

Journal of Visualized Experiments

The swimmeret system of crayfish: A practical guide for the dissection of the nerve cord and extracellular recordings of the motor pattern --Manuscript Draft--

Manuscript Number:	JoVE52109R3
Full Title:	The swimmeret system of crayfish: A practical guide for the dissection of the nerve cord and extracellular recordings of the motor pattern
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	crustacean; dissection; extracellular recording; fictive locomotion; motor neurons; locomotion
Manuscript Classifications:	8.1.158.610: Neurosciences; 95.51.3: animal biology; 95.53.4: behavioral sciences
Corresponding Author:	Carmen Ramona Smarandache-Wellmann, Ph.D. University of Cologne Cologne, NRW GERMANY
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	carmen.wellmann@uni-koeln.de
Corresponding Author's Institution:	University of Cologne
Corresponding Author's Secondary Institution:	
First Author:	Henriette Andrea Seichter
First Author Secondary Information:	
Other Authors:	Henriette Andrea Seichter Felix Blumenthal
Order of Authors Secondary Information:	
Abstract:	<p>Here we demonstrate the dissection of the crayfish abdominal nerve cord. The preparation comprises the last two thoracic ganglia (T4, T5) and the chain of abdominal ganglia (A1 to A6). This chain of ganglia includes the part of the central nervous system (CNS) that drives coordinated locomotion of the pleopods (swimmerets): the swimmeret system. It is known for over five decades that in crayfish each swimmeret is driven by its own independent pattern-generating kernel that generates rhythmic alternating activity. The motor neurons innervating the musculature of each swimmeret comprise two anatomically and functionally distinct populations 4. One is responsible for the retraction (power-stroke, PS) of the swimmeret. The other drives the protraction (return-stroke, RS) of the swimmeret. Motor neurons of the swimmeret system are able to produce spontaneously a fictive motor pattern, which is identical to the pattern recorded in vivo 1.</p> <p>The aim of this report is to introduce a model system interesting for studying rhythm generating networks and coordination of independent microcircuits that is convenient for students' practical laboratory courses. The protocol provided includes step-by-step instructions for the dissection of the crayfish's abdominal nerve cord, pinning of the isolated chain of ganglia, desheathing the ganglia and recording the swimmerets fictive motor pattern extracellularly from the isolated nervous system.</p> <p>Additionally, we can monitor the activity of swimmeret neurons recorded intracellularly from dendrites. Here we also describe briefly these techniques and provide some examples. Furthermore, the morphology of swimmeret neurons can be assessed using various staining techniques. Here we provide examples of intracellular (by iontophoresis) dye-filled neurons and backfills of pools of swimmeret motor neurons. In our lab we use this preparation to study basic functions of fictive locomotion, the effect of sensory feedback on the activity of the CNS and coordination between microcircuits</p>

	on a cellular level.
Author Comments:	<p>Dear Dr. Nguyen,</p> <p>Thank you for supporting our manuscript "The swimmeret system of crayfish: A practical guide for the dissection of the nerve cord and extracellular recordings of the motor pattern" (JoVE52109R2). We revised the manuscript carefully and also reconsidered the highlighted sections. The highlighted Protocol reads now as a cohesive story.</p> <p>Sincerely,</p> <p>Carmen Smarandache-WellmannHenriette Seichter andFelix Blumenthal.</p> <p>Editorial comments: The manuscript has been modified by the Science Editor to comply with the JoVE formatting standard. Please maintain the current formatting throughout the manuscript. The updated manuscript (52109_R2_051414.docx) is located in your Editorial Manager account under "File Inventory." Please download the .docx file and use this updated version for any future revisions.</p> <p>Changes made by the Science Editor:</p> <p>1. There have been edits made to the manuscript. Thank you for the helpful revisions. Changes to be made by the Author(s): 1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version. We revised the manuscript carefully</p> <p>2. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. The highlighted steps should form a cohesive narrative with a logical flow from one highlighted step to the next. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader. We highlighted 2.75 pages of the Protocol with headings and spacing, see also attached document of the highlighted protocol.</p> <p>3. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). The highlighted Protocol forms a cohesive narrative with logical flow. There are only complete sentences highlighted.</p>
Additional Information:	
Question	Response
If this article needs to be "in-press" by a certain date to satisfy grant requirements, please indicate the date below and explain in your cover letter.	
If this article needs to be filmed by a certain date to due to author/equipment/lab availability, please indicate the date below and explain in your cover letter.	

Title:

The swimmeret system of crayfish: A practical guide for the dissection of the nerve cord and extracellular recordings of the motor pattern

Authors:

Henriette A. Seichter
Emmy Noether Group / Institute of Zoology
University of Cologne
Cologne, Germany
hseichte@uni-koeln.de

Felix Blumenthal
Emmy Noether Group / Institute of Zoology
University of Cologne
Cologne, Germany
f.blumenthal87@gmail.com

Carmen R. Smarandache-Wellmann
Emmy Noether Group / Institute of Zoology
University of Cologne
Cologne, Germany
carmen.wellmann@uni-koeln.de

Corresponding Author:

Carmen Smarandache-Wellmann
University of Cologne
Emmy Noether Group / Institute of Zoology
Zuelpicher Strasse 47b
Cologne, Germany
Email: carmen.wellmann@uni-koeln.de
TEL: +49-(0) 221-470-8068

Keywords:

crustacean; dissection; extracellular recording; fictive locomotion; motor neurons; locomotion

Short abstract:

Here we describe the dissection of the crayfish abdominal nerve cord. We also demonstrate an electrophysiological technique to record fictive locomotion from swimmeret motor neurons.

Long abstract:

Here we demonstrate the dissection of the crayfish abdominal nerve cord. The preparation comprises the last two thoracic ganglia (T4, T5) and the chain of abdominal ganglia (A1 to A6). This chain of ganglia includes the part of the central nervous system (CNS) that drives coordinated locomotion of the pleopods (swimmerets): the swimmeret system. It is known for over five decades that in crayfish each swimmeret is driven by its own independent pattern-generating kernel that generates rhythmic alternating activity¹⁻³. The motor neurons innervating the musculature of each swimmeret comprise two anatomically and functionally distinct populations⁴. One is responsible for the retraction (power-stroke, PS) of the

swimmeret. The other drives the protraction (return-stroke, RS) of the swimmeret. Motor neurons of the swimmeret system are able to produce spontaneously a fictive motor pattern, which is identical to the pattern recorded *in vivo*¹.

The aim of this report is to introduce an interesting and convenient model system for studying rhythm generating networks and coordination of independent microcircuits for students' practical laboratory courses. The protocol provided includes step-by-step instructions for the dissection of the crayfish's abdominal nerve cord, pinning of the isolated chain of ganglia, desheathing the ganglia and recording the swimmerets fictive motor pattern extracellularly from the isolated nervous system.

Additionally, we can monitor the activity of swimmeret neurons recorded intracellularly from dendrites. Here we also describe briefly these techniques and provide some examples. Furthermore, the morphology of swimmeret neurons can be assessed using various staining techniques. Here we provide examples of intracellular (by iontophoresis) dye-filled neurons and backfills of pools of swimmeret motor neurons. In our lab we use this preparation to study basic functions of fictive locomotion, the effect of sensory feedback on the activity of the CNS, and coordination between microcircuits on a cellular level.

Introduction:

The swimmerets of crayfish serve a function in posture control and beat rhythmically when the animals swim forward, ventilate their burrows or females aerate their eggs^{5,6}. The swimmerets of the signal crayfish, *Pacifastacus leniusculus*, occur in pairs from the second to the fifth abdominal segment, with one limb on each side of the abdomen⁷. The central nervous system produces on its own the rhythmic motor pattern which drives the swimmeret movement in the intact animal as well as in the isolated nerve cord preparation. When there is no sensory feedback or descending input present the rhythmic motor pattern produced is called fictive locomotion^{1,2}. In the swimmeret system this motor pattern does not differ in any parameter from the activity of the swimmerets measured in the intact animal.

The movement of each swimmeret is driven by a microcircuit that is located in and restricted to one corresponding hemiganglion¹⁻³. In each microcircuit there is a pattern-generating kernel that comprises five identified non-spiking interneurons. They can be functionally characterized as being either Inhibitor of Power-Stroke (IPS) or Inhibitor of Return-Stroke (IRS)⁸. These IPS and IRS interneurons are not endogenous oscillators, rather their alternating activity is driven by reciprocal inhibition⁹. Because these interneurons inhibit the swimmeret motor neurons directly, the alternating PS-RS movement is generated¹⁰.

Locomotion however, does not only require the generation of activity, but also coordination of the different independent microcircuits. In the swimmeret system such coordination is established by the coordinating microcircuit which ensures that limbs are active at correct times. This microcircuit is built by three identified neurons in each segment¹¹⁻¹⁵.

This protocol provides for the first time a step-by-step dissection guide to isolate the chain of ganglia (T4 to A6, Figure 1). We show how to pin the isolated abdominal nerve cord and desheath each ganglion. In this isolated nervous system preparation, the neurons responsible for swimmeret movement are ready for use in electrophysiological and morphological experiments. The second part of this protocol demonstrates the main features of the swimmeret motor pattern. This includes a step-by-step guide to extracellularly record the activity of swimmeret motor neurons. Axons of RS motor neurons project through the anterior branch of nerve N1, while axons of PS motor neurons project through the posterior

branch of the same nerve (Figure 1) ⁴. Therefore their activity can be recorded from these branches with differential pin electrodes.

[Place Figure 1 here]

This dissection procedure and the electrophysiological technique demonstrated are convenient for undergraduate students and may complement student practical courses in physiology. The isolated chain of ganglia has been used in a number of experiments to study nervous system function, coordination, or modulation of swimmeret microcircuits ⁶ as well as neuronal control of adaptive behavior in locomotion ^{16,17}. The crayfish swimmeret system thus provides an enormous amount of interesting teaching or training opportunities which all begin with the dissection of the ventral nerve cord of crayfish and extracellular recording of the fictive motor pattern.

Protocol:

This dissection procedure is in accordance with the European Communities Council Directive of 22nd September 1986 (1986/609/EEC).

1. Preparation

1.1) Obtain crayfish, *Pacifastacus leniusculus* (Dana), of both sexes ≥ 8 cm in size. Ensure that the animals are vital and the abdomen and abdominal limbs are intact.

1.2) Take care to inspect the carapace and that this cuticle is hard and rigid. Pre- and postmolt animals have a soft carapace and are not suited for experiments because during the molting process many parameters change (*e.g.*, decrease in locomotor activity).

1.3) Assemble all tools and materials used during the dissection, pinning and desheathing of the nerve cord shown in Figure 2 and listed in the supplements provided.

[Place Figure 2 here]

2. Gross Dissection

2.1) Anesthetize animal on ice for 15-20 min. Carry out the first part of the gross dissection at a lab bench near the sink since it includes an exsanguination step and the specimen needs to be rinsed regularly with crayfish saline during the procedure.

2.2) Hold animal ventral side up and use strong scissors to cut both claws at their bases near the thorax (Figure 3-1). Remove left and right uropod (Figure 3-2).

2.3) Place the animal ventral side up in the dissection dish lined with black sylgard. Elevate cephalothorax by inserting ice underneath and pin the abdomen at the telson (Figure 4 A).

2.4) Fill the saline dispenser with ~60 ml chilled crayfish saline. Perfuse crayfish with cold saline through the claw opening (Figure 4 B). Excess saline will drain off through the cuts at the uropods. Cover crayfish with ice during exsanguination

2.5) Decapitate the animal with a single transverse cut just posterior to the animal's eyes using strong scissors (Figure 5 A). Remove all walking legs near the base joints as indicated in Figure 5 B.

2.6) Isolate the abdomen with the last thoracic segments from the rest of the cephalothorax. Make a first cut at the level of the second walking legs (thoracic segment 3) by inserting the tip of the scissors into the opening of the second walking leg and cutting to the opposite side. (Fig. 6 A-1).

2.7) Extend this first cut to both sides through the cephalothorax (Figure 6 A-2).

2.8) Flip the animal open to make some of the internal organs visible. Push the prominent digestive gland (Figure 6 B-3) to the anterior part of the specimen and use forceps to remove the reproductive organs from the abdominal cavity.

2.9) Remove the anterior part of the cephalothorax (Figure 6 C). Use lateral cuts to remove the lateral parts of the carapace, which cover the gills, on both sides of the remaining thorax (Figure 6 D-4). Remove the gills and rinse the specimen with cold saline.

2.10) Continue the dissection with a cut through the whole length of the sternal plate as indicated in Figure 6 E-5. Make this cut at maximal lateral positions between pleuron and swimmerets (Figure 6 E red markings). Proceed on the other side with a same cut. Rinse the specimen with cold saline.

2.11) Carry out the remaining dissection under a dissection microscope. Place the crayfish's abdomen ventral side up in the dissection dish lined with black sylgard and filled with crayfish saline so that it covers the specimen.

Note: The following steps (2.12 – 4.8) contain directional instructions that apply to right-handed experimenters. In the following steps (2.12 - 6.8) it is important to replace crayfish saline in regular intervals, every 20-30 min with cold saline, to keep the nervous system healthy.

2.12) Fix the specimen with insect pins posteriorly at the telson and anteriorly at the remains of the carapace. Place the specimen so that the telson points to the left and is parallel to the table's edge.

2.13) Use coarse forceps in the left hand to grab through a walking leg opening (Figure 7 A) and pull the specimen open (Figure 7 B white arrow). Identify the large two dorsal flexor muscle strands (Figure 7 B-1 and C -1) and cut their ventral basis as shown in Figure 7 B.

2.14) Identify the sternal artery (Figure 7 C-2), that descends from dorsal (the heart) to ventral, at the 4th thoracic segment. This artery lies right above the nerve cord (Figure 7 C-3), before it projects under the nerve cord, forming the ventral artery.

2.15) Transect the sternal artery. As demonstrated in Figure 7 C, lift the artery first, using one blade of the scissors, and only cut when the ventrally located nerve cord is visible.

2.16) Fix the dorsal flexor muscles anteriorly (to the right side). This should be done in maximum stretched position with pins so they will not block vision and the specimen remains

stretched. When the dorsal muscle strands are fixed (Figure 8-1), the first abdominal ganglia, A1 and A2, with associated nerves N1, N2, and N3 are visible (Figure 8).

2.17) Use forceps in the left hand to grab the specimen at one opening of the walking legs. Throughout the following dissection steps pull gently to keep the specimen open.

2.18) Transect the nerves N3 at the most distant position from the nerve cord. (Figure 8-3).

2.19) Cut the flexor muscles close to the ventral apodeme as shown in Figure 8-4. Take care not to damage the nerve cord or the nerves N2.

2.20) Repeat the steps 2.18 and 2.19 for the nerves N3 and the flexor muscles of the remaining abdominal ganglia A2 to A5.

2.21) At the last abdominal ganglion, A6, cut the dorsal flexor muscles from the ventral apodeme and the specimen should look as shown in Figure 9.

2.22) Cut the sternal plate posterior to the nerves of A6 (Figure 9-1) and keep the ventral part (Figure 9-2). Discard the dorsal part with flexor muscles (Figure 9-3). Fix the sternal plate anteriorly with pins through the openings of the walking legs, and posteriorly to A6.

3. Fine Dissection

3.1) Place the specimen under the microscope with the anterior part directed away and the posterior part towards the tables' edge.

3.2) Use forceps as shown in Figure 10 A to remove the most anterior parts of the cephalothoracic sterna.

Note: The thoracic ganglia and associated nerves are partly covered by the leg musculature and cephalothoracic sterna. The cephalothoracic sterna form a skeleton that separates the laterally located cavities of the walking legs from each other and from the medial cavity in which the ventral nerve cord resides.

3.3) Cut the muscles between the remaining exoskeletal structures as indicated in Figure 10 B-1 and B2. Use forceps to grab and lift the anterior end of the ventral nerve cord (Figure 10 C).

Note: The nerve cord will be damaged in the process so avoid picking up the nerve cord multiple times.

