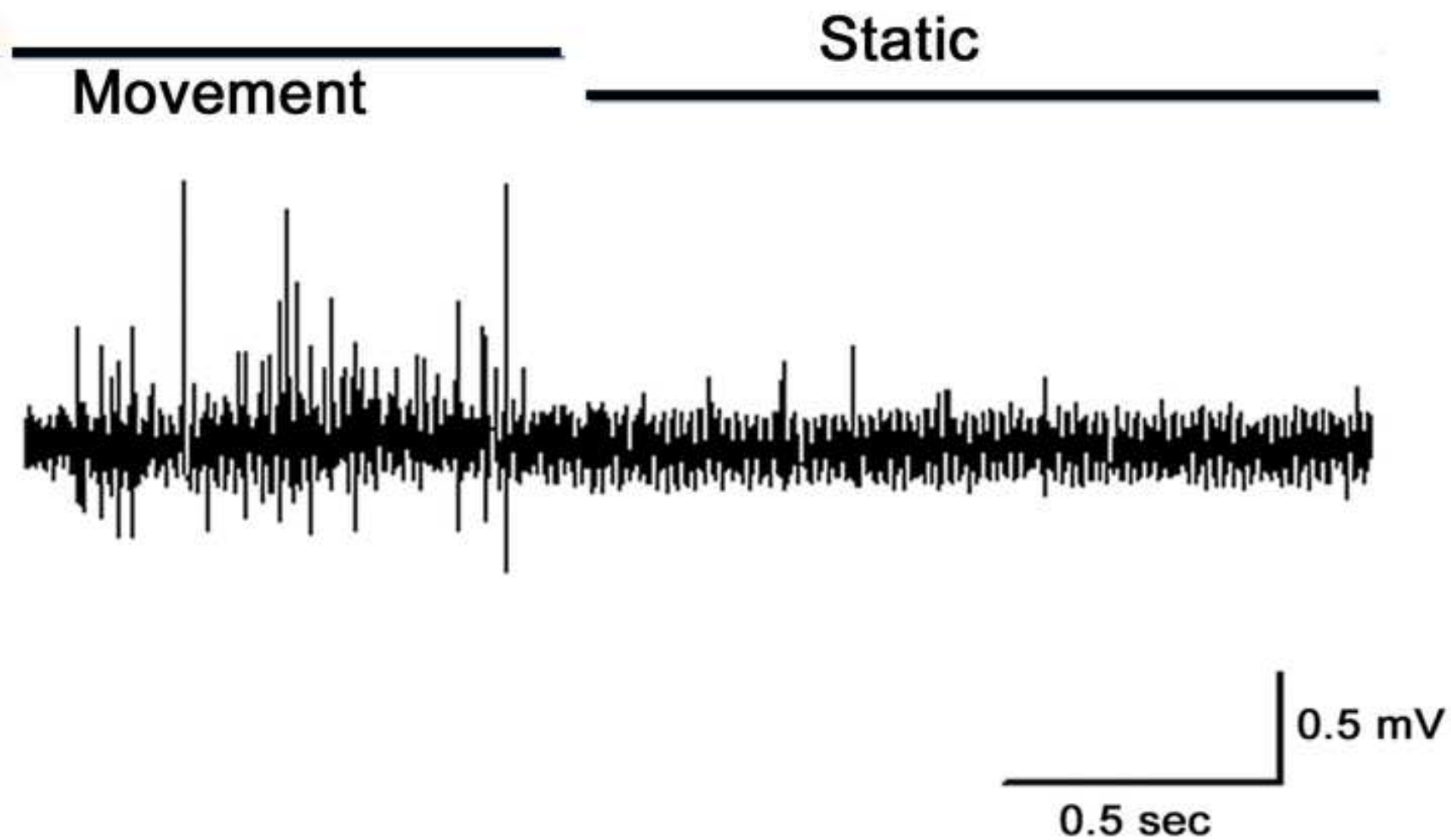


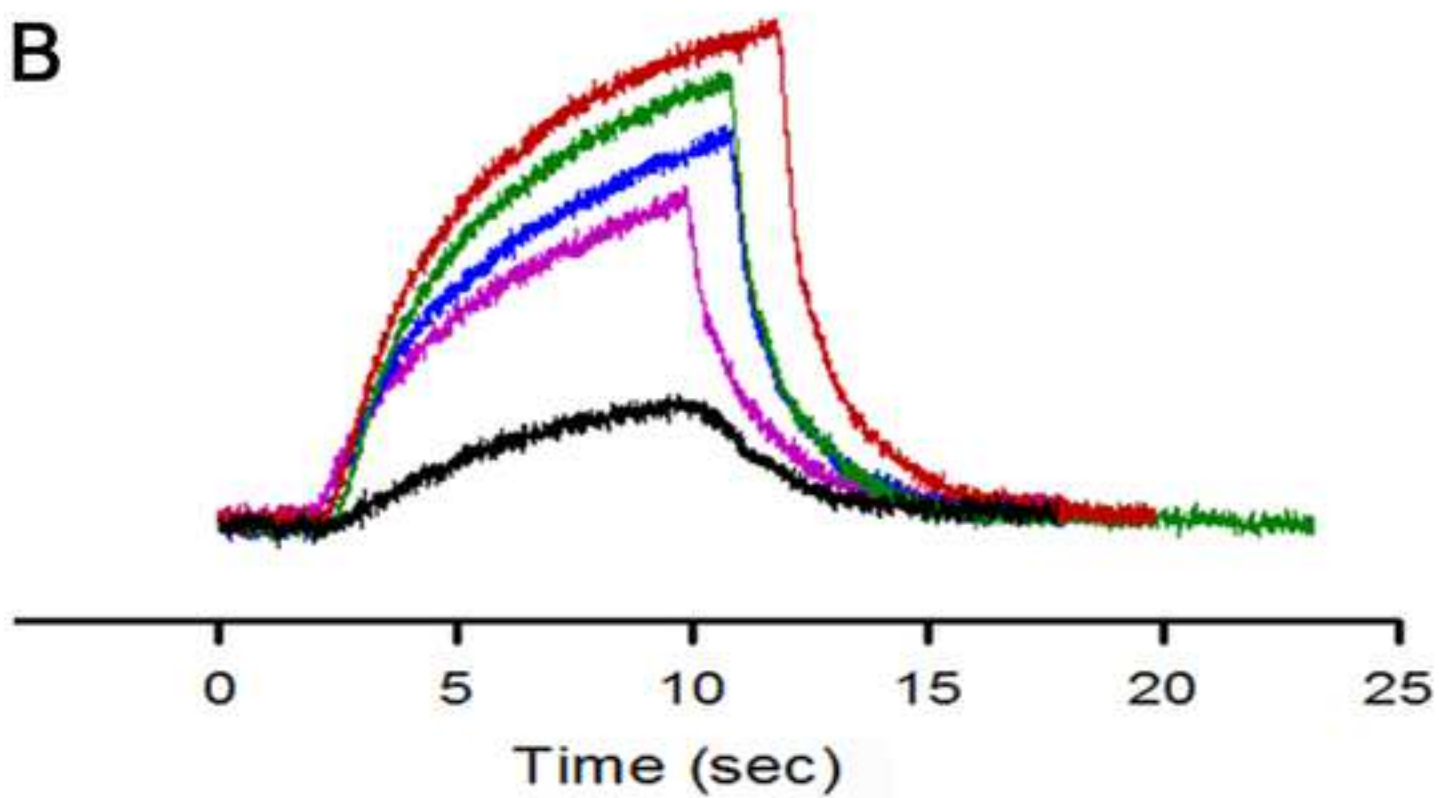