3.4) Cut the thoracic nerves laterally while lifting the nerve cord (Figure 10 C-3). Keep these nerves at suitable length for pinning. Remove the squeezed part of the chain of ganglia, which was picked up with forceps, by cutting away all the tissue anterior to T4 (Figure 10 C-4).

3.5) Place the specimen with the anterior part to the left and focus on A1. Cut the nerves N1 and N2 of A1 at a suitable length (max. 1 cm) for pinning them out.

3.6) Focus on A2 and identify the nerves N1, N2, and N3 of this segment (Figure 11). The nerves N1 of abdominal ganglia A2-A5 reside between two sternal cuticular infoldings in each segment (Figure 11 A-1) and are covered by musculature. Make one cut along the posterior sternal cuticular infolding. Start at the lateral rim of the abdomen and proceed towards the midline as shown in Figure 11 A.

3.7) If the target N1 is still covered with tissue as shown in Figure 11 B (red arrow), cut across the muscle bundle, but this time anterior to both sternal cuticular infoldings and the nerve N1 (Figure 11 B-2).

3.8) Cut nerve N1 as distally as possible (Figure 11 C-3). Nerve N1 is fully visible and the anterior and posterior branch can be identified (Figure 11 C).

3.9) Proceed to the contralateral nerve N1 and first cut the muscles along the posterior sternal cuticular infolding, starting medially, near the ganglion (Figure 11 D). If the nerve is still covered by tissue, cut across the muscle bundle, but this time anterior to both sternal cuticular infoldings and the nerve N1, similar to Figure 11 B-2. Cut the nerve N1 as distally as possible.

3.10) Cut the nerves N2 of this ganglion to a suitable length (approx. 0.5 cm) for pinning.

3.11) Repeat steps 3.7-3.11 for the nerves of A3-A5.

3.12) Cut the nerves of ganglion A6 as distally as possible (Figure 12 A). Use forceps to grab multiple nerves of A6 to lift this ganglion and start isolating the chain of ganglia from the sternal plate.

3.13) While lifting the nerve cord, pull it gently in the anterior direction as demonstrated in Figure 12 B (white arrow). As the individual ganglia are lifted, remove the ventral artery that may be attached to the ventral side of the nerve cord (Figure 12 C). Continue this (gently) pull-cut sequence until the nerve cord is completely isolated.

3.14) Transfer the isolated chain of ganglia to a Petri dish lined with clear sylgard and filled with crayfish saline (Figure 12 D).

4. Pinning the nerve cord into the Petri dish

Note: Use small pins cut from stainless steel wire (see supplements) to pin the nerve cord. Touch only the nerve ending with the forceps and do not squeeze the connectives or ganglia.

4.1) Pin the chain of ganglia in a straight line, while applying gentle stretch.

4.2) Arrange the nerve cord in the Petri dish with the dorsal side facing upwards (Figure 13, black line). The ventral side of the ganglia can be identified by its convexity; the dorsal side is flat. Pin the thoracic nerves to the sides. Continue with the nerves of A6, stretching the nerve cord along its longitudinal axis.

4.3) Pin out the nerves of A1 at a 90° angle relative to the nerve cord.

4.4) Proceed to A2 and pin the nerves N2 at an angle of 35 – 45° relative to the nerve cord (Figure 1 A).

4.5) Separate the nerves N1 in their anterior and posterior branches before pinning as demonstrated in Figure 14. Use two pairs of fine forceps to pick up with one pair of forceps the anterior and with the other the posterior branch of the nerve N1. Take care to pick only the most distal ends of the nerve branches. Now pull them carefully apart.

4.6) Pin the anterior branch of the nerve N1 at a 90° angle relative to the nerve cord (Figure 1 A). Pin the posterior branch of nerve N1 between the anterior N1 branch and the nerve N2.

4.7) Repeat the steps 4.4 – 4.6 for the nerves of ganglia A3 – A5. While fixating the nerve cord stretch it in longitudinal as well as transversal directions.

5. Desheathing the ganglia

5.1) Place the preparation in such a manner that the experimenter's hands are always resting on a stable plane to avoid shaking. In order to desheath the ganglia illuminate the nerve cord from below.

5.2) Focus on any abdominal ganglion A1 to A5. Use fine spring scissors to make a small lateral cut through the ganglion sheath, posterior to the ganglion and between the nerves N2 and N3 (Figure 15 A red arrow).

5.3) Pick up the ganglion sheath using very fine forceps and cut transversely across the sheath above the connectives, as indicated in Figure 15 A-1. Take care not to squeeze or cut the nerve cord with the scissors.

5.4) Still holding and lifting the ganglion sheath with forceps continue to cut it along the lateral borders of the ganglion (Figure 15 B-2 and -3). Remove the sheath. Alternatively pin it to both sides of the connectives in such a way that it is fixed but the nerve cord is not squeezed.

5.5) Repeat steps 5.2- 5.4 for all abdominal ganglia A1 to A5.

5.6) Desheath the thoracic ganglia and the ganglion A6 in a similar fashion. In order to desheath A6 start anterior to the ganglion and proceed in posterior direction. Pin the ganglion sheath of A6 to the posterior end of the chain of ganglia.

6. Extracellular recordings from motor neurons

6.1) Assemble all tools and materials used for extracellular recordings shown in Figure 16 B and listed in the supplements. An overview of the recording setup is shown in Figure 16 A. Start all electronic equipment used in this experiment (Figure 16 C), so that the amplifiers can warm up for at least 30 min prior to recording. Turn on the computer and start the recording software.

6.2) Place the chain of ganglia on the microscope table and illuminate from below. Insert the recording electrode into the sylgard close to the target nerve and the reference electrode at

a position nearby, but lateral to the ganglia (Figure 17 A and B). Bend the target nerve around the recording electrode (Figure 17 C).

6.3) Stretch the nerve slightly, to ensure contact between the electrode and the nerve and pin it to the side (Figure 17 D). Fix the electrode cables to the microscope table using modeling clay, so they stay in the desired position.

6.4) Use a syringe filled with petroleum jelly and a 20 gauge needle (with rounded tip) (Figure 17 E-3) to isolate the target nerve from the bathing solution. First dab some petroleum jelly on the sylgard around the recording electrode. The result is a layer of petroleum jelly covering the sylgard in the proximity of the recording electrode (Figure 17 E-4). Take care not to directly dab onto the nerve and avoid air bubbles in this layer.

6.5) Seal the recording electrode with petroleum jelly from all sides up to the surface level of the saline (Figure 17 F).

6.6) Repeat this procedure for all target nerves whose activity should be monitored.

6.7) **Start recording.** Use a continuous or gap-free acquisition mode and a sampling rate of 5 kHz. Set the extracellular amplifier to the following parameters; gain to 1000 (amplifies the signal 1000 times, take care to include this amplification parameter in the acquisition software settings) and a bandpass filter range of 300 Hz (low cut) to 2000 Hz (high cut).

Representative Results:

With the simultaneous extracellular recordings from RS and PS, motor neurons of one ganglion, the alternating activity of these motor neuron pools, can be monitored (Figure 18), representing the fictive locomotion pattern.

[Place Figure 18 here]

The extracellular PS activity of ipsilateral PS 2 to PS 5 motor neurons highlights interesting aspects of intersegmental coordination in the swimmeret system (Figure 19 A).

First, the activity always starts with a PS burst in A5 (Figure 19 A, 4th trace) and the excitation wave propagates in the anterior direction (Figure 19 A, green line). Second, the period (which is the time measured from the onset of one burst to the onset of the next burst) in each segment remains constant (Figure 19 A, cyan arrow). This is true, as long as the information flow from one ganglion to the next is intact and the experimental conditions do not change.

In different preparations or under different experimental conditions the periods and burst durations can vary substantially: from periods of 0.25 to 1 sec. In order to compare these parameters between different preparations, they need to be normalized and graphed in a phase histogram (Figure 19 B). This reveals the third interesting aspect of the motor pattern: the phase-lag between segments, the intersegmental delay, remains constant and independent of the frequency of the rhythm.

To calculate a phase histogram, the following measurements and calculations are necessary: First, a reference trace has to be chosen. Usually we use the PS trace of the most posterior ganglion (*e.g.*, PS 5) as a reference (being time 0) for a phase histogram of the swimmeret rhythm recorded across several segments (Figure 19A). Then, measure the period (in ms) of

the rhythm from the beginning of one PS 5 burst (time 0, Figure 19 A, orange dotted line) to the beginning of the next PS 5 burst (Figure 19 A, cyan arrow). Within this cycle, measure the latencies (in ms) between the start of the PS 5 burst (time 0) and the start (burst onset, Figure 19 A and B, orange arrows) and end (offset Figure 19 A and B, violet arrows) of the PS burst in each of the other ganglia. Therefore to calculate the phase onset and offset for each burst the latencies are divided by the concurrent period. These measurements will result in numeric values between 0 and 1. Finally, plot these values in a phase histogram as shown in Figure 19 B.

The resulting phase histogram (Figure 19 B) contains information about the duty cycle of the PS burst in each segment. And the intersegmental phase-lag of ~ 0.25 between neighboring segments is clearly visible.

[Place Figure 19 here]

For more advanced experimenters, intracellular recordings with sharp microelectrodes from the dendrites of motor neurons can be useful. The membrane potentials of individual intracellularly recorded PS motor neurons show in-phase oscillations with the extracellularly recorded activity of all PS motor neurons of the same hemiganglion (Figure 20, gray panels). Alternatively, RS motor neurons or interneurons can be recorded intracellularly in the same way (data not shown).

[Place Figure 20 here]

For identification of intracellularly recorded neurons (interneurons or motor neurons), cells can be dye-filled using sharp microelectrodes and iontophoresis (see discussion). In Figure 21, two intracellularly dye-filled PS motor neurons are shown. Their ventral somata are located posterior to the base of the nerve N1. The primary neurite projects anteriorly (Figure 21 B-2) and branches within the Lateral Neuropil (Figure 21 A and B-3); the axon projects through the posterior branch of the nerve N1 to the swimmeret musculature (Figure 21 B-4).

[Place Figure 21 here]

For less advanced experimenters, or those who want to study the morphology of the abdominal ganglion, backfills from the PS and RS motor neuron pools via the nerve N1, are an interesting exercise. In Figure 22, the RS and PS motor neurons of one hemiganglion were stained with Co^{2+} (RS) and Ni^{2+} ions (PS), respectively. Somata of PS motor neurons are located posterior to the base of the nerve N1 (Figure 22 -1). All somata of RS motor neurons are located anterior to the base of the nerve N1 (Figure 22 -2).

[Place Figure 22 here]

Figure 1: Isolated nervous system from thoracic ganglion 4 (T4) to abdominal ganglion 6 (A6) and a schematic diagram of it. T4: thoracic ganglion 4; T5: thoracic ganglion 5; A1, A2 ... A6 abdominal ganglion 1, abdominal ganglion 2 ... abdominal ganglion 6; N1: nerve N1; N2: nerve N2; N3: nerve N3; PS: power-stroke; RS: return-stroke. Directional abbreviations: A = anterior; P = posterior.

Figure 2: Materials and tools used for the dissection, pinning and desheathing of the nerve cord.

(1) big bucket filled with ice; (2) crayfish saline; (3) saline dispenser; (4) dissection microscope; (5) dissection dish; (6) strong scissors; (7) forceps (8) spring scissors; (9) Petri dish lined with clear sylgard; (10) fixing pins; (11) cold lamp source.

Figure 3: Removal the claws (1) and uropods (2). Red lines (1) and (2) indicate positions of cuts. Bar = 5 cm.

Figure 4: A. The animal is pinned ventral side up and fixed at the telson. B. The crayfish is exsanguinated through the claw opening with cold saline. Bars = 5 cm.

Figure 5: A. Decapitation and B. removal of walking legs. Red lines indicate positions of cuts. Bars = 1 cm.

Figure 6: A., B., C. and D. Removal of the anterior and the lateral parts of the cephalothorax. E. Opening of the abdomen along the lateral side. Red lines (1), (2), (4), and (5) indicate positions of cuts. (3) Digestive gland. Red arrows point to the already opened abdominal cavity. Bars = 1 cm.

Figure 7: A. The specimen is grabbed at an opening of the walking legs (red arrows). B. The dorsal muscle strands (1) are transected while the specimen is opened. White arrow depicts the direction in which the sternal plate is pulled. C. Overview of the abdominal cavity with the sternal artery (2) and nerve cord (3).

Figure 8: Transverse view of the crayfish abdomen with fixed dorsal muscle strands (1) and sternal artery (2); Red lines (3) and (4) indicate positions of cuts. Bars = 5 mm.

Figure 9: Dorsal and ventral parts of the abdomen are separated from each other. (1) Red line indicates position of cut. Sternal plate (2) with ventral nerve cord; dorsal part of the abdomen (3).

Figure 10: A. Positions of the forceps to remove the anterior cephalothoracic sterna. B. Red lines (1) and (2) indicate where to cut the muscles between the remaining cephalothoracic sterna. C. The thoracic nerves are transected at the red lines (3) to isolate the thoracic ganglia from the surrounding tissue. Red line (4) indicates the cut between T3 and T4. Bars = 5 mm.

Figure 11: A. and B. View of the sternal plate at A2. The nerve N1 is isolated from the tissue with a cut along the posterior sternal cuticular infolding (1) and anterior to both sternal cuticular infoldings (B. 2). C. Higher magnification of the region around A2, with the isolated nerve N1. D. Isolation of the nerve N1 on the contralateral side with similar cuts. Red lines (2) and (3) indicate positions of cuts; (1) anterior and posterior sternal cuticular infoldings; Bars = 5 mm.

Figure 12: A. and B. A6 is isolated from the tissue and the nerve cord can be lifted at the nerves of A6. C. All connections of the abdominal ganglia to the ventral abdominal artery are transected. D. Isolated nerve cord is transferred into a Petri dish lined with clear sylgard and filled with saline. Red lines indicate positions of cuts. Bars = 5 mm.

Figure 13: Lateral view of one ganglion. Identification of the dorsal and ventral side (black line) of the nerve cord. Bar = 5 mm

Figure 14: The anterior branch of the nerve N1 is separated from the posterior branch. Bar = 5 mm.

Figure 15: Directions for desheathing of an abdominal ganglion A. Position of the first small cut through the sheath of the connectives (red arrow), placed lateral and posterior to the ganglion. The subsequent cut through the sheath above the connective is marked by the red line (1). B. Red lines (1) and (2) mark the cuts along the lateral borders of the ganglion. The sheath can afterwards be pulled from the ganglion. Bars = 1 mm.

Figure 16: A. Overview of the recording setup. B. Materials and tools needed for extracellular recordings. C. Equipment necessary for electrophysiological recording. (1) cold lamp source; (2) faraday cage; (3) stereo microscope; (4) air-table; (5) microscope table; (6) mirror; (7) differential pin electrodes; (8) syringe filled with petroleum jelly; (9) modeling clay; (10) forceps; (11) liquid waste disposal; (12) digitizer; (13) extracellular amplifier; (14) computer, equipped with recording software and monitor.

Figure 17: A. and B. Position of recording (1) and reference (2) pin electrode. C. and D. The posterior branch of the nerve N1 is bend around the recording electrode. E. The base (4) of the recording electrode is isolated with petroleum jelly. F. The complete recording electrode is isolated with petroleum jelly from the bath solution. (3) Syringe filled with petroleum jelly. Bars = 2 mm.

Figure 18: Schematic of one ganglion and placement of differential pin electrode. Extracellular recording of RS motor neurons (upper trace) and PS motor neurons (lower trace).

Figure 19: A. Schematic of electrode placement and extracellular recording of ipsilateral PS 5 to PS 2. B. Measurements and calculations needed for the phase histogram. The phase histogram calculated from ten consecutive bursts ($n = 10$) shows the intersegmental phase delay of ~ 0.25 .

Figure 20: Schematic of electrode placement. The membrane potential of one intracellularly recorded PS motor neuron (upper trace) oscillates in phase (gray panels) with the extracellularly recorded PS activity (lower trace).

Figure 21: A. Schematic of an abdominal ganglion. B. Two intracellularly stained PS motor neurons. (1) somata; (2) primary neurites; (3) dendritic branching in the lateral neuropil; (4) axons project through the posterior branch of the nerve N1; Bar = 100 μm .

Figure 22: Backfills from the anterior (orange, Co^{2+}) and posterior (blue, Ni^{2+}) branch of nerve N1. (1) Somata of PS motor neurons; (2) somata of RS motor neurons. Bar = 100 μm .

Discussion:

The anatomy of crayfish and their abdominal ganglia has been described previously^{5, 18, 19, 20} and it is recommended to become familiar with them prior to the dissection in order to avoid cutting of important nerves.

It is critical to keep the preparation at temperatures below 23 °C to prevent degradation of the isolated nerve cord. This can be achieved easily by replacement of the bathing solution every

20 – 30 min with cold crayfish saline. Under these circumstances the chain of ganglia can be used for electrophysiological experiments for up to 12 hours.

Sometimes swimmeret motor neurons are inactive and show no spontaneous bursting activity. The cholinergic agonist carbachol is used to induce bursting activity in these preparations. Dissolved in cold crayfish saline, bath applications of 1-3 μM carbachol are sufficient to activate the swimmeret rhythm²¹.

The fictive locomotion and especially the coordination of the limbs recorded in this system provide a lot of information about the cellular properties of rhythmic activity and coordination. In this system the neuronal components⁸⁻¹⁰ of the local microcircuits are identified and additionally the coordinating network is known in cellular detail^{11, 14, 15}. The results drawn from the experiments performed with extracellular recordings, allow already predictions for mathematical models and for roboticists.

All swimmeret neurons show dendritic branching within the Lateral Neuropil and can be intracellularly recorded from these dendrites. In Figure 20, an intracellular recording of a PS motor neuron is shown. For this a sharp microelectrode (see supplements) filled with 1 M KAc + 0.1 M KCl (electrode resistance between 35 - 45 M Ω) is placed above the Lateral Neuropil. The oscillating membrane potentials as well spikes from the dendrites of PS and RS motor neurons can be recorded when the electrode is inserted near the base of nerve N1.

To stain individual neurons (*e.g.*, motor neurons) intracellularly as shown in Figure 21, the sharp microelectrode must be additionally filled with fluorescent dye (*e.g.*, 1% dextran Texas Red, see supplements) dissolved in 1 M KAc + 0.1 M KCl. To fill motor neurons intracellularly by iontophoresis, polarizing pulses of 1 nA with a duration of 250 ms at 2 Hz are applied for at least 10 min. For positively charged dyes use depolarizing pulses and for negatively charged dyes use hyperpolarizing pulses. Longer fills result in a stronger labeled neuron. For visualization the nerve cord must be fixed, dehydrated, and mounted²².

One method to stain a pool of motor neurons is to use backfills from the nerve N1 (Figure 22)^{4, 23}. Place a well of petroleum jelly around the posterior branch of the nerve N1 to backfill the pool of PS motor neurons. To stain the pool of RS motor neurons a well is placed on the anterior branch of the nerve N1. The crayfish saline in the well is replaced by ddH₂O. Then cut this nerve inside the well and leave it for 15 minutes in ddH₂O at room temperature (RT). Thereafter, replace the ddH₂O in the wells with either 5% CoCl₂- or 5% NiCl₂- solution (dissolved in ddH₂O) and close wells with petroleum jelly. The sample needs to be incubated for at least 15 hours at 4 °C. Open the wells, remove the dye and rinse the sample 3 times with saline. Precipitate the Ni²⁺ or Co²⁺ ions with 10 drops of Dithiooxamid (saturated solution dissolved in 100% EtOH) for every 10 ml crayfish saline in the Petri-dish, under the hood for 20 min at RT. Ni²⁺- ions will precipitate to blue Ni(SCH) and Co²⁺-ions to orange CoS. The nerve cord must be fixed, dehydrated, and mounted in order to acquire images of the labeled cells under a light microscope equipped with a camera.

Acknowledgements:

We thank Jos Burgert for helping with some of the figures. We are grateful to Ingo Selbach (and the group “Edelkrebsverein NRW”) for his efforts to supply the lab with experimental animals. We thank Anna C. Schneider for proofreading first versions of the manuscript. This research was supported by an Emmy Noether DFG grant SM 206/3-1 and a startup grant of the University of Cologne for female faculty.

Disclosures:

The authors declare that they have no competing financial interests.

References:

- 1 Hughes, G. M. & Wiersma, C. A. G. The Co-Ordination of Swimmeret Movements in the Crayfish, *Procambarus-Clarkii* (Girard). *J Exp Biol.* **37** (4), 657-670 (1960).
- 2 Mulloney, B. & Smarandache, C. Fifty Years of CPGs: Two Neuroethological Papers that Shaped the Course of Neuroscience. *Front Behav Neurosci.* **4**, 45, DOI: 10.3389/fnbeh.2010.00045 (2010).
- 3 Murchison, D., Chrachri, A. & Mulloney, B. A Separate Local Pattern-Generating Circuit Controls the Movements of Each Swimmeret in Crayfish. *J Neurophys.* **70** (6), 2620-2631 (1993).
- 4 Mulloney, B. & Hall, W. M. Functional organization of crayfish abdominal ganglia. III. Swimmeret motor neurons. *J Comp Neurol.* **419** (2), 233-243, DOI: 10.1002/(SICI)1096-9861(20000403)419:2<233::AID-CNE7>3.0.CO;2-Y (2000).
- 5 Davis, W. J. Lobster Righting Responses and Their Neural Control. *Proc R Soc Ser B-Bio.* **170** (1021), 435-456, DOI: 10.1098/rspb.1968.0049 (1968).
- 6 Mulloney, B. & Smarandache-Wellmann, C. Neurobiology of the crustacean swimmeret system. *Prog Neurobiol.* **96** (2), 242-267, DOI: 10.1016/j.pneurobio.2012.01.002 (2012).
- 7 Huxley, T. H. *The crayfish: An introduction to the study of zoology*. MIT Press, Cambridge, MA (1980).
- 8 Smarandache-Wellmann, C., Weller, C., Wright, T. M. & Mulloney, B. Five types of nonspiking interneurons in local pattern-generating circuits of the crayfish swimmeret system. *J Neurophys.* **110** (2), 344-357, DOI: 10.1152/jn.00079.2013 (2013).
- 9 Skinner, F. K., & Mulloney, B. Intersegmental coordination of limb movements during locomotion: mathematical models predict circuits that drive swimmeret beating. *J Neurosci.* **18** (10), 3831-3842 (1998).
- 10 Mulloney, B. During fictive locomotion, graded synaptic currents drive bursts of impulses in swimmeret motor neurons. *J Neurosci.* **23** (13), 5953-5962, DOI: 10.1523/JNEUROSCI.2642-13.2014 (2003).
- 11 Smarandache-Wellmann, C., & Grätsch, S. Mechanisms of coordination in distributed neural circuits: Encoding coordinating information. *J Neurosci.* **34** (16), 5627-5639, DOI: 10.1523/JNEUROSCI.2670-13.2014 (2014).
- 12 Mulloney, B. & Hall, W. M. Local commissural interneurons integrate information from intersegmental coordinating interneurons. *J Comp Neurol.* **466** (3), 366-376, DOI: 10.1002/cne.10885 (2003).
- 13 Mulloney, B., Harness, P. I. & Hall, W. M. Bursts of information: Coordinating interneurons encode multiple parameters of a periodic motor pattern. *J Neurophys.* **95** (2), 850-861, DOI: 10.1152/jn.00939.2005 (2006).
- 14 Smarandache, C., Hall, W. M. & Mulloney, B. Coordination of Rhythmic Motor Activity by Gradients of Synaptic Strength in a Neural Circuit That Couples Modular Neural Oscillators. *J Neurosci.* **29** (29), 9351-9360, DOI: 10.1523/JNEUROSCI.1744-09.2009 (2009).
- 15 Smarandache-Wellmann, C., Weller, C. & Mulloney, B. Mechanisms of Coordination in Distributed Neural Circuits: Decoding and Integration of Coordinating Information. *J Neurosci.* **34** (3), 793-803, DOI: 10.1523/JNEUROSCI.2642-13.2014 (2014).
- 16 Chrachri, A., Neil, D. & Mulloney, B. State-Dependent Responses of 2 Motor Systems in the Crayfish, *Pacifastacus leniusculus*. *J Comp Physiol A.* **175** (3), 371-

- 380, DOI: 10.1007/BF00192996 (1994).
- 17 Chrachri, A. & Neil, D. M. Interaction and Synchronization between 2 Abdominal Motor Systems in Crayfish. *J Neurophys.* **69** (5), 1373-1383 (1993).
- 18 Skinner, K. The Structure of the 4th Abdominal-Ganglion of the Crayfish, *Procambarus-Clarki* (Girard) II. Synaptic Neuropils. *J Comp Neurol.* **234** (2), 182-191, DOI: 10.1002/cne.902340205 (1985).
- 19 Skinner, K. The Structure of the 4th Abdominal-Ganglion of the Crayfish, *Procambarus-Clarki* (Girard) I. Tracts in the Ganglionic Core. *J Comp Neurol.* **234** (2), 168-181, DOI: 10.1002/cne.902340204 (1985).
- 20 Mulloney, B., Tschuluun, N. & Hall, W. M. Architectonics of crayfish ganglia. *Microsc Res Techniq.* **60** (3), 253-265, DOI: 10.1002/jemt.10265 (2003).
- 21 Braun, G. and B. Mulloney, Cholinergic modulation of the swimmeret motor system in crayfish. *J Neurophys.* **70** (6), 2391- 2398 (1993).
- 22 Davis, W. J. Motoneuron Morphology and Synaptic Contacts - Determination by Intracellular Dye Injection. *Science.* **168** (3937), 1358-1360, DOI: 10.1126/science.168.3937.1358 (1970).
- 23 Altman, J. S., & Tyrer, N. M. Filling Selected Neurons with Cobalt through Cut Axons. In: Strausfeld N.J., Miller T.A. (editors); *Neuroanatomical Techniques*, 373–402. Springer, New York, NY (1980).

Figure 01
[Click here to download high resolution image](#)

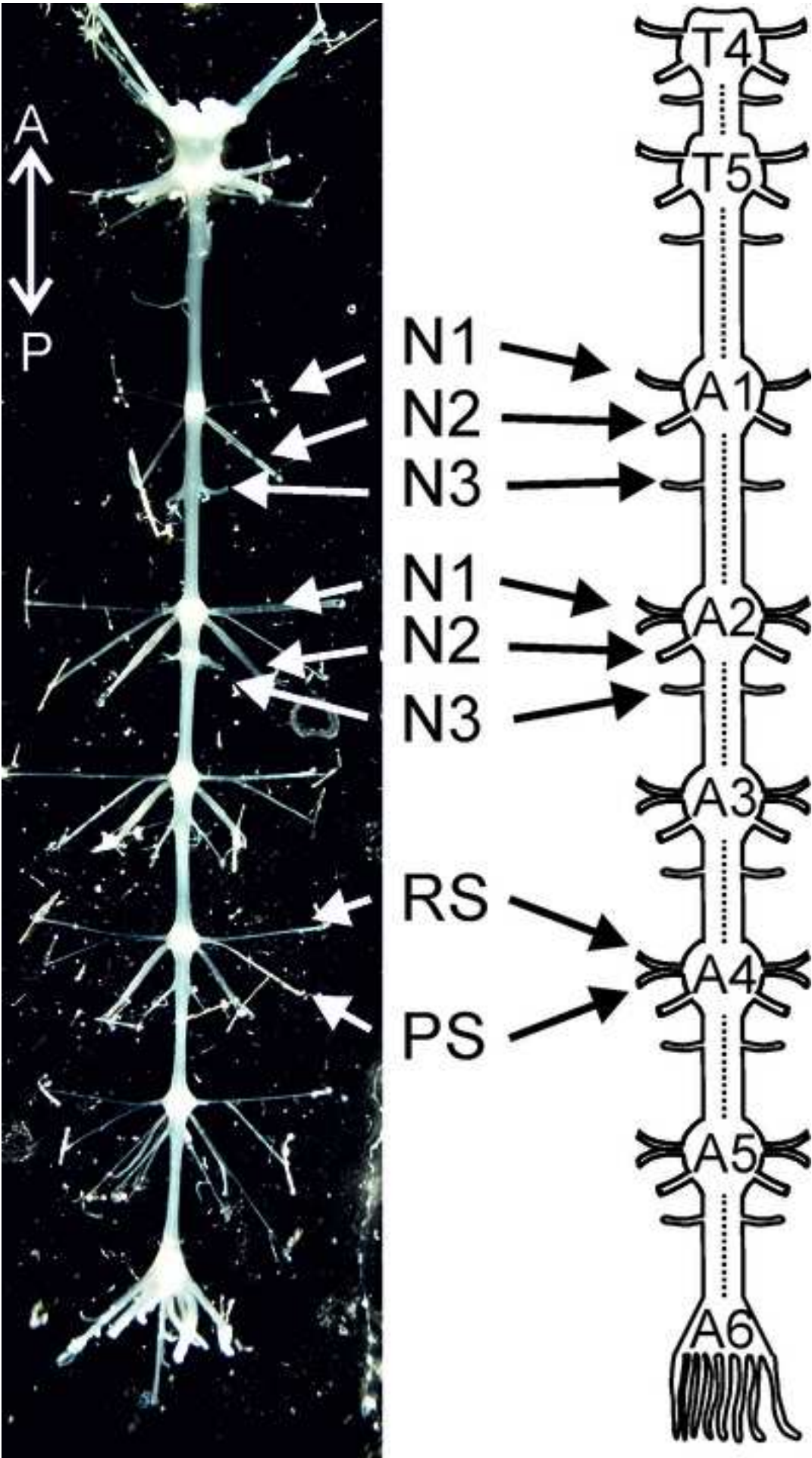


Figure 02
[Click here to download high resolution image](#)

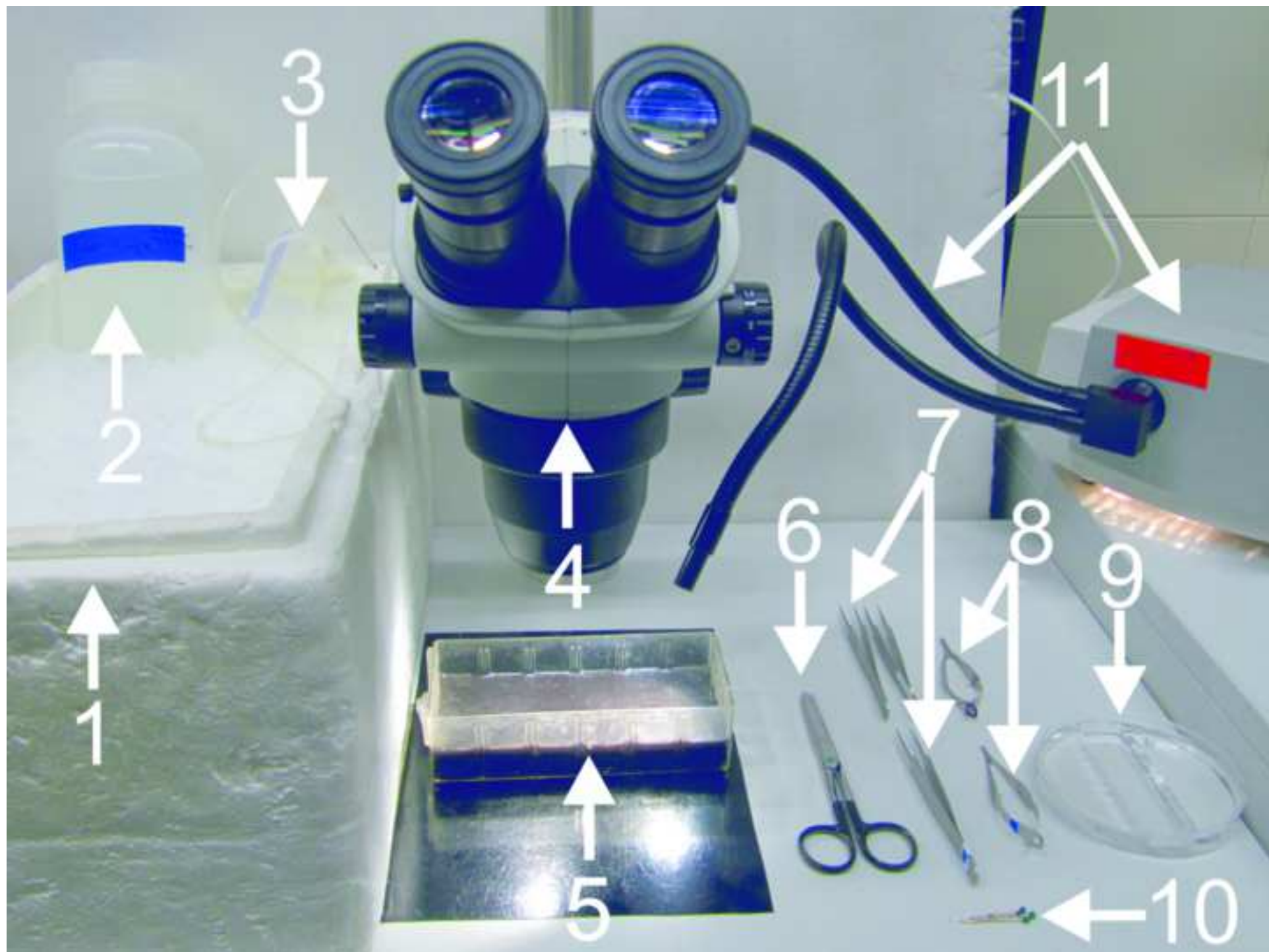


Figure 03
[Click here to download high resolution image](#)