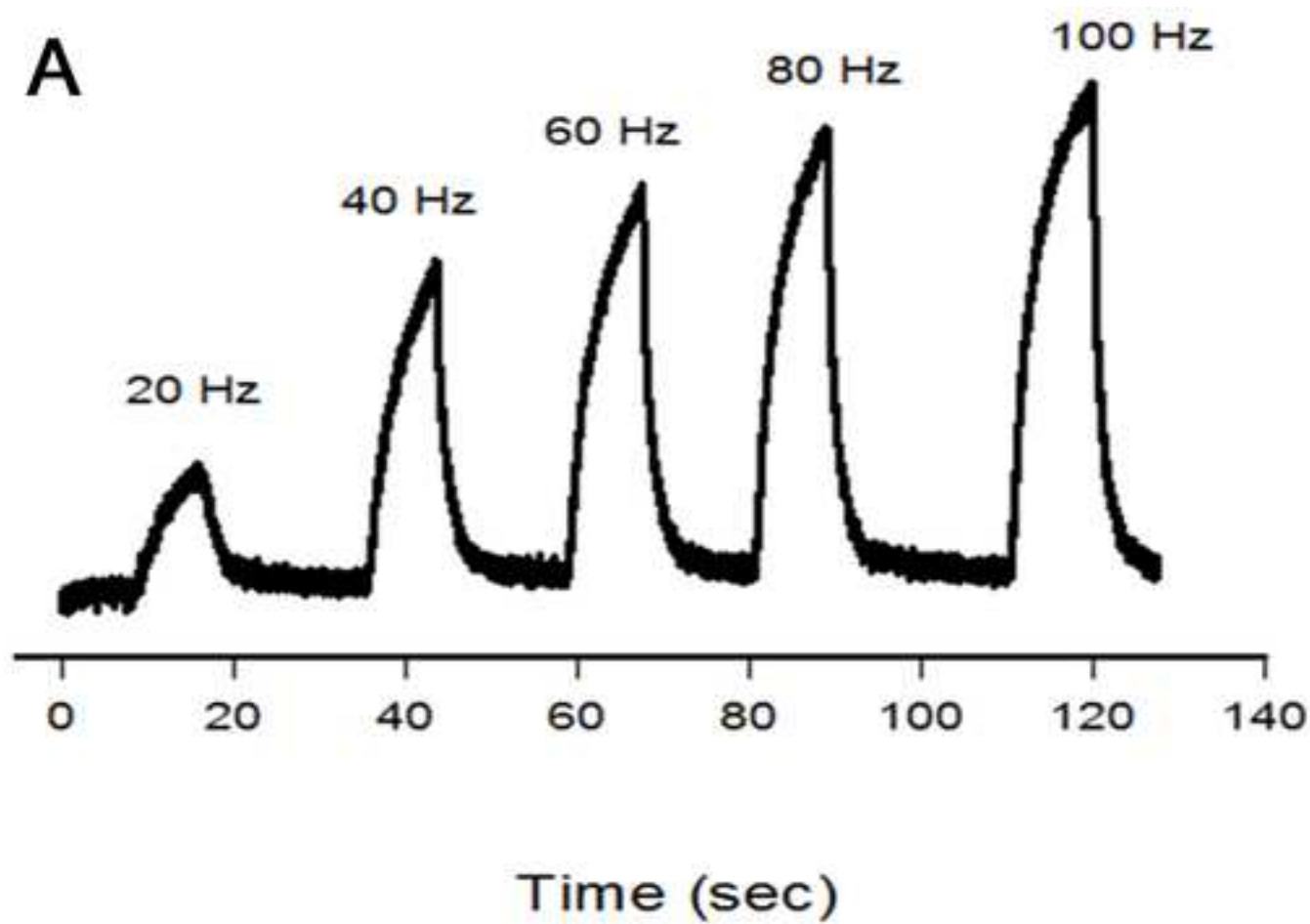