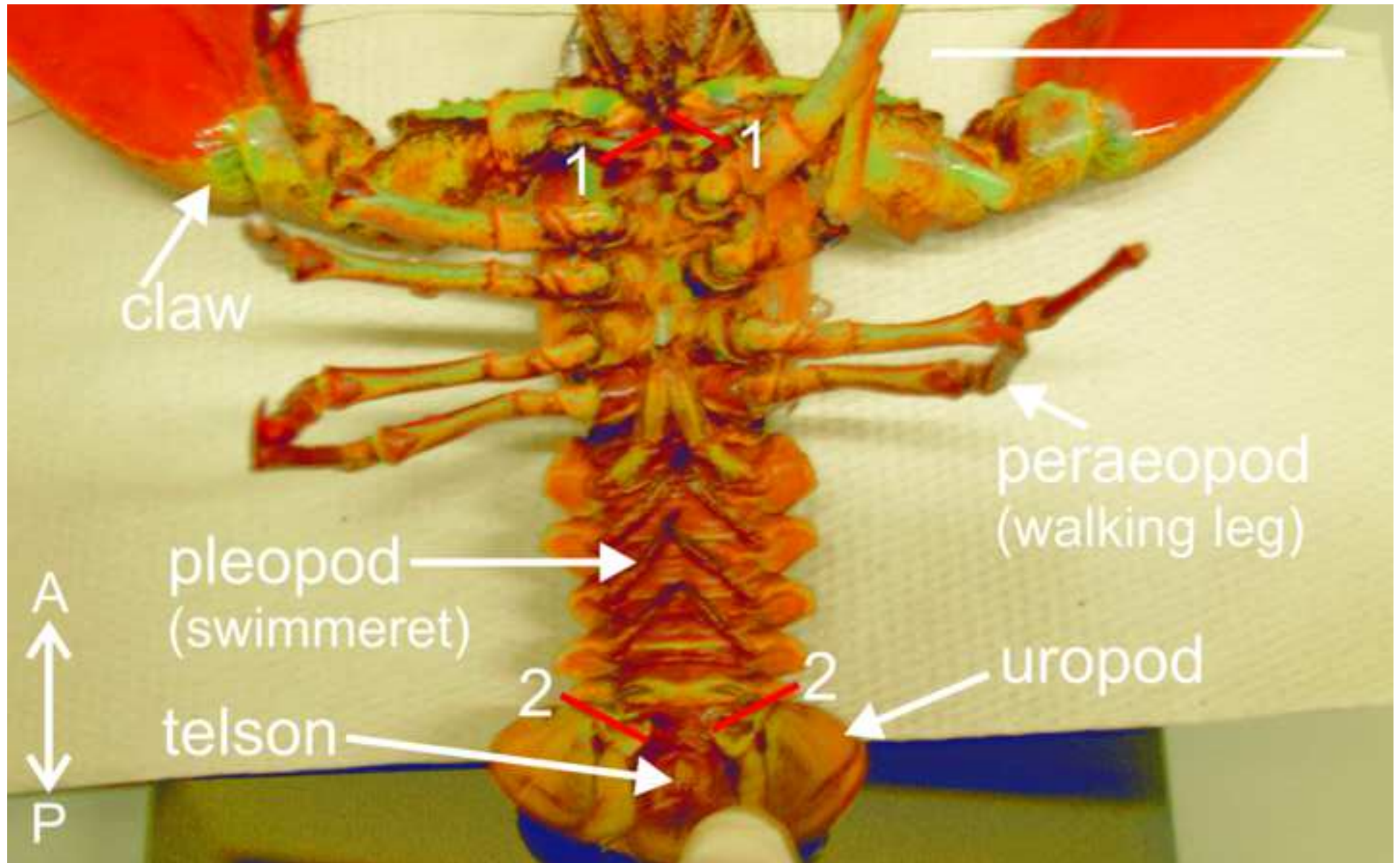


Figure 04
[Click here to download high resolution image](#)

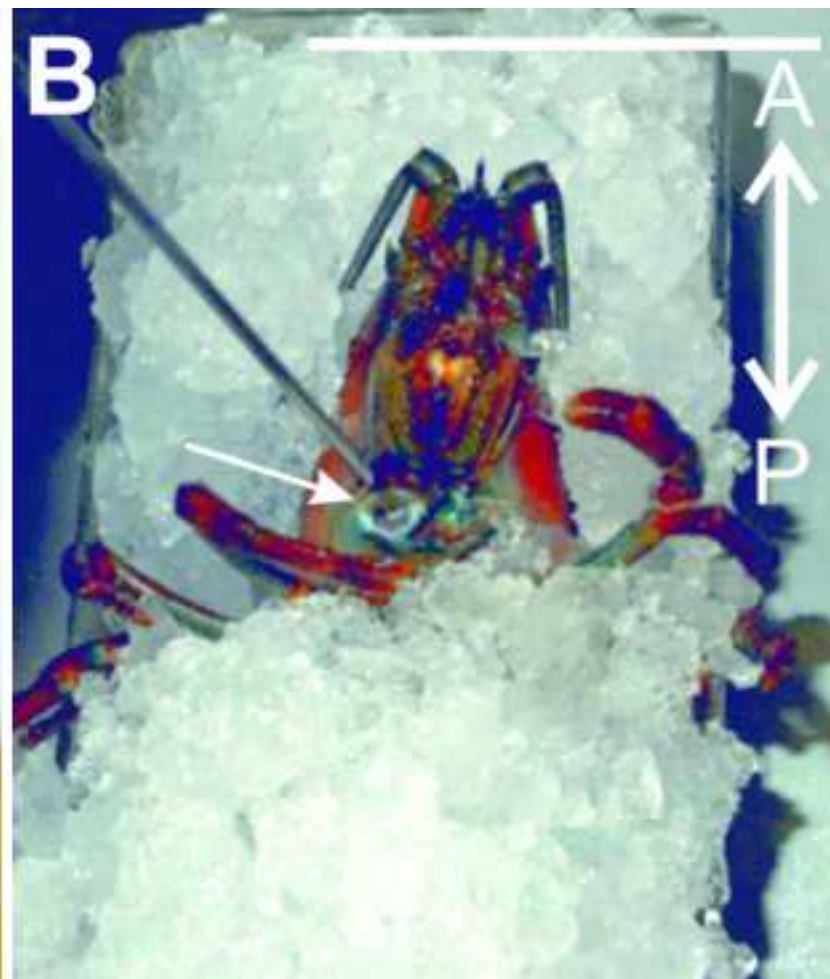
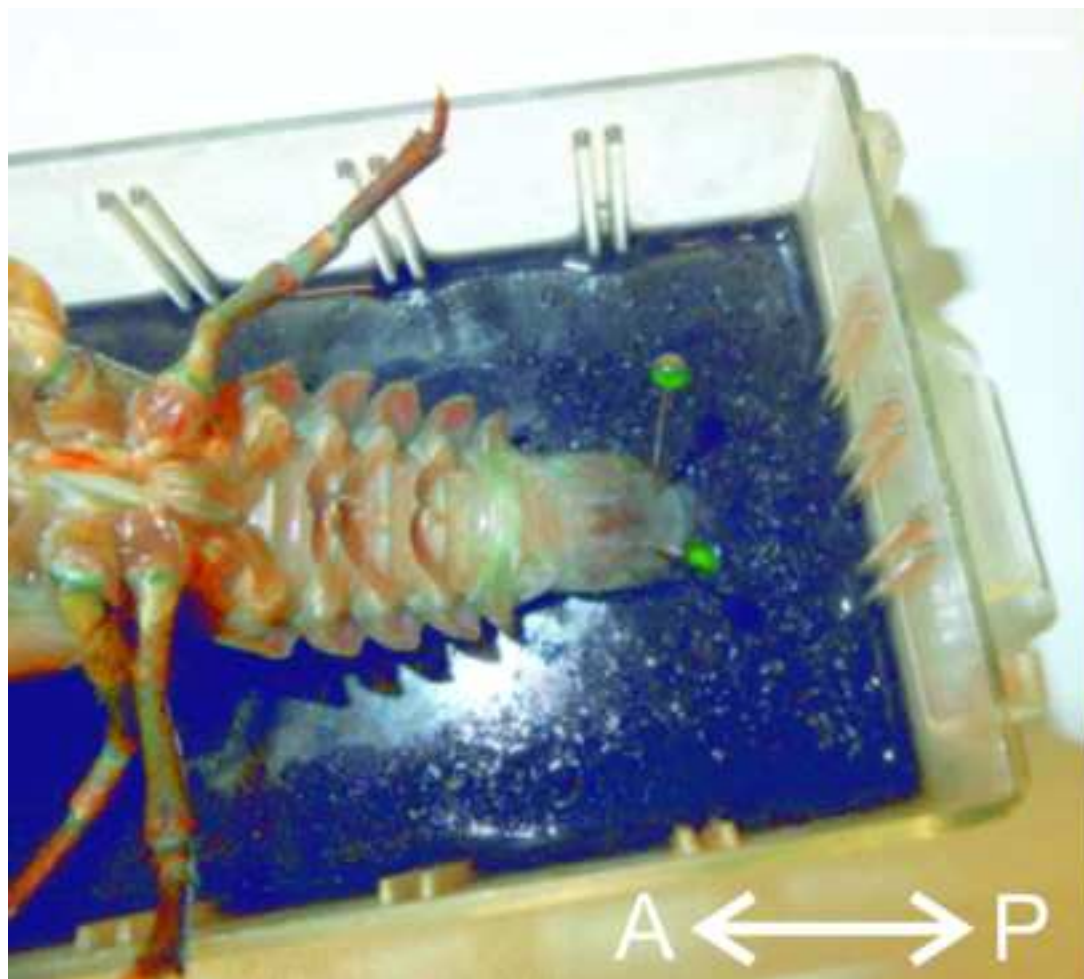


Figure 05
[Click here to download high resolution image](#)

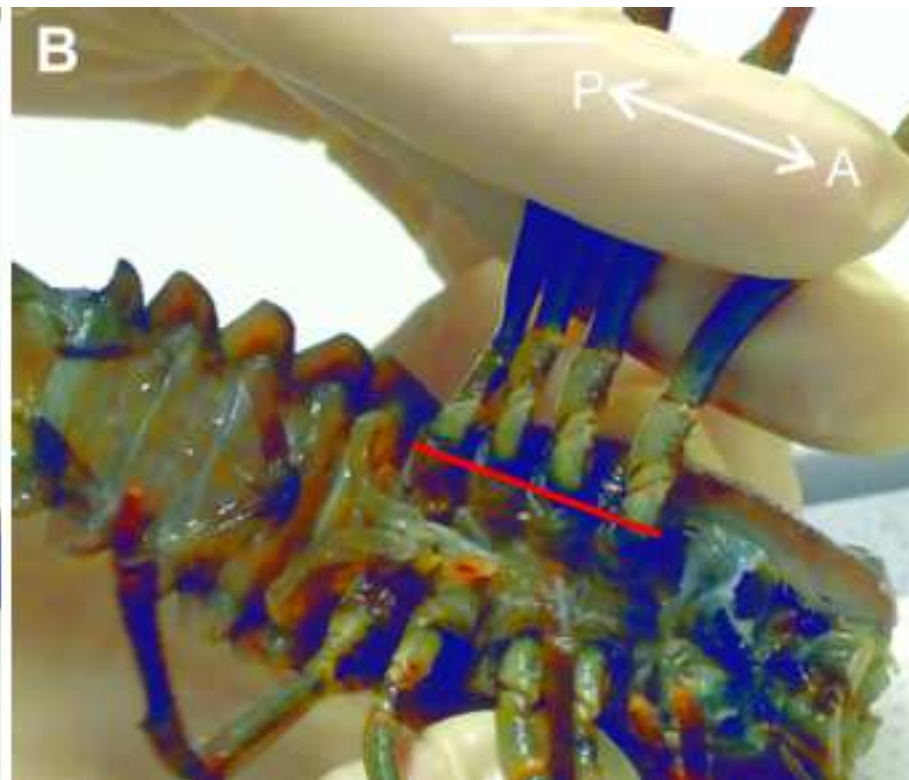
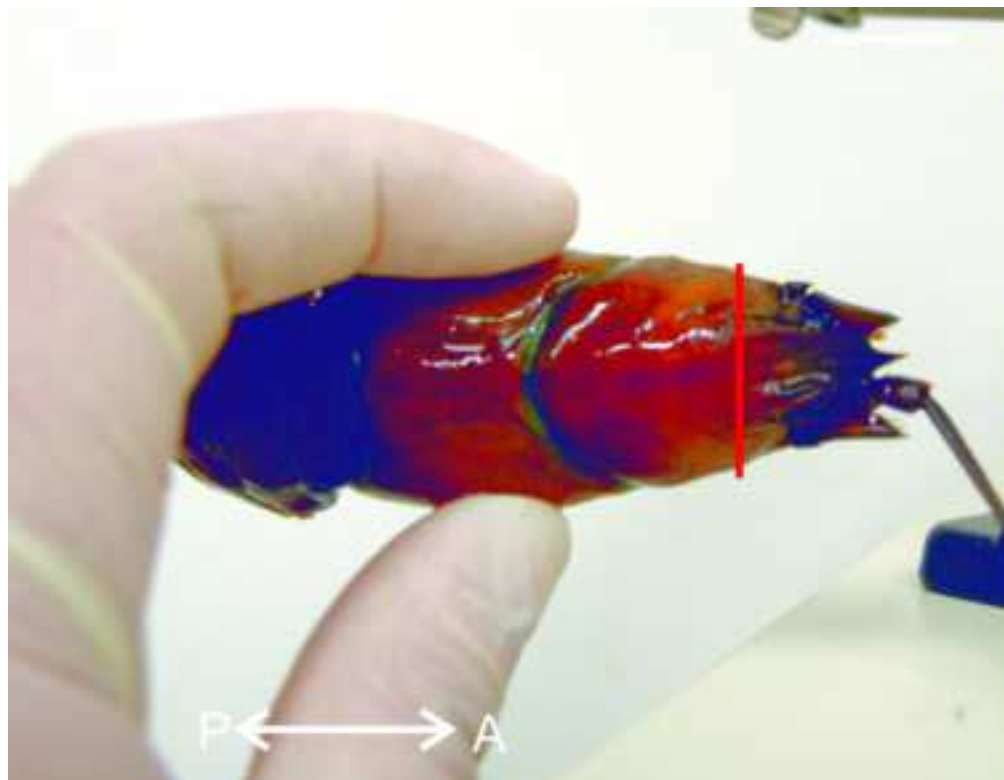


Figure 06
[Click here to download high resolution image](#)

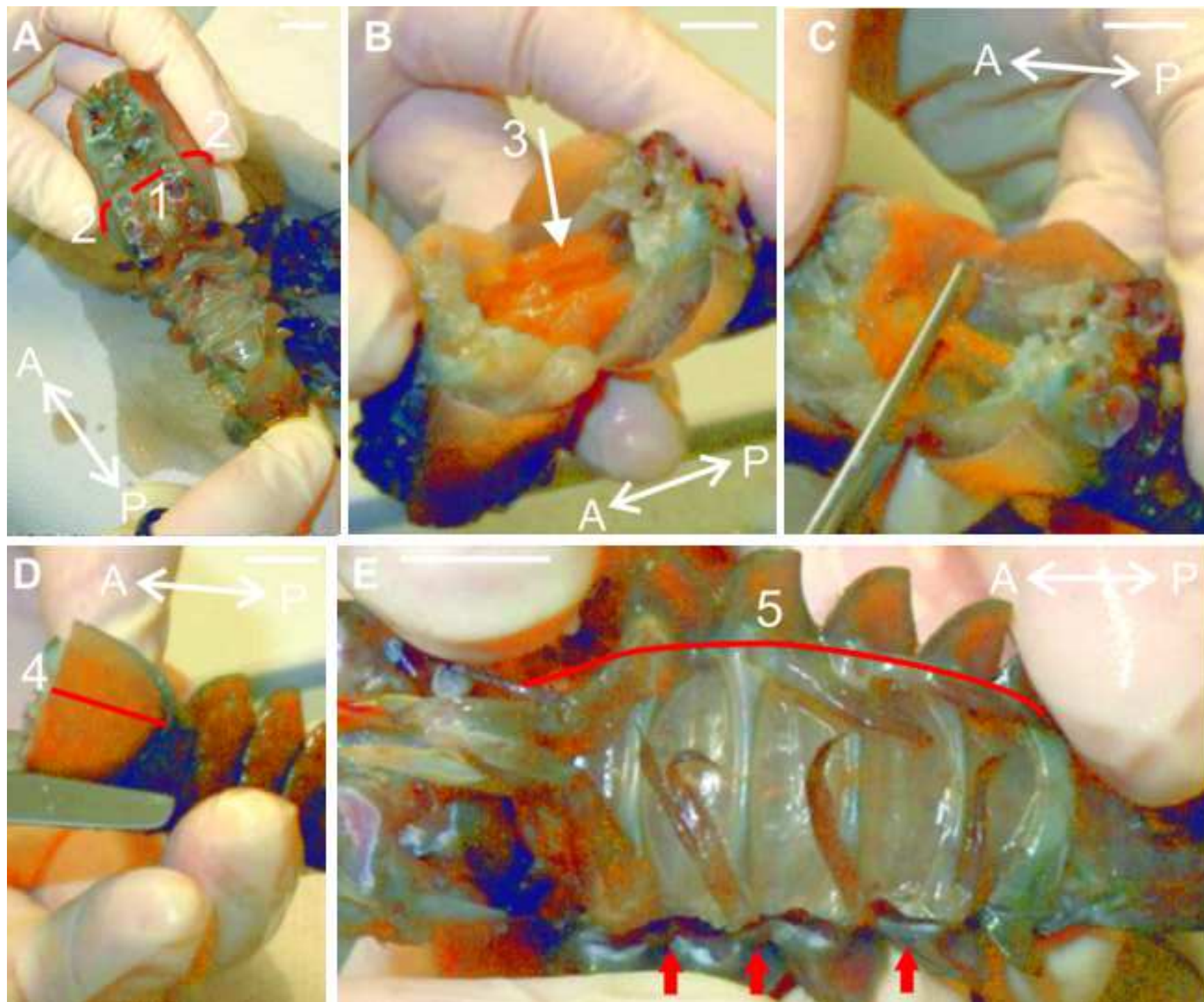


Figure 7
[Click here to download high resolution image](#)

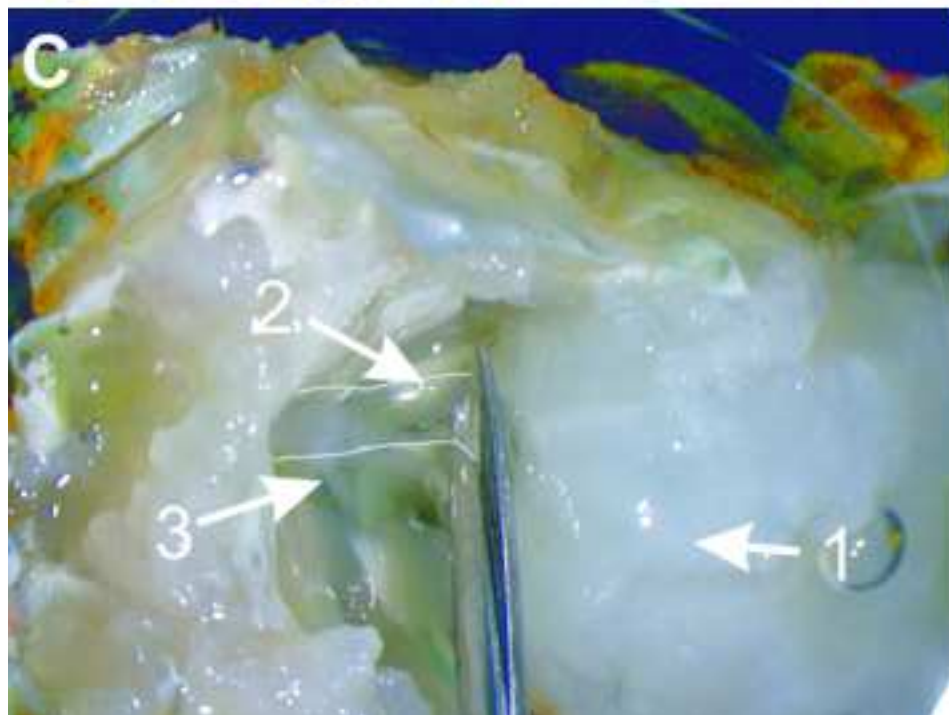
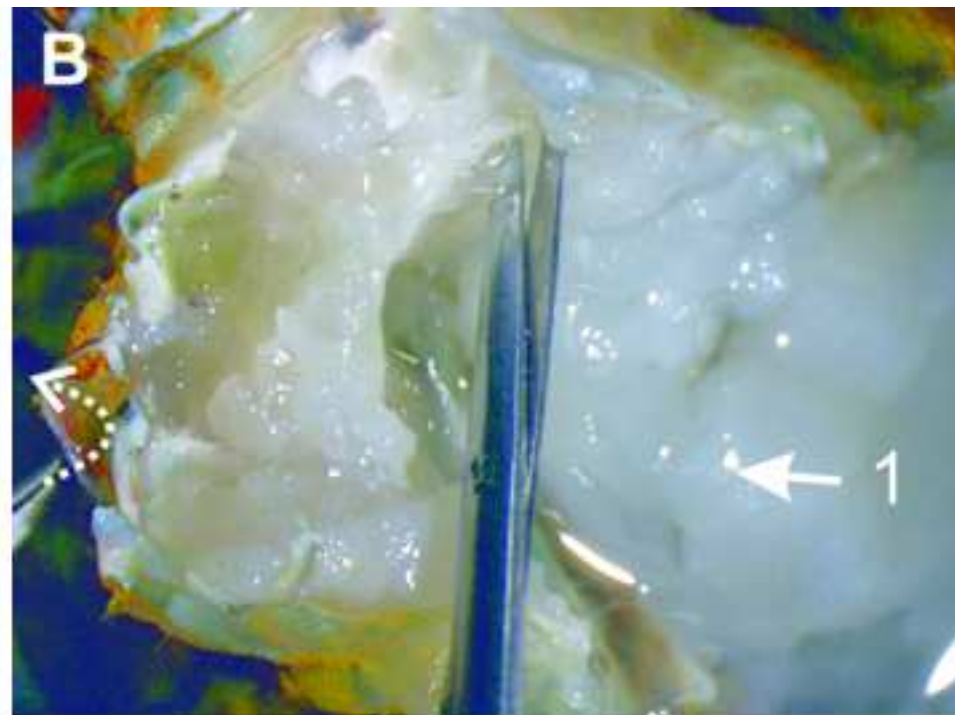
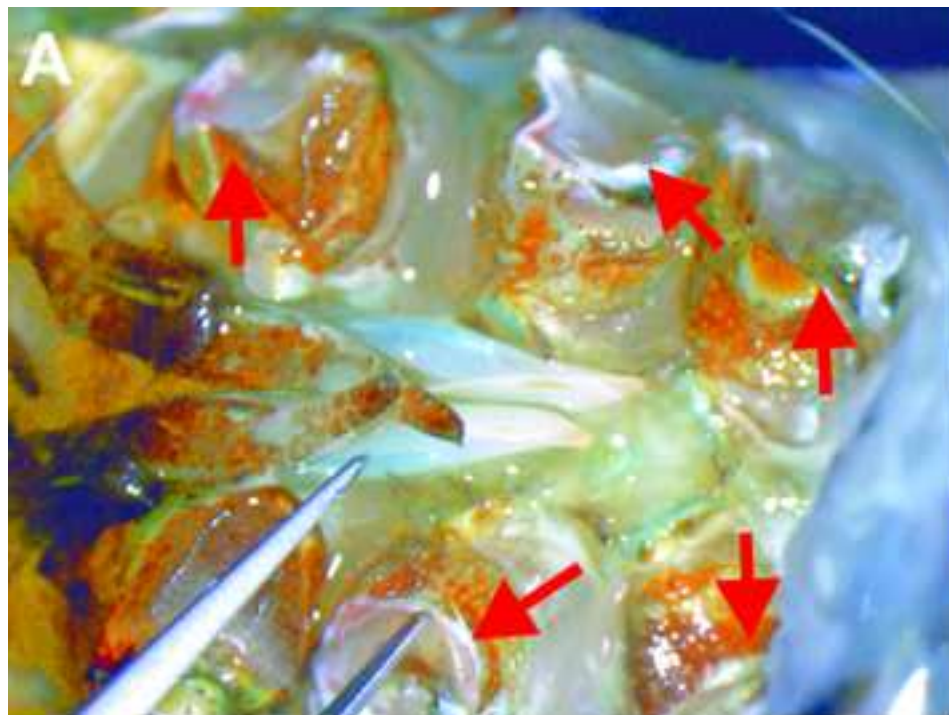


Figure 08
[Click here to download high resolution image](#)

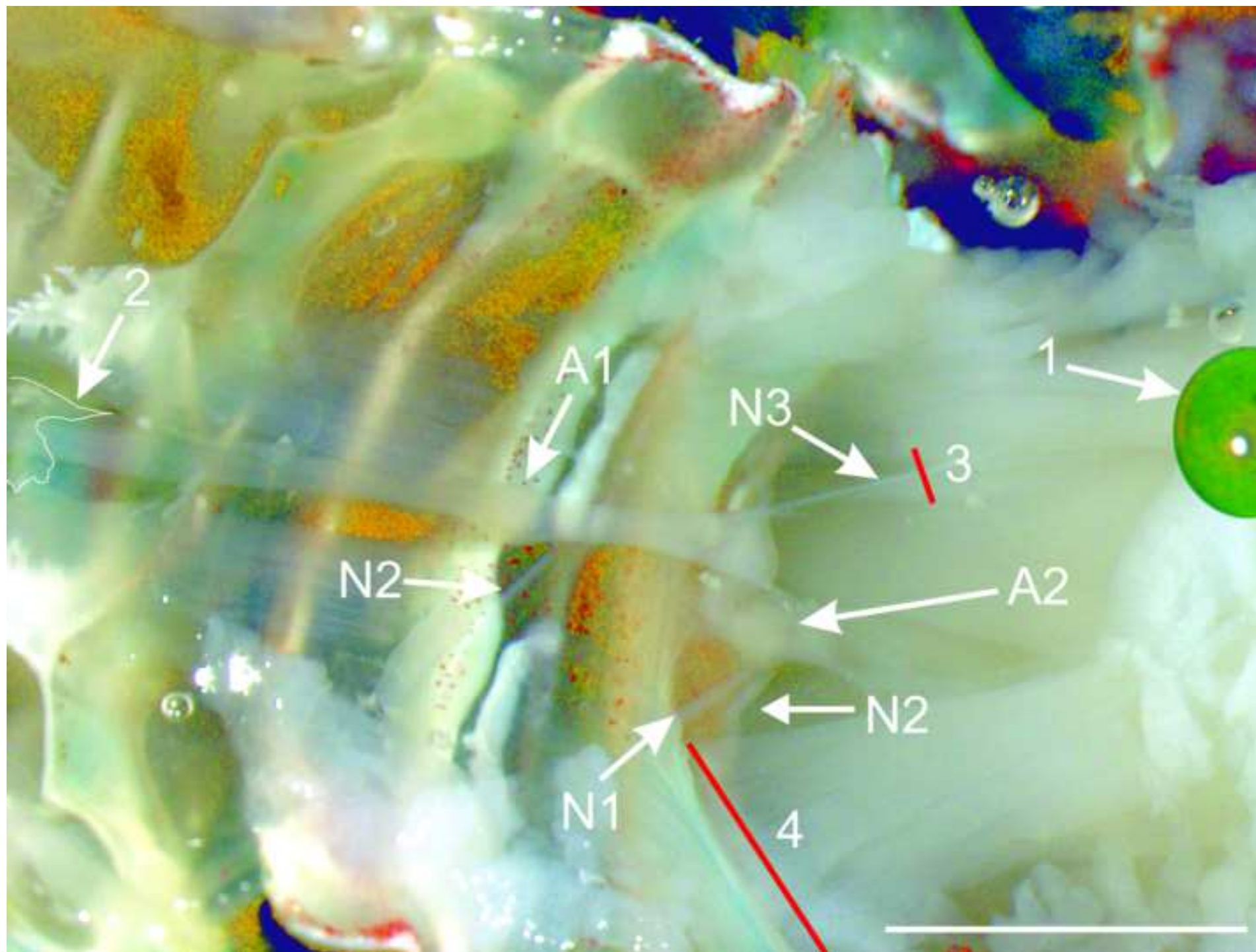


Figure 09
[Click here to download high resolution image](#)

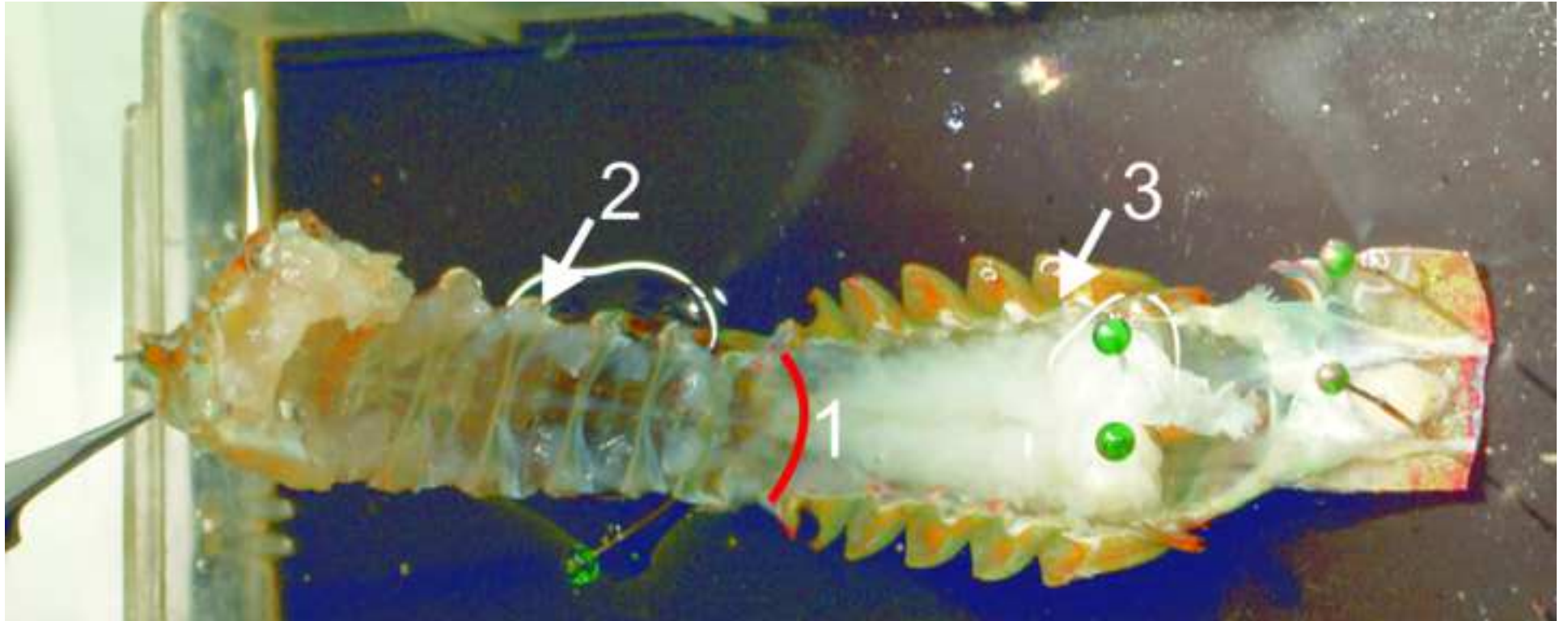


Figure 10
[Click here to download high resolution image](#)