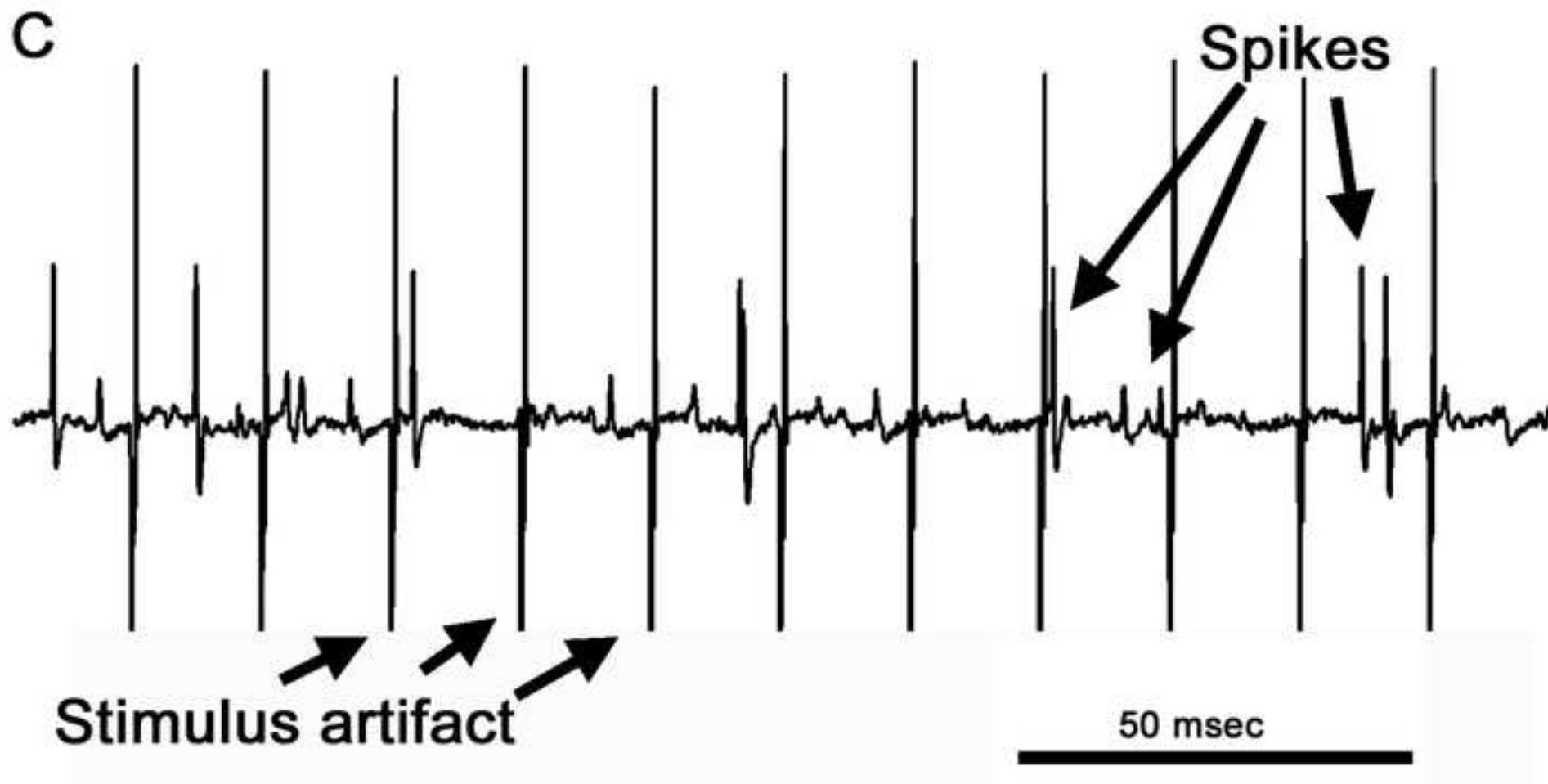
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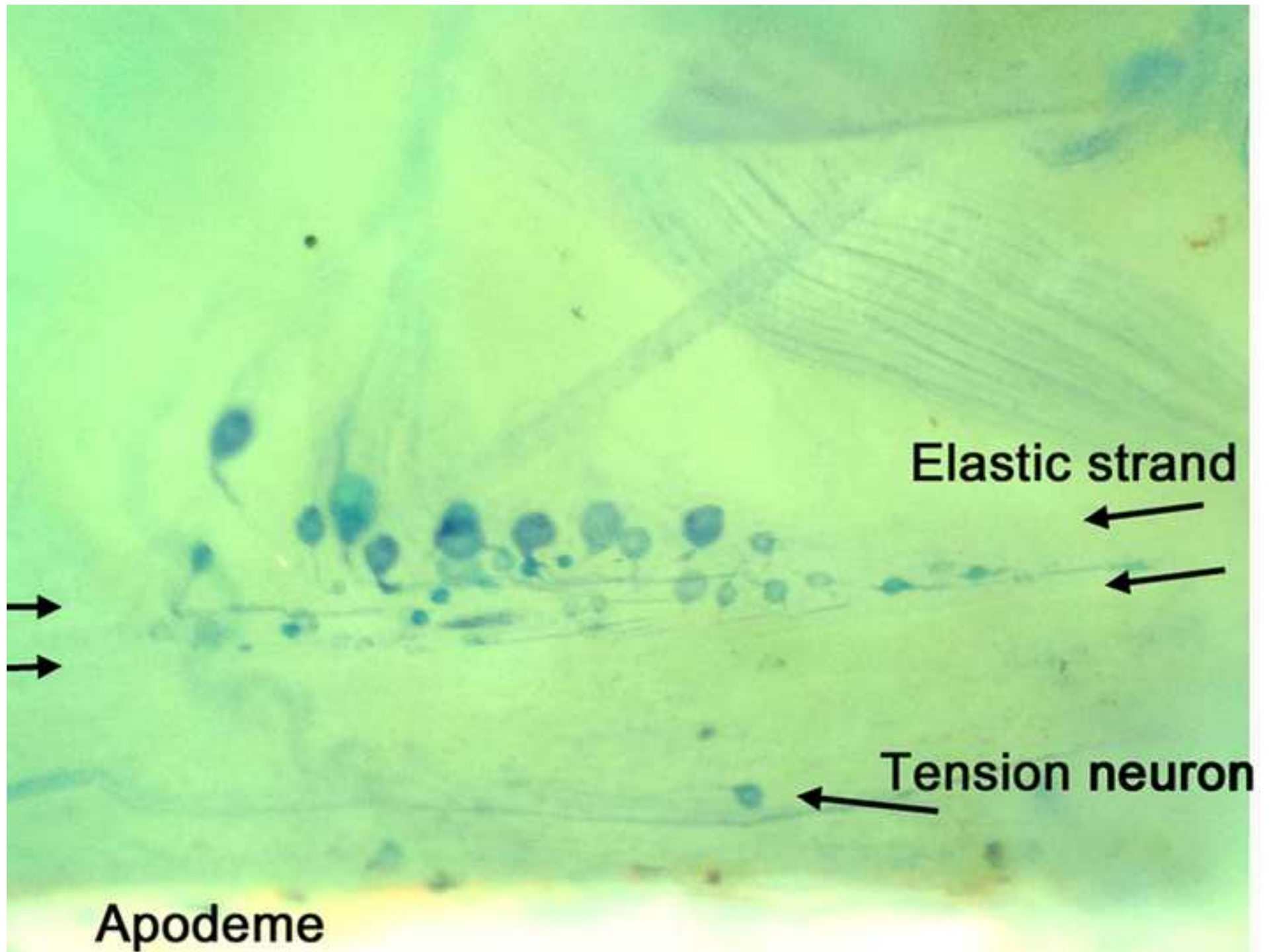
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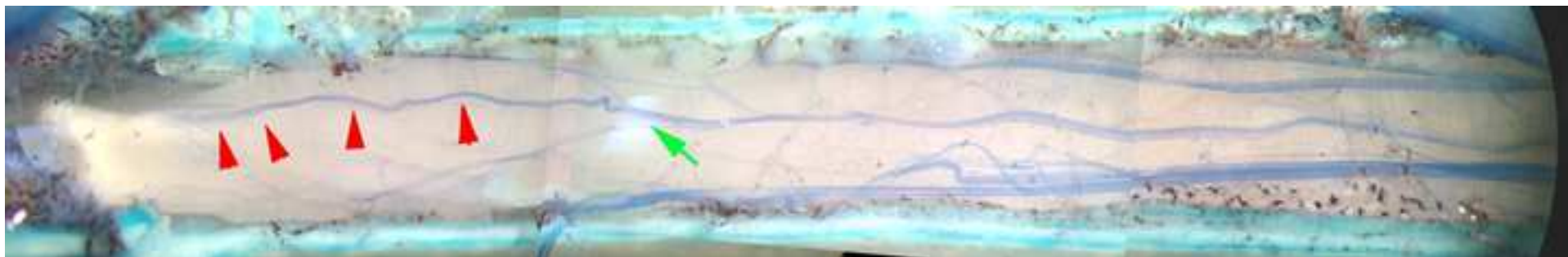