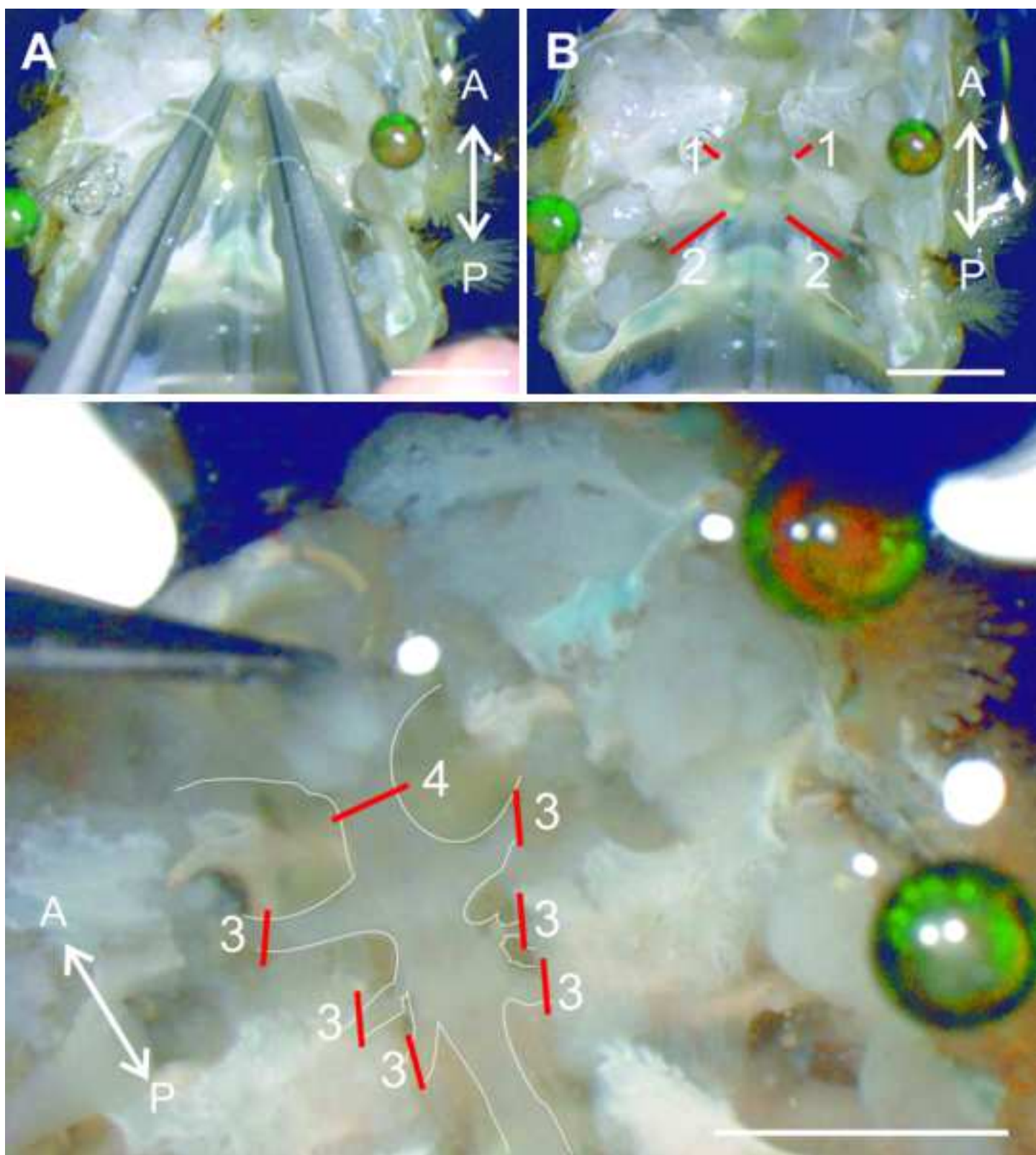


Figure 11
[Click here to download high resolution image](#)

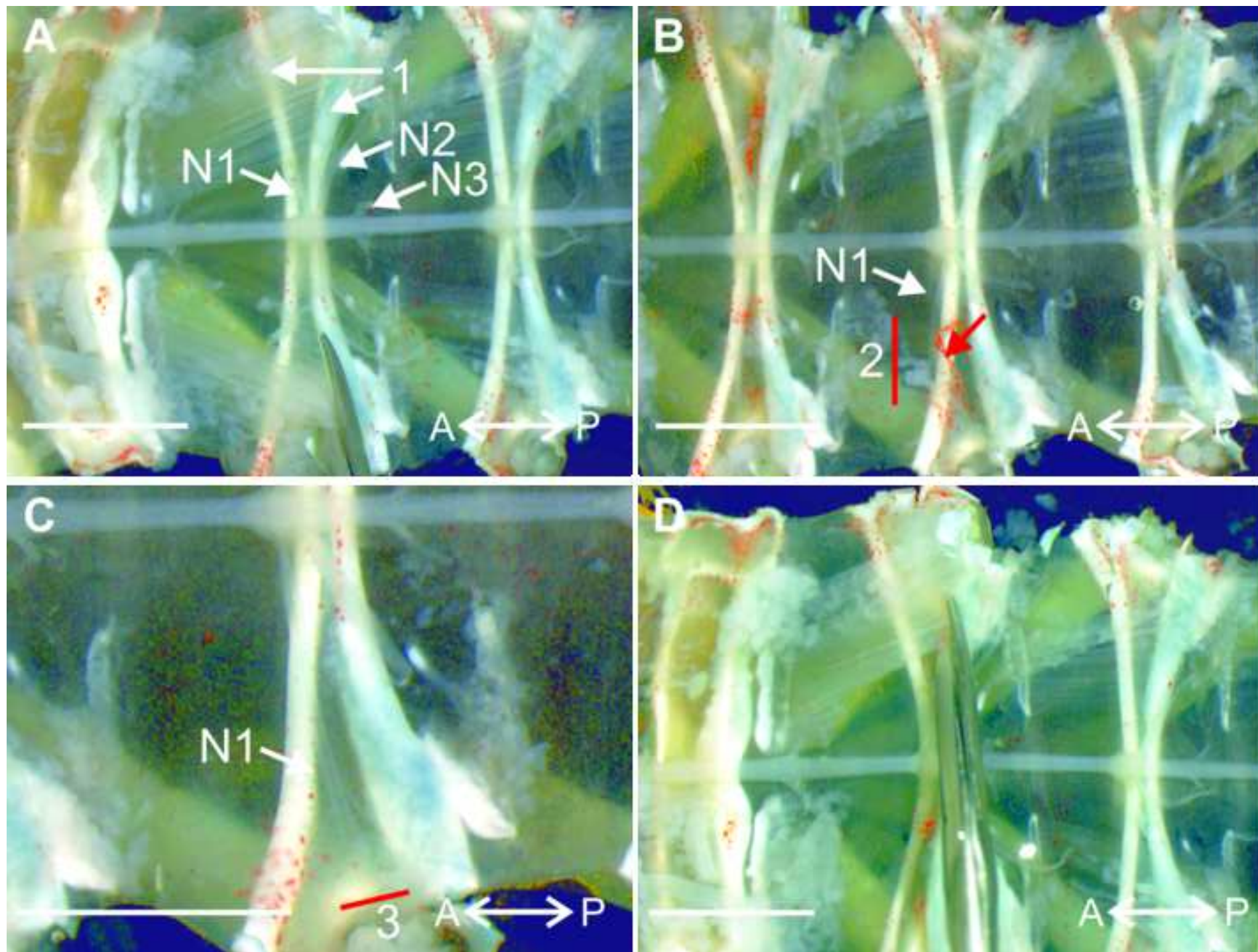


Figure 12
[Click here to download high resolution image](#)

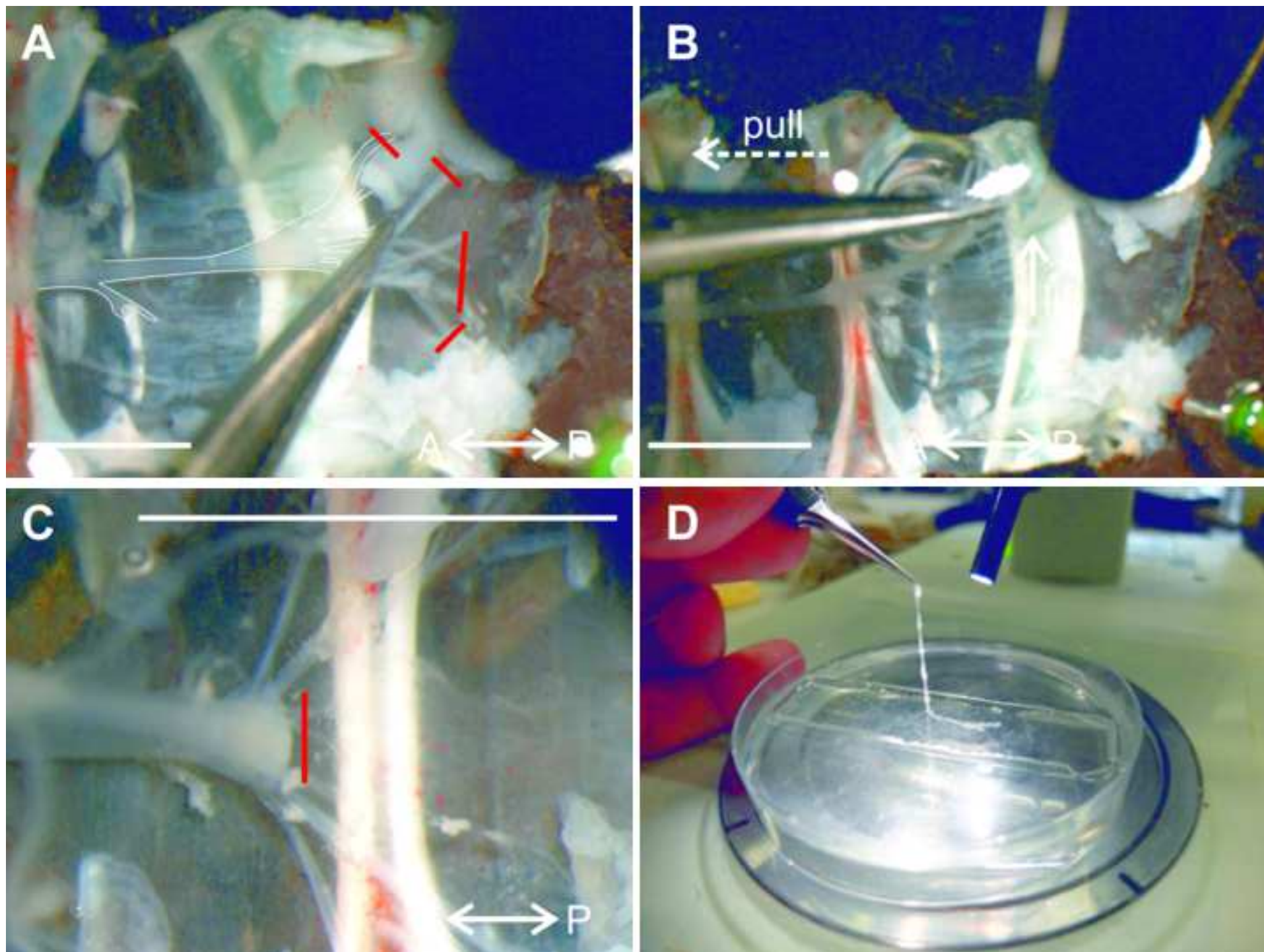


Figure 13

[Click here to download high resolution image](#)

Figure 14
[Click here to download high resolution image](#)

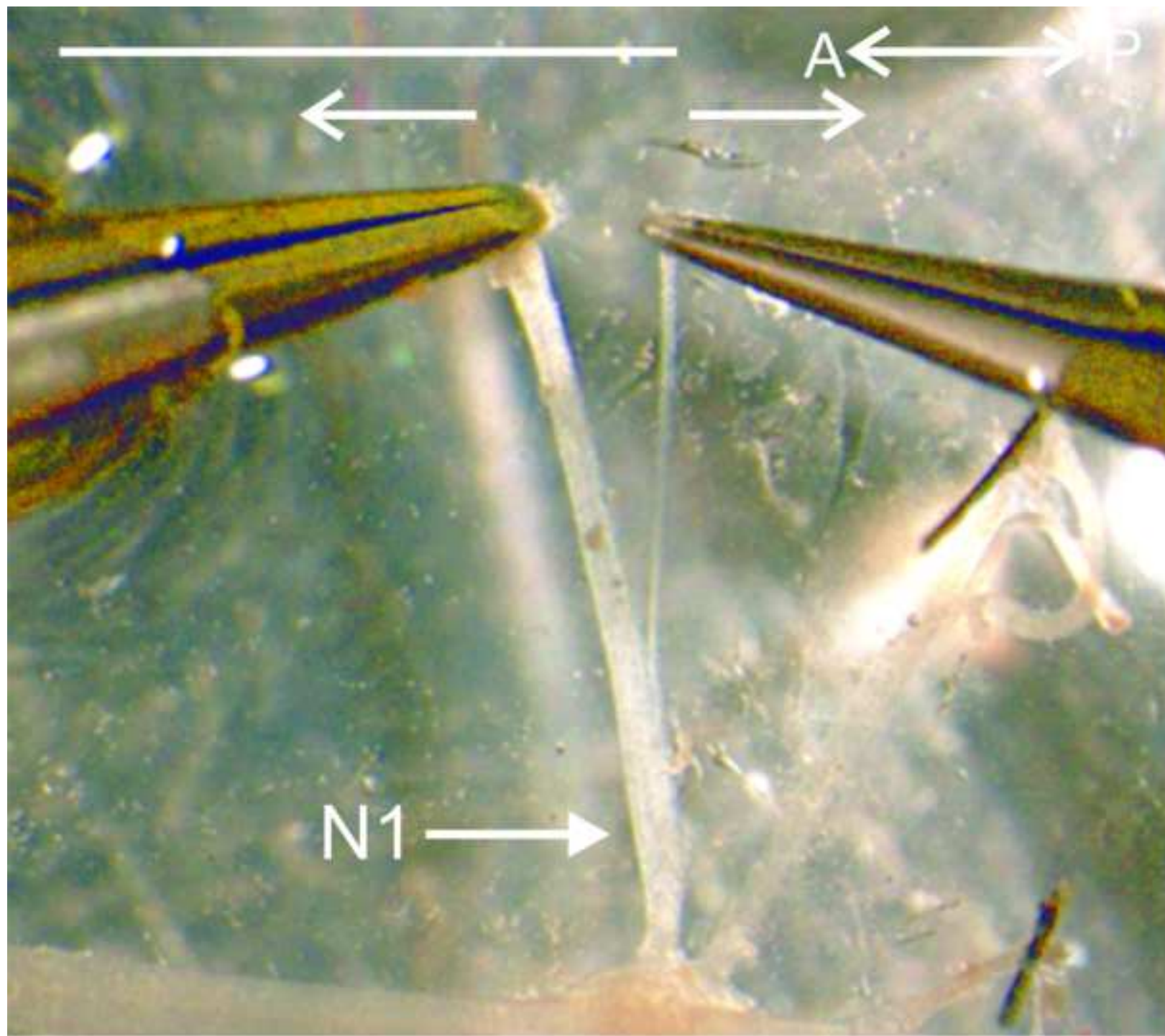


Figure 15
[Click here to download high resolution image](#)