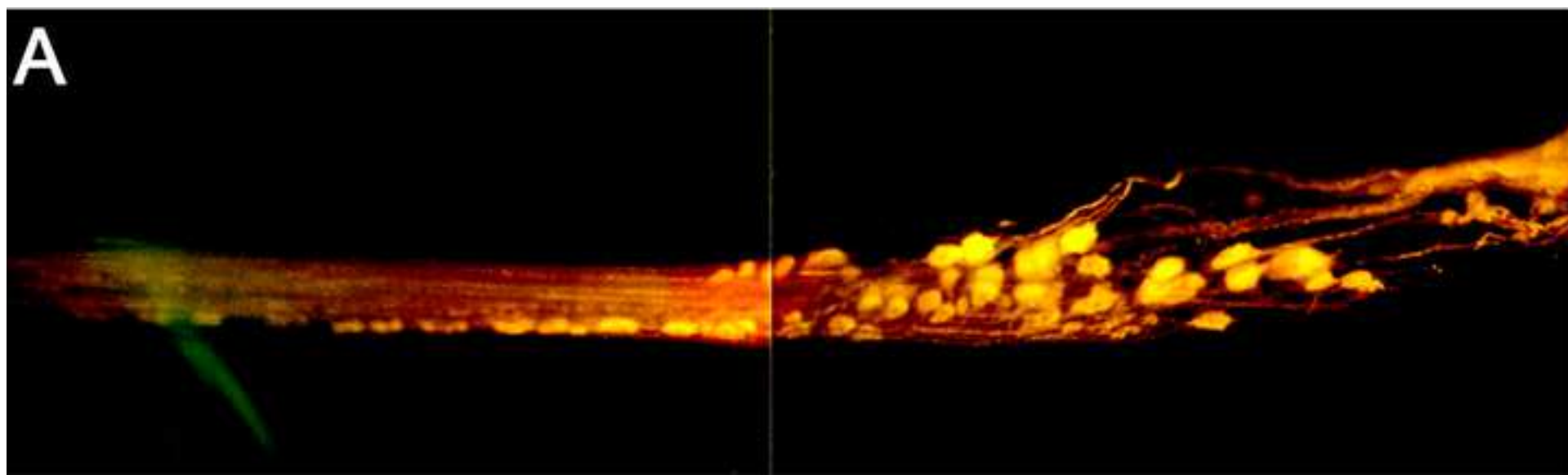


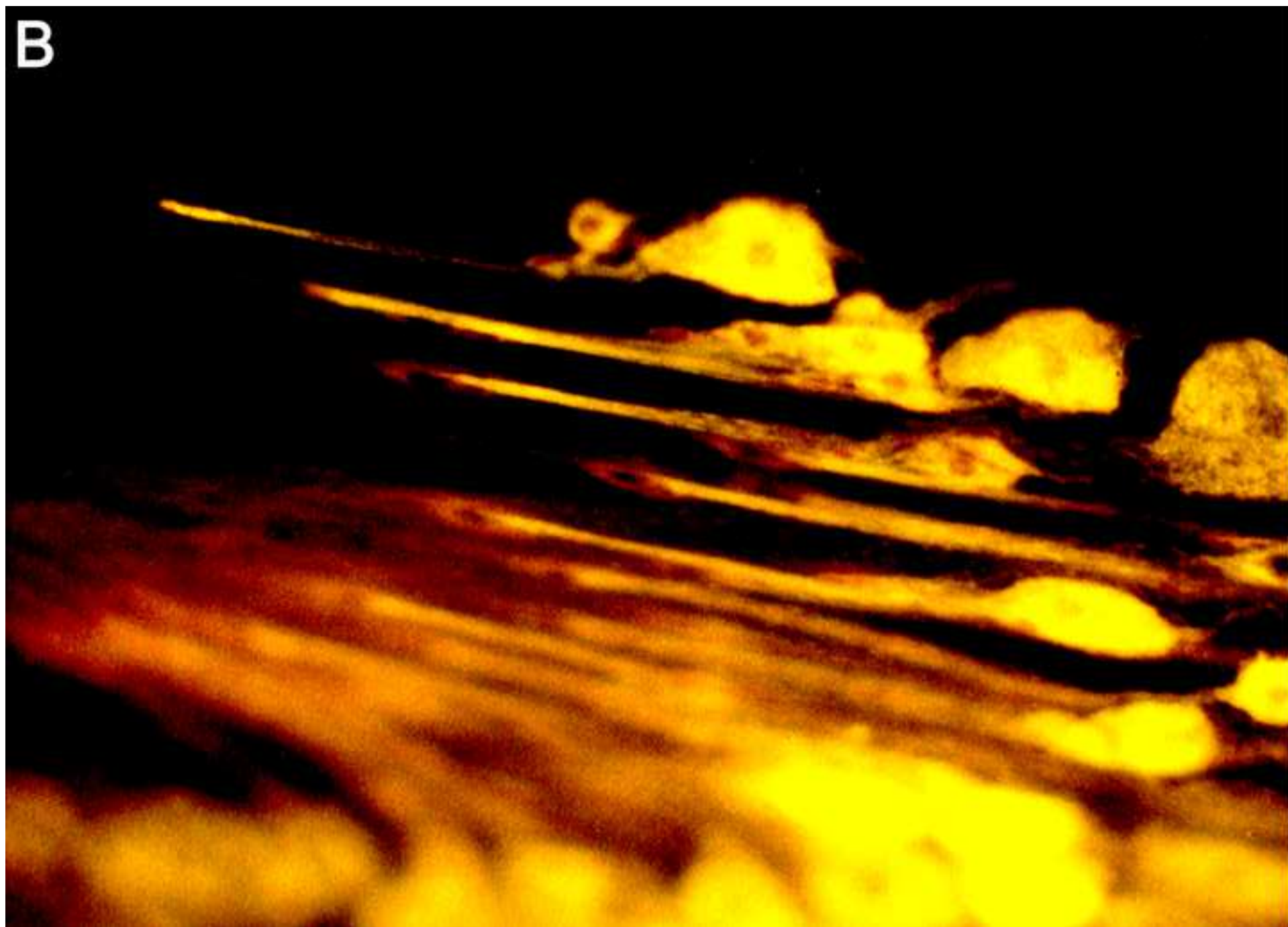
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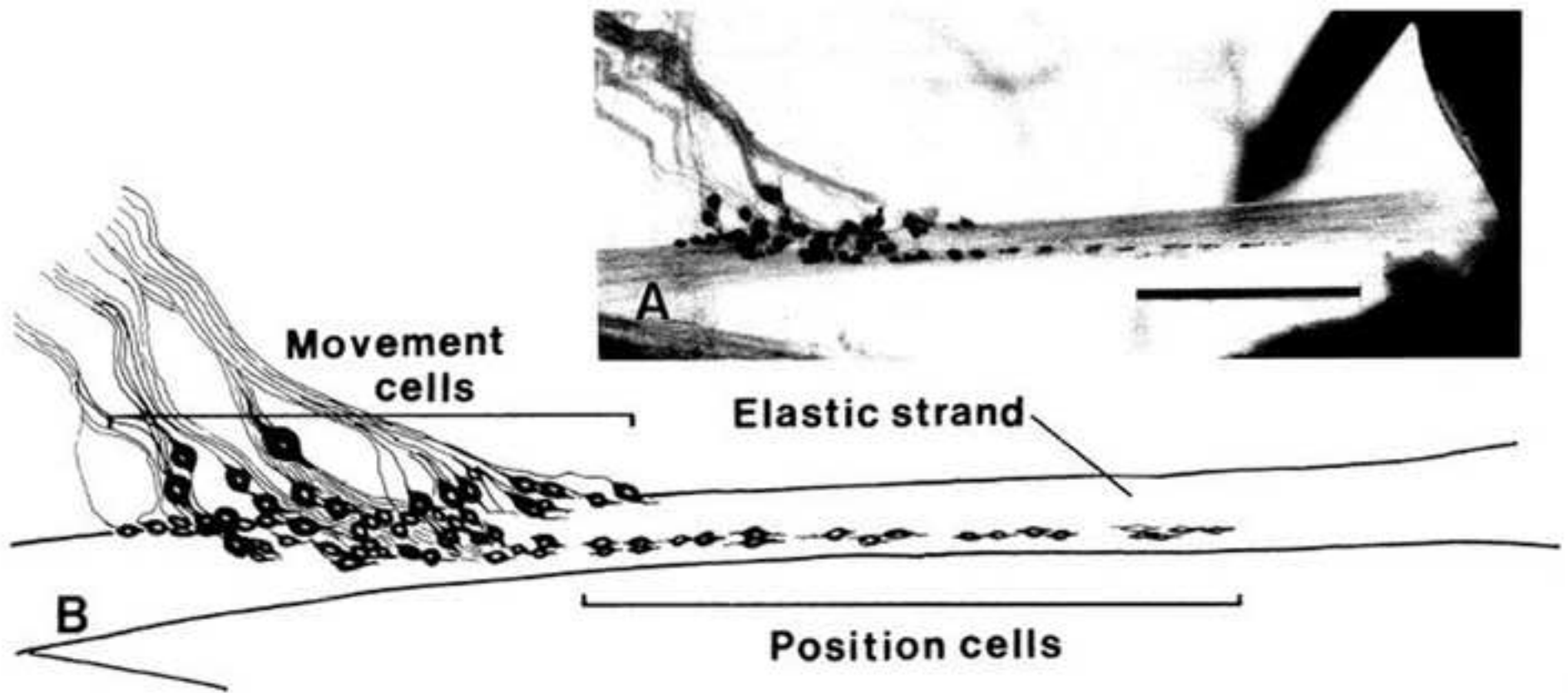
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Saline for *C. sapidus* ²⁴