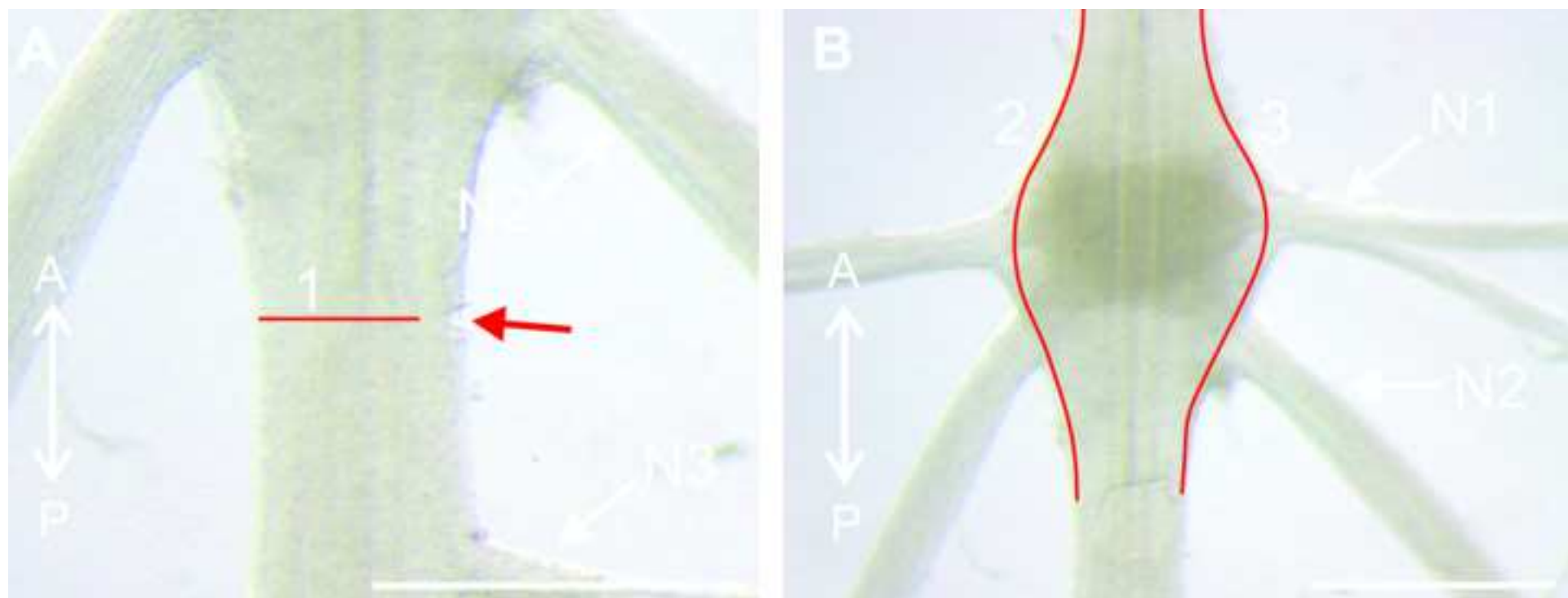


Figure 16
[Click here to download high resolution image](#)

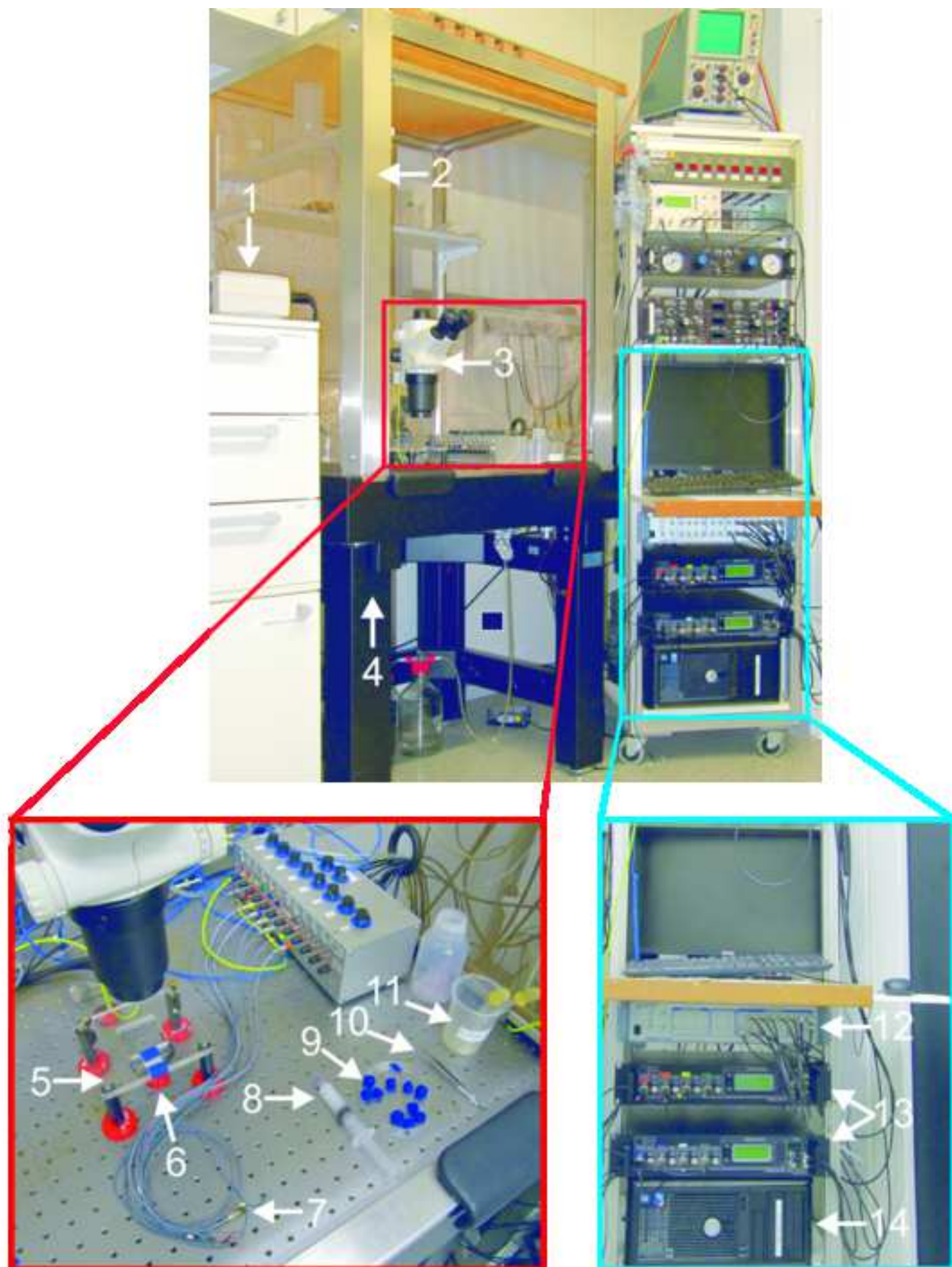


Figure 17
[Click here to download high resolution image](#)

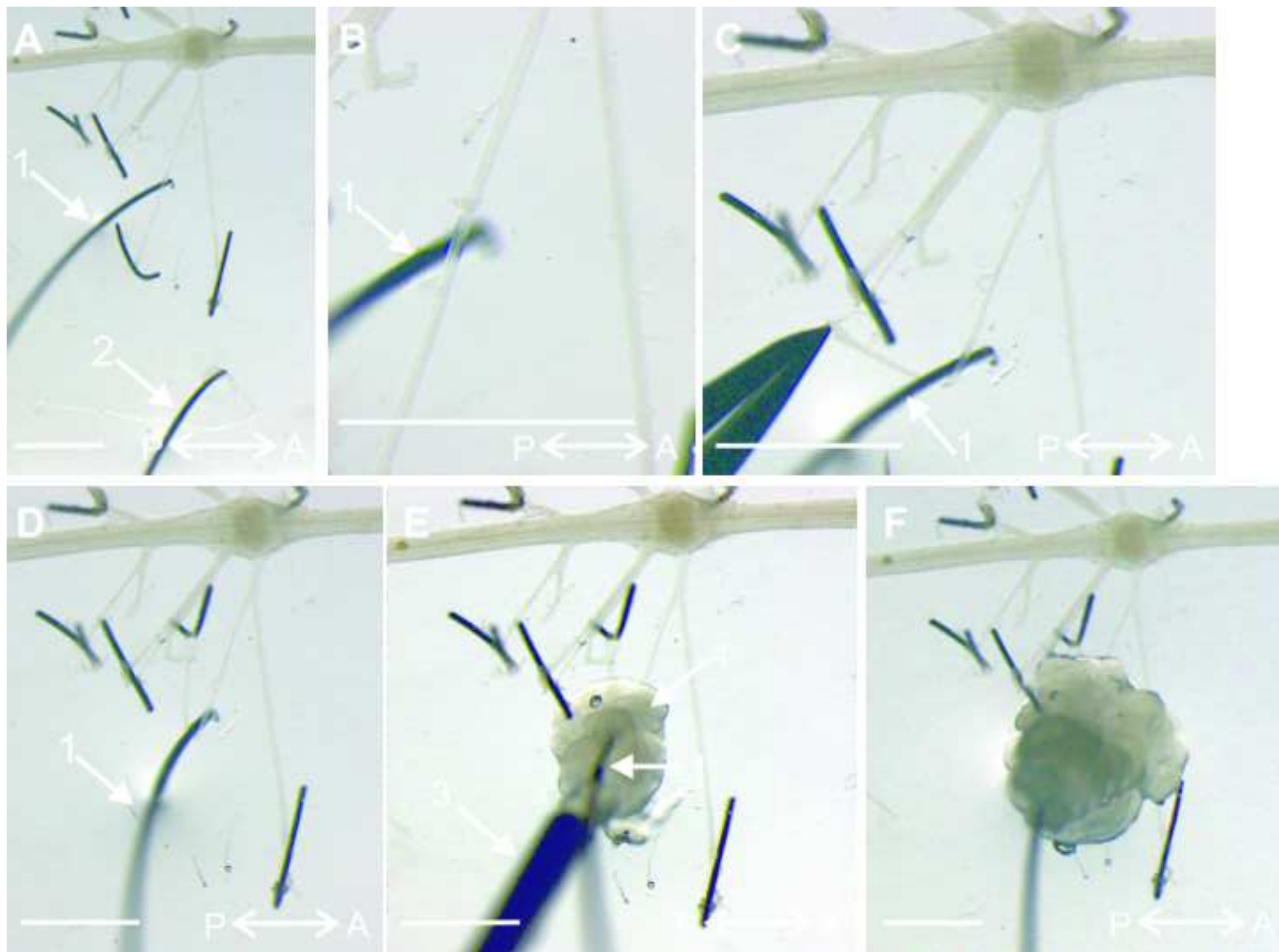


Figure 18
[Click here to download high resolution image](#)

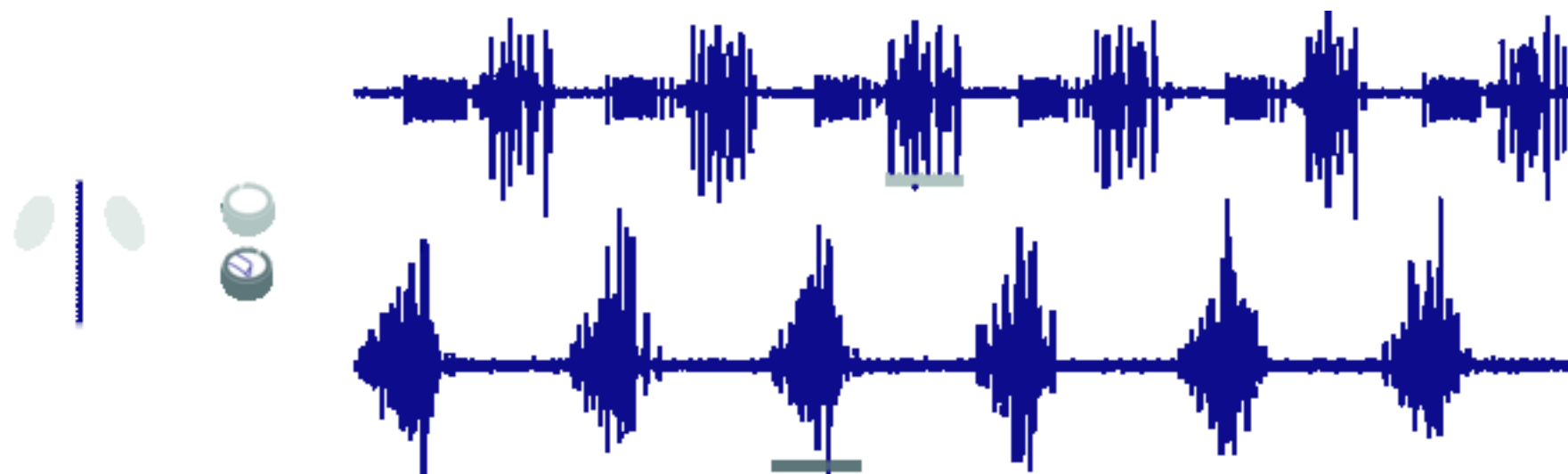


Figure 19
[Click here to download high resolution image](#)

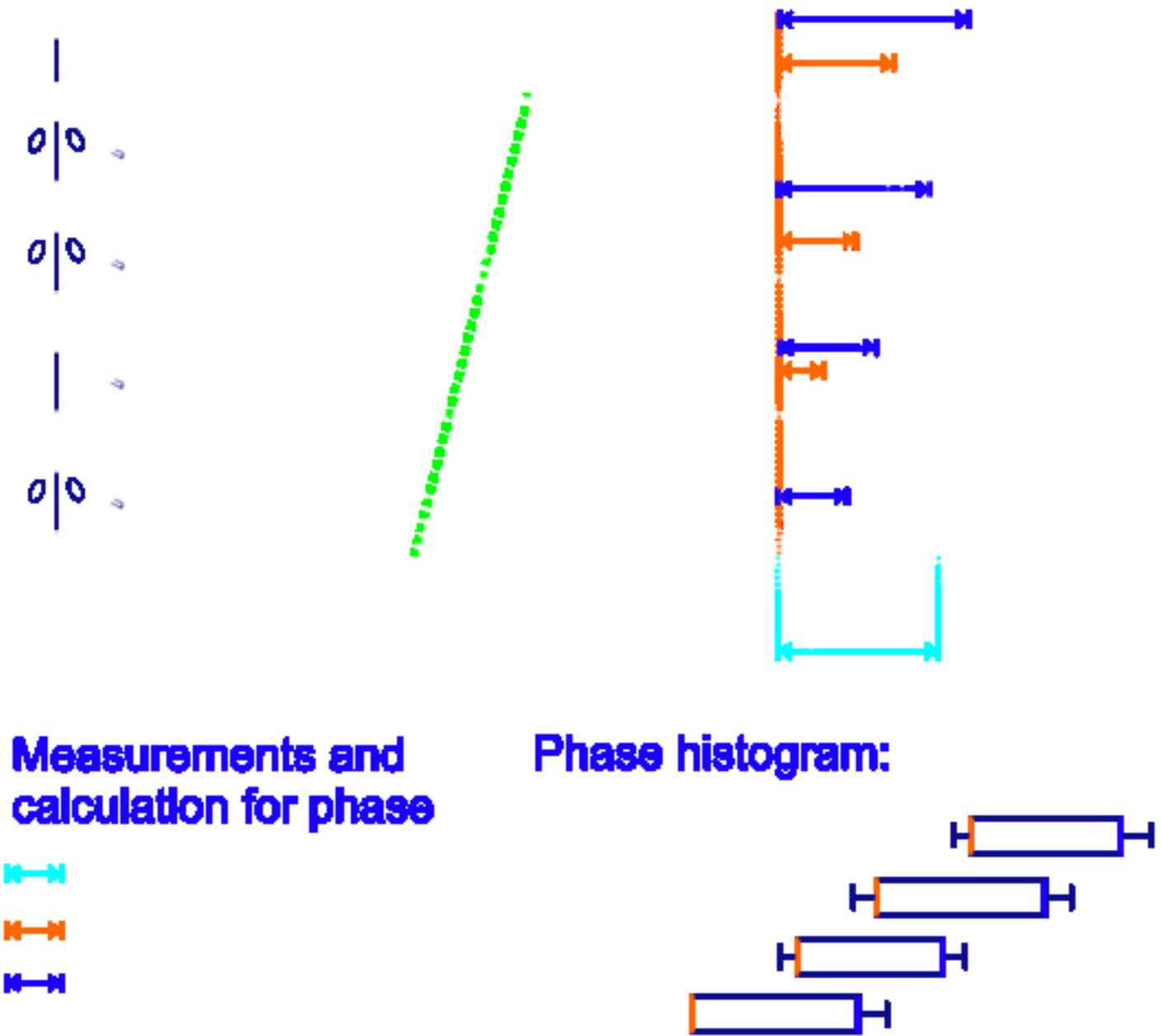


Figure 20
[Click here to download high resolution image](#)

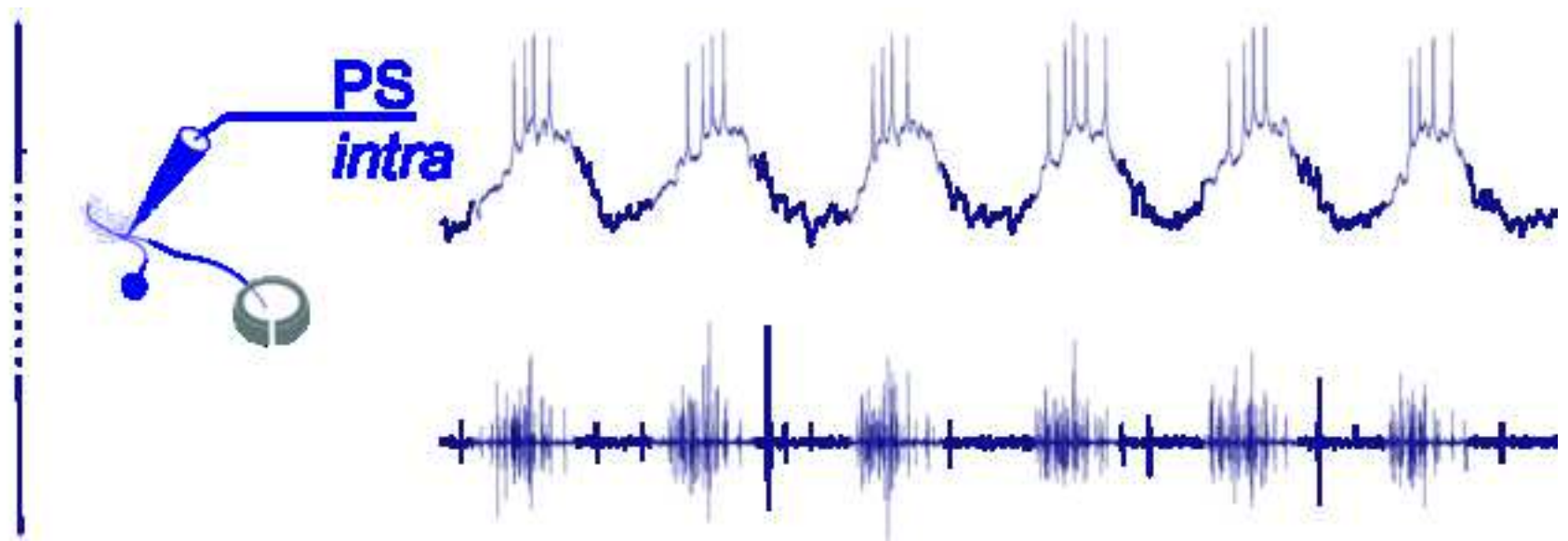


Figure 21
[Click here to download high resolution image](#)

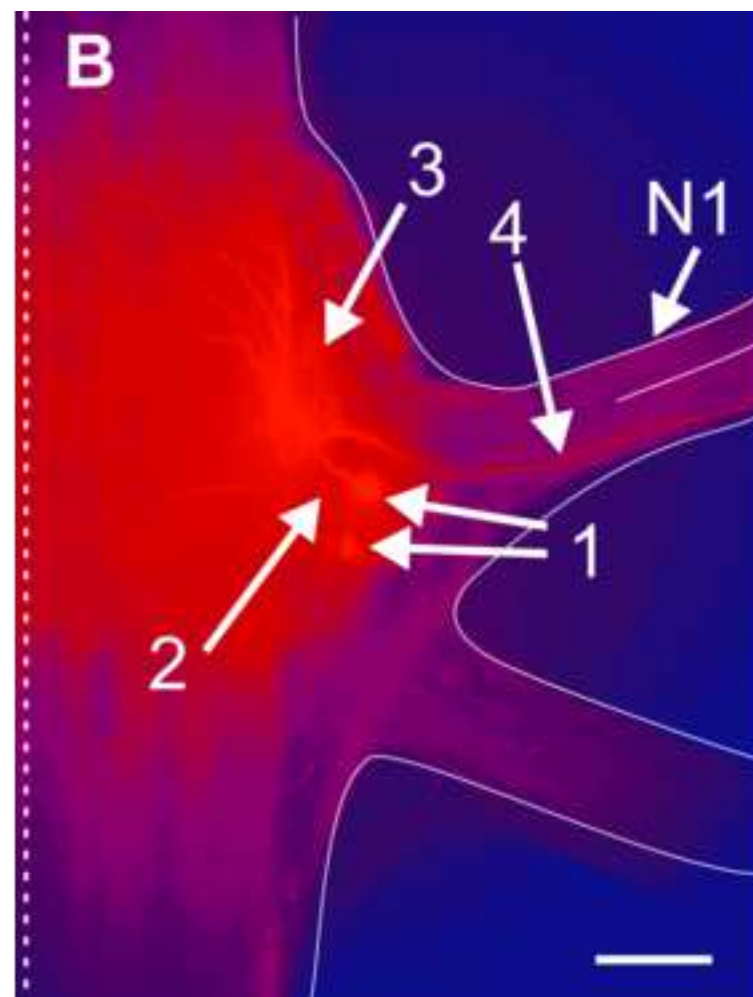
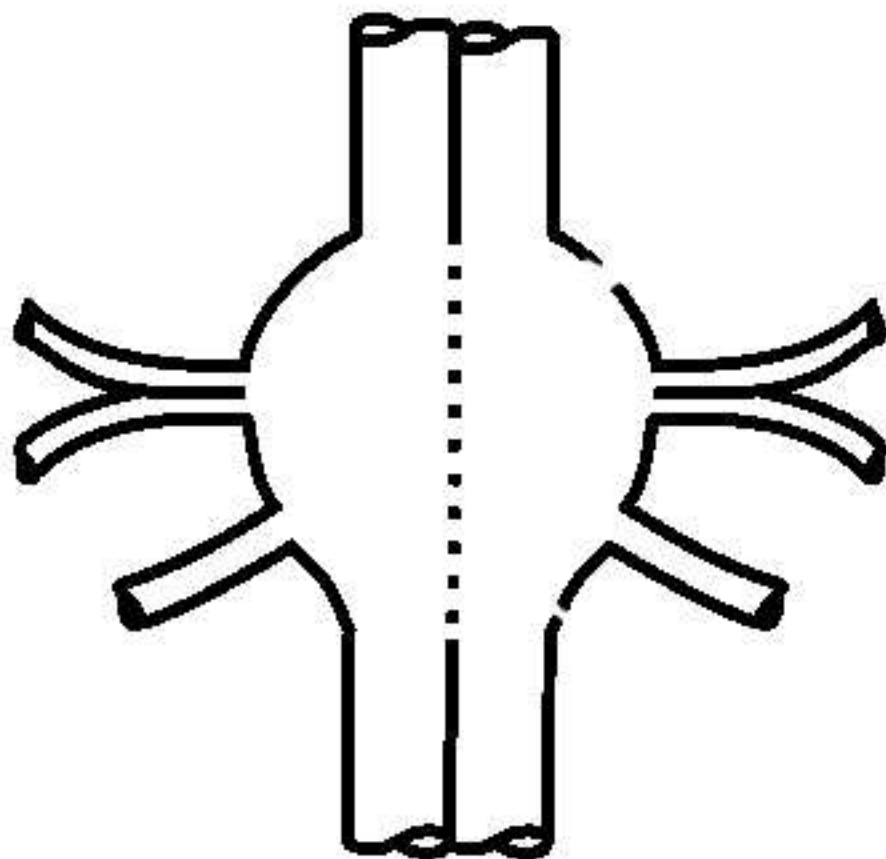
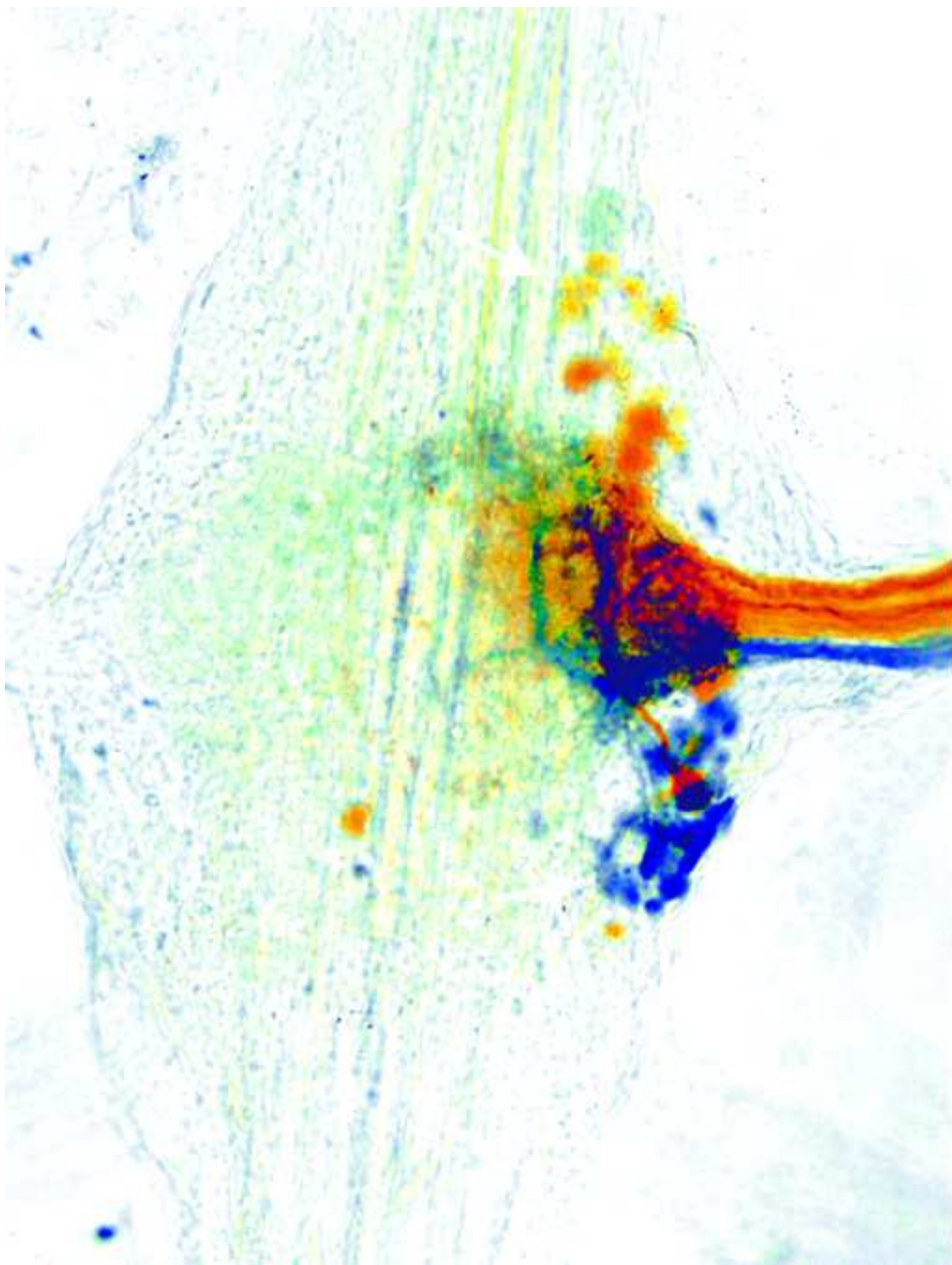


Figure 22
[Click here to download high resolution image](#)



Name of Material/ Equipment	Type	Company	Catalog Number	Comments/ Description
4-channel extracellular amplifier: MA 102	Amplifier	Elektroniklabor, Zoologie, Universität zu Köln, Germany		for extracellular recording
air-table		Technical Manufacturing Corporation (TMC) a unit of AMETEK Ultra Precision Technologies, Peabody, MA, USA	63-534	for intracellular recording
Axon Digidata 1440A	Digitizer	Axon Instruments, Molecular Devices Design, Union City, CA	DD1440A	digitizes recorded signals
big bucket				filled with ice
Clampex & Clampfit	pClamp 10, recording and analysis software	Molecular Devices Design, Union City, CA	pClamps 10 Standard	for extracellular recording
cold lamp source	with flexible light guide (fiber optic bundle)	Euromex microscopes holland, Arnhem, BD	LE.5211 & LE.5235	
computer and monitor	equipped with recording software			for extracellular recording
container and pipette for liquid waste				
crayfish saline	contains (in mM): 5.4 KCl, 2.6 MgCl ₂ , 13.5 CaCl ₂ , and 195 NaCl, buffered with 10mM Tris base and 4.7mM maleic acid; aerated for 3 hours. Adjust at pH of 7.4.			always keep at temperatures ~ 4° C
dextran, Texas Red (3000MW, lysine fixable)	fluorescent dye, lysine fixable	Life Technologies GmbH, Darmstadt, Germany	D3328	for intracellular dyefill of neurons
differential pin electrodes	made from stainless steel ϕ 0.2 mm			for extracellular recording
dissection dish	(l x w x h) 15x7x5 cm; lined with black silicone			used in the gross dissection
faraday cage				for extracellular recording
fixing pins				for pinning the specimen

forceps (biology, Dumont #5)	Forceps: Biology, tip 0.05 x 0.02 mm, length 11cm, INOX	Fine Science Tools (FST), Germany	11252-20	fine forceps: used to pick nerves
forceps (biology, Dumont #55)	Forceps: Biology, tip 0.05 x 0.02 mm, length 11cm, INOX	Fine Science Tools (FST), Germany	11255-20	extra fine forceps: used for desheathing
forceps (electronic, Dumont #5)	Forceps: Standard, tip 0.1 x 0.06 mm, length 11cm, INOX	Fine Science Tools (FST), Germany	11251-20	coarse forceps: used to grab specimen and pins
intracellular electrode	Borosilicate glass capillaries (outer/inner diameter: 1mm/0.5mm), with filament	Sutter Instruments, Novato, CA	BF100-50-10	for intracellular recording and dyefill of neurons
Leica S8 Apo StereoZoom	Dissection Microscope Zoom 1x - 8x	Leica, Germany	10446298	for extracellular recording
microscope table				for extracellular recording
mirror	to illuminate preparation from below			for extracellular recording
modeling clay				for extracellular recording
Olympus SZ61	Dissection Microscope Zoom 0.67x - 4.5x	Olympus, Germany		for the dissection