Saline	Grams/liter
NaCl	27.47
KCl	0.59
MgCl ₂ 6H ₂ O	1.421
CaCl ₂ 2H ₂ O	2.205
Dextrose (D-Glucose)	1.982
HEPES acid	1.19
HEPES salt	1.3
Adjust to pH 7.5 with NaOH or HCl	

Saline for *C. magister* ^{3,19}

Saline	Grams/liter
NaCl	27.29
KCl	0.81
MgSO ₄ 7H ₂ O	4.81
CaCl ₂ 2H ₂ O	1.85
Na ₂ SO ₄ 10H ₂ O	0.97
Dextrose (D-Glucose)	1.982
HEPES acid	0.476
HEPES salt	2.08
Adjust to pH 8.1 with NaOH or HCl	

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Chemicals			
Sylgard	Dow Corning	182 silicone kit	
NaCl	Sigma -Aldrich	S7653	
KCl	Sigma -Aldrich	P9333	
CaCl2	Sigma -Aldrich	C5670	
HEPES acid	Sigma	3375	Free acid, crystalline
HEPES base	Sigma		
D-Glucose	Sigma	G7021	
MgSO ₄ 7H ₂ O	Sigma	M2643	
Na ₂ SO ₄ 10H ₂ O	Sigma	246980	
Bouin’s solution fixative	Sigma	HT10-1-32	Caution: Hazardous material (Special shipping cost required) Caution: Hazardous material. Please follow proper disposal according to local and federal regulations.
CoCl ₂	Sigma		
Methylene blue chloride	Matheson Co., Inc	Basic Blue 9, C.I. 52015	
4-Di-2-ASP	Molecular Probes, Eugene, OR		4-(4-diethylaminostyryl)-N-methylpyridinium iodide
Bleach	Sigma -Aldrich		To chloride silver wire
NaOH	Sigma -Aldrich	221465	To adjust pH
HCl	Sigma -Aldrich	H1758	To adjust pH
Materials			
Dissecting tools	World Precision Instruments	assortment	

Intracellular electrode probe			
Faraday cage			
Insect Pins	Fine Science Tools, Inc	26001-60	
Dissecting microscope (100x)			
Fiber optic lamp			
Small adjustable mirror			To direct light beam toward the preparation.
Glass electrodes	Sigma -Aldrich	CLS7095B5X	Box of 200, Suction electrodes
Micromanipulator	World Precision Instruments	MD4-M3-R	Can fix for base or on a metal rod
Raised preparation stand			
Silver wire (10/1000")	A-M Systems	782500	
Computer	Any company		
AC/DC differential amplifier	A-M Systems	Model 3000	
PowerLab 26T	AD Instruments	27T	
Force transducer	AD Instruments	0-50g	MLTF050/ST
Head stage	AD Instruments		Comes with AC/DC amplifier
LabChart7	AD Instruments		
Electrical leads	Any company		
Glass tools	Make your self		For manipulating nerves
Cable and connectors	Any company		
Pipettes with bulbs	Fisher Scientific	13-711-7	Box of 500
Beakers	Any company		
Wax or modeling clay	Any company or local stores		
Stimulator	Grass Instruments	SD9 or S88	
Plastic tip for suction electrode	Local hardware store (Watt's brand)	¼ " OD x 0.170" ID	Cut in small pieces. Pull out over a flame and Cut back the tip to the correct size

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Author(s):

Zana M. Ajeed, Josh Tilton, H. Bernard Hartman, Robin L. Cooper *laboratory exercises*

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Dear Dr. Cooper,

Your manuscript JoVE51050 'Proprioception and tension receptors in crab limbs: Student laboratory exercises.' has been editorially reviewed and the following comments need to be addressed.

Please use the "track-changes" function in Microsoft Word or change the text color to identify all of your manuscript edits. When you have revised your submission, please also upload a list of changes, where you respond to each of the comments individually, in a separate document at the same time as you submit your revised manuscript.

Editorial comments:

* Protocol text:

1) Please simplify steps so that individual steps contain only 1-2 actions per step. We recommend that you split your longer steps into sub-steps numbered accordingly, i.e. step 1 is followed by sub-step 1.1 then 1.1.1 as necessary (an example step is indicated in green font, see attached document).

The sets are simplified and divided into sub-sections. Now there are one or two actions in each step.

2) Please skip a line between each step in your protocol (for example, section 1.2)

A line is skipped between the steps of the protocol.

3) Please re-write your protocol section in imperative tense, as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) (see underlined text and sample step 2.1.1)

The protocol is written in imperative tense.

4) Please highlight less than 3 pages of text to identify which portions of the protocol are most important to film; i.e. which steps should be visualized to best supplement the written section of the protocol. Please see JoVEs instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

The important steps in the protocol that should appear in movie are highlighted.

5) Please revise the protocol text to avoid the use of any pronouns (i.e. "we", "you", "our" etc.). If you feel it is very important to give a personal example, you may use the royal "we" sparingly and only as a "NOTE:" after the relevant protocol step.

We have tried to remove these pronoun as possible as we can.

*Figures embedded in the text is fine for review. However, please also upload each figure file separately.

OK

*Representative results need to be in its own section and not included in the protocol text.

The representative results are in their own section now.

*Figure numbering (Figure 11 is used twice).

This figure (9) is different than the other figure (8). First figure (8) shows PD nerve inside the well before it has been cut. But figure (9) shows PD nerve inside the well after it has been cut.

*Please place figure legend between representative results and discussion.

The legends are in figure legend section.

*Please review document formatting. Numerical headings are only used for protocol text.

It is formatted now.

*Please reformat Reference section.

The references are reformatted.

*Please see the attached version of your manuscript. We have made a few minor modifications and notes to the author.

Your revision is due by **Apr 15, 2013**.

To submit a revision, go to the [JoVE submission site](#) and log in as an author. You will see a menu item called 'Submission Needing Revision'. You will find your submission record there.

Sincerely,

Susan Rasakham, Ph.D.

Science Editor

[Journal of Visualized Experiments](#)

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Response to review

Editorial comments:

*Protocol text:

- 1) The protocol has about 3.25 pages highlighted, and much of the highlighting is not in complete sentences (for instance, 3.4.4 has only "Bouin's solution fixative" highlighted). Please review the highlighted sections of your protocol and ensure complete action statements are highlighted.

****OK these sections have been corrected. I thought we just needed headings for the sections.**

For instance, please review section 3.4.2 of your protocol and clarify if all steps will be videotaped. The number of steps that are most important to film should be less than 3 pages of text (~2.75 pages); i.e. which steps should be visualized to best supplement the written section of the protocol. The shorter length requirement is to allow our scriptwriter 0.25 pages to make transitions and references to the omitted text. Please see JoVEs instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

****OK we have limited the highlighted portion of the protocol to 2.75 pages.**

- 2) As mentioned previously, please remove embedded figures and upload each figure file separately. To denote figure placement throughout the manuscript use brackets [Place figure 1 here].

****OK**

- 3) The manuscript will benefit from copyediting, as there are a number of errors throughout. Please thoroughly review the manuscript and edit any errors that you may find.

****OK . The abbreviations of PD and others will be corrected. The fragmented sentences were due to our misunderstanding of listing topics/sections and not statements for the step wise fashion of the methodology. These steps were also made by the JoVE editor in breaking the parts to steps. We will double check for typos etc... Sorry about the oversight.**

Reviewers' comments:

Reviewer #1:

Concerns:

1. Introduction: The significance of proprioception and the type of information processed by these neurons should be addressed in more depth rather than simply saying that an animal needs to know where its limbs are and that monitoring force is important. The relationship between chordotonal organs and their individual sensory neurons could also be addressed in more detail (roughly how many, why the need for multiple, etc.).

Response: We agree with the reviewer. We just tried keeping the text as short as possible. The following text has been added to address this point:

"...these receptors convey information about joint position, direction, speed, muscle tension and muscle-length. The crab leg has six joints and each joint has one or two proprioceptive chordotonal organs (Alexandrowicz, 1972) . Typically a chordotonal organ has 60-100 or more sensory neurons embedded within an elastic strand to monitor movement or changes in the strand. This information allows the animal to receive a fine degree of sensory information related to each joint. In addition, the tension receptors provide information on the force being generated to hold various joint positions or to move a joint. The various threshold of activation for the sensory receptors provide a range fractionation of movements. The input is then centrally processed for all the joints for the animal."

2. Results: How will the data be analyzed and quantified? For example, the data shown in Fig. 10 - spike counting could determine (significant) differences between movement and static. In Fig. 12 - spike sorting would allow to identify individual neurons, etc.

Why is this not part of the experiment/protocol?

Response: Good points. We do have students do just this type of analysis. For the JoVE report we just referenced to other studies that have conducted such analysis.

4.2) Further analysis of the spikes can be readily approached by sorting the relative amplitudes. This is an approach to demonstrate different populations of sensory neurons being recruited for positions or types of movements (Cooper, 2008). In sorting of amplitudes a frequency of occurrences in the various size ranges can also be graphically represented for analysis.

3. Discussion: Reads like an introduction. Maybe adding a sentence or two how these measurements provide insights that generalize beyond the arthropods? Proprioceptors play a major role in all organisms (as briefly mentioned in the introduction); basic principles are common among most organisms and results obtained from crabs will help understand these basic mechanisms. Elaborate on this.

Response: Yes we agree. The reviewer might not be aware of the space limitations we were restricted to with this large multi preparation and experimental design protocol. We are delighted to expand on these points. The following text has been added to the discussion:

“Proprioception and tension reception of skeletal muscles are sensory modalities that enable coordinated behaviors and responses to external and internal environment for all animals, not just crustaceans. The capacity to record from single neurons or scale up to entire sensory nerve bundles provides a wide range of physiological inquiry to convey the basic concepts of sensory reception. These relatively simple crustacean preparations allow one to address many fundamental aspects of proprioception and tension monitoring and to potentially address central integration of inputs that are relatively inaccessible in mammalian preparations.

Procedure (general suggestions):

4. Individual sections would probably benefit from a brief summary of the general objective of each part appearing beforehand.

Response: I am not sure this would fit the JoVE format. This format is a bit different with 1.2.3 procedures. I am going to refer to the JoVE Science Editor here as I am not sure of the format for including this approach.

5. Methodological considerations relevant to both procedures are sometimes omitted from one of the procedures. They should either appear in both or be discussed beforehand.

Response: I am not sure exactly all the considerations in the procedures the reviewer is addressing. Now I think the major points are addressed for the joint receptor part and the tension receptor parts.

6. A brief discussion of mechanisms, such as how the various stains work, or the logic or importance of certain steps, would be useful.

Response: The general uses of the stains are now described.

“Methylene blue provides some contrast of tissues (muscles and nerves) and can be visualized with visible light. More selective neuronal staining with a brief exposure to 4-di-2-ASP provides a definitive outline of the neurons but requires fluorescence imaging. Filling of the neurons for CoCl_2 does not require fluorescence imaging and the targeted neurons will remain stained after fixation for long periods of time. However this staining process is more labor and time intensive, limitations that make it difficult to complete this procedure in a single instructional laboratory setting.”

7. Are there any health risks or hazards involved in the preparation or stains? If yes, they should be mentioned.

Response: I am going to refer to the JoVE Science Editor here for how much content needs to be listed here. All the chemicals are well labeled on the bottles for their potential hazards such as the CoCl_2 .

Spelling:

8. There are typos in the manuscript.

Response: Very sorry for the typo oversights. The manuscript was carefully examined in the revised version.

a) "Detailed electrophysiological studies began in the 1930's and still being carried out today" - are still being carried out today

Response: corrected

b) "Very little is known in the segmental connections of proprioceptors" - about the segmental connections

Response: corrected

c) "around the Southern and Southeastern boarder of the United States" - border not boarder

Response: corrected

Specific comments:

9. Starting with the Introduction, "PD" is never defined for the PD chordotonal organ (I am assuming it means: Propodite-Dactylopodite).

Response: corrected

10. 1.1.) Was saline added to the first preparation? When?

Response: corrected. Yes. See step 1.1.4

11. 1.1.1.) It appears that both *C. sapidus* and *C. magister* were used. It's not obvious from the text which animal was used for which protocol.

Response: Either crab species can be used. We state at the end of the Introduction

"Use of the blue crab (*C. sapidus*) in this procedure is for the reasons that they are readily accessible all around the Southern and Southeastern border of the United States. In addition this species likely serves as a representative of the chordotonal and tension nerve arrangements found in most crabs." However we show some stained preparations of *C. magister* for comparative purposes.

The text states:

"4.5) 4-Di-2-ASP staining of a PD preparation from a different crab (*Cancer magister*) (**Figure 16**). If one has a high enough magnification the sensory endings can be seen inside of the supportive scolopales^{12,13,19} (**Figures 17**)."

12. 1.1.3.2) "condyle" should be explained.

Response: corrected

13. Figure 3: An arrow pointing to the nerve would be helpful.

Response: corrected

14. 2.1.2) I found this part confusing. Maybe rephrase.

Response: corrected

This section now reads,

"2.1.2) In order to stimulate the opener muscle the stretcher motor nerve in the carpopodite region is isolated and stimulated with a suction electrode. The proximal part of the leg is removed by transecting the meropodite

with scissors.

15. 1.2.6) How are suction electrodes made?

Response: corrected

This section now reads,

“1.2.6) Position the micromanipulator so the attached suction electrode assembly will have easy access to the saline bath and preparation. The suction electrode is constructed as shown in an on line video (Baierlein et al. 2011).”

16. Figure 5: Maybe consider using a diagram here rather than pictures. If pictures are kept, an arrow pointing to the nerve (branching from the main nerve) would be helpful.

Response: corrected. Arrows now shown in figures.

2.1.3.2) Find the nerve branching from the main nerve bundle to the stretcher muscle (Figure 5E, at arrow) and this can be cut close to the muscle and pulled into a suction electrode to stimulate (Figure 5F&G, arrow depicts the branch).

17. 2.2.9.1) How much (volume) of the neuromodulators should be applied?

Response: corrected.

2.2.9.1) Use a pipette and just drip 1 or 2 ml over the preparation.

18. 3.2.1) What is the stock solution?

Response: corrected.

“3.2.1) Dilute one part methylene blue chloride stock solution (0.25%) with two parts of distilled water. “

19. 3.3.2) What volume is used?

Response: corrected.

3.3.2) Use a 10 μ M concentration of 4-Di-2-ASP solution and leave the preparation in the refrigerator for 15 minutes. Use just enough solution to cover the preparation.

20. Figure 13: Should be labeled A) and B). What is on the y-axis? What do the different colors mean? More explanation is needed.

Response: corrected.

Figure 13: The relative forces that are developed with the joint fully flexed and stimulated at the various frequencies. A, The relative forces are shown with each stimulation frequency. B, The traces in panel A are superimposed in different colors for ease in comparison.

Reviewer #2:

Manuscript Summary:

This paper presents a very thorough demonstration of a preparation which is outstanding for teaching purposes.

Response: Thank you.

Major Concerns:

While the content of the paper is excellent, the writing “requires editing.” (sic).

The fragmented sentences were due to our misunderstanding of listing topics/sections and not statements for the step wise fashion of the methodology. These steps were also made by the JoVE editor in breaking the parts to steps. We will double check for typos etc... Sorry about the oversight.

Minor Concerns:

P1 p5 line 2 adding a comma after proprioception makes the sentence easier to read

P1 p 5 line 7 delete "of a chordotonal organ"

p2 p1 line 3 a methodology "for recording from primary"...

P2 p 1 line 4 "... (individually or as a group)..."

P2 p10 line 10 "...which neurons "carry" out..."

P2 p1 line 10 "...relate "this" to the location..."

P2 p1 line 16 ... "are" still being carried out..."

Fig 13 add scale bars and label traces

Fig 14 add scale bars

Response: Corrections made and manuscript proof read by 3rd party.

It's probably a good idea to mention that blue crabs are rather aggressive, and very fast, particularly since they can actually draw blood if they get a good pinch in.

Response: Good idea. Thank you. The following caution has been added to the methods section:

1.1) Dissection

1.1.1) Hold the crab across the carapace from behind, and avoiding the claws, pinch the proximal part of the meropodite with forceps. The leg will autotomize. This avoids the animal from bleeding to death. Blue crabs are rather aggressive and very fast one needs to use caution when handling.

Additional Comments to Authors:

N/A

Reviewer #3:

Manuscript Summary:

This manuscript shows the recording and staining techniques for a sense organ in a crab. The paper describes is not one, but a suite of experimental procedures including dissection, extracellular recording, stimulation, and cellular staining. Tasks include:

1. Isolating a small sensory organ and a small motor nerve;
2. Recording from the sensory organ while the limb is being moved passively;
3. Recording from the sensory organ while the limb is actively moving from neural stimulation;
4. Recording muscle tension caused by varying neural stimulation;
5. Two staining methods to visualize the sense organ. **NOTE: We used three staining methods.**

Major Concerns:

This seems to be a challenging preparation for teaching proprioception. Sticking with decapod crustaceans, the abdominal muscle receptor organ can be recorded in crayfish (smaller, more readily available), is a simpler dissection, shows many of the same physiological properties (e.g., neurons responding to movement and position), and is well documented in widely distributed teaching materials (e.g., the Crawdad Project; <http://www.crawdad.cornell.edu/>). Given that, this article needs to make a stronger case for the benefits of using this particular preparation compared to others. Perhaps emphasizing the possible role of these sensory organs in active walking, swimming, and so on, might provide justification for studying these particular sensory structures.

Response: We agree that crayfish have some advantages but one can not record from joint receptors within the walking legs as well in crayfish preparations as compared to the crab legs. We do have a video recording of the methodology for recording from the crayfish MRO presented for educational purposes.

Leksrisawat, B., Cooper, A. S., Gilberts, A. B., Cooper, R. L. Muscle Receptor Organs in the Crayfish Abdomen: A Student Laboratory Exercise in Proprioception . *J. Vis. Exp.* (45), e2323, doi:10.3791/2323 (2010).

Good point on justifying the crab leg for relating to understanding sensory organs in active walking and swimming etc...

This text was added to the Introduction:

"The muscle receptor organ in the abdomen of the crayfish is also a well documented preparation (e.g., the Crawdad Project; <http://www.crawdad.cornell.edu/>) for teaching purposes of proprioception with only two neurons per abdominal hemi-segment (Leksrisawat et al., 2010); however the chordotonal organ in the joints of walking legs in crabs allows one to emphasize recruitment of neurons in range fractionation as well as sensory processing as part of locomotion."

"3.3.2) Use a 10 μ M concentration?" How much dye should be used per volume of saline?

Response:

3.3.2) Use a 10 μ M concentration of 4-Di-2-ASP solution and leave the preparation in the refrigerator for 15 minutes. Use just enough solution to cover the preparation.

Minor Concerns:

There is some specialized terminology that may be unfamiliar to novices. E.g., PD organ is not defined (though can probably be figured out from context); condyle.

Response: Corrections made.

"In this demonstration we use the Propodite-Dactylopodite (PD) chordotonal organ."

"1.3.2) Using the same technique cut a smaller window on the pigmentless (medial) side of the propodite, but leaving the condyle (the socket joint or hinge between segments) attachment intact."

"3.4.1.2) ? If any CoCl_2 spills into the saline bath the entire preparation will stain black, and the preparation should be discarded." If students are aware of the spill, a quick and complete change of saline can prevent everything from staining.

Response: Corrections made. Good point.

"3.4.1.2) Make a petroleum jelly well to hold the CoCl_2 . If any CoCl_2 spills into the saline bath the entire preparation will stain black, and the preparation should be discarded. **NOTE: that may work with crayfish but doesn't work with crab preps.**

"3.4.2.2) ? Good metal tools are not to be used to handle the preparation after this step (you should use specific tools for this)." This may be cryptic to new readers; what sort of tools? I assume the authors mean using forceps that have already been exposed to fixatives, etc.

Response: Corrections made. Good point.

"3.4.2.2) Transfer the isolated preparation to a small glass Petri dish containing about 10 ml of crab saline. The neurons are washed and the following steps are done in situ. Good metal tools are not to be used to handle the preparation after this step (you should use specific tools that are not to be used at a later time for physiology). "

"3.4.3.2) Make certain that the development solution you have poured into the sink drain is followed by running tap water for a few minutes." A waste bottle might be preferred by some safety officers rather than the sink.

Response: Corrections made. Good point.

"3.4.3.2) Make certain that the development solution you have poured into the sink drain is followed by running tap water for a few minutes or in a waste bottle with a lid."

"3.4.4.2) ? With time the filled cells will become more apparent because the surrounding tissue will become clearer." Worth noting that the stained neurons will also fade unless intensified.

Response: Corrections made. Good point.

"3.4.4.2) After about 10-15 minutes in two changes in 100 % alcohol, clear the tissue by replacing alcohol with 100% methyl salicylate. The preparation will stay in this solution permanently for repeated viewing. With time the filled cells will be come more apparent because the surrounding tissue will become clearer. Intensification methods can be used to help prevent fading over time (Delaney and Gelperin, 1990)."

The introductory review is somewhat cursory. "Detailed electrophysiological studies [are] still being carried out today for research and for teaching purposes.5,6,7" has no references past the 1950s, which does not support the claim that this prep is actively being used. More recent references might include:

Bévengut M, Simmers AJ, Clarac F. 1983. Central neuronal projections and neuromuscular organization of the basal region of the shore crab leg. *The Journal of Comparative Neurology* 221(2): 185-198.
<http://dx.doi.org/10.1002/cne.902210207>

El Manira A, Cattaert D, Clarac F. 1991. Monosynaptic connections mediate resistance reflex in crayfish (*Procambarus clarkii*) legs. *Journal of Comparative Physiology A* 168(3): 337-349.
<http://dx.doi.org/10.1007/BF00198353>

Ray DL, Clarac F, Cattaert D. 1997. Functional analysis of the sensory motor pathway of resistance reflex in crayfish. I. Multisensory coding and motor neuron monosynaptic responses. *Journal of Neurophysiology* 78(6): 3133-3143. <http://jn.physiology.org/cgi/content/abstract/78/6/3133>

Le Bon-Jego M, Cattaert D. 2002. Inhibitory component of the resistance reflex in the locomotor network of the crayfish. *Journal of Neurophysiology* 88(5): 2575-2588. <http://jn.physiology.org/cgi/content/abstract/88/5/2575>

Response: Corrections made. Good references are added.

Additional Comments to Authors:

This is good material, but may be best targeted at advanced students.

Reviewer #4:

Manuscript Summary:

In this paper the authors present the interest of the crab leg preparation to demonstrate function of proprioceptors. The presentation is clear and the practical informations will be helpful for teaching. In addition,

procedures used to stain sensory neurons and nerves are given with all details very useful for replication with students. I found the paper very interesting for the amount of practical details it provides.

Major Concerns:

Page 4 Figure 1: indicate cut line between carpopodite and propodite

Response: Corrections made.

Page 9, Figure 6

Fig 6A: The limit of the removed cuticle section is hardly visible.

The difference between figures 6B and 6C is not obvious.

Response: Corrections made. This is really Figure 5.

“2.1.3.1) Cut the apodeme of the bender muscle and remove the muscle carefully as not to pull the main leg nerve out of the leg cavity (**Figure 5B, C**- note the arrows where to bender apodeme is separated).”

Fig 6F: indicate the motor nerve with an arrow.

Response: Corrections made.

Page 17, Figure 13: Add indication of opening and closing movements of the joint

Response: Corrections made. This is now Figure 11.

Page 18, Figure 14, bottom: indicate the significance of colors?

Response: Corrections made.

Page 18, § 4.3: "? nerve can be correlated ?" (insert "be").

Response: Corrections made.

Change "Figure 11" into "Figure14"

Response: Corrections made.

Page 19: Figure 11 is indeed Figure 14

Response: Corrections made.

Page 20: Figure 12 is indeed Figure 15

Response: Corrections made.

Page 20: Figure 13 is indeed Figure 16

Response: Corrections made.

Page 20: Figure 14 is indeed Figure 17

Response: Corrections made.

Page 20, Line 2: (and Figure 8) Indicate in Figure 8 which area is magnified in Figure12 15

Response: Corrections made.

Minor Concerns:

Page 1, Long Abstract, last line : « ? receptoris ? » : insert a space « ?receptor is ? »

Response: Corrections made.

Page 8, §2.1.2, L2: suppress the full stop after carpopodite

Response: Corrections made.

Additional Comments to Authors:

N/A

Your revision is due by **Jun 17, 2013**.

To submit a revision, go to the *JoVE* [submission site](#) and log in as an author. You will see a menu item called 'Submission Needing Revision'. You will find your submission record there.

Sincerely,

Susan Rasakham, Ph.D.

Science Editor

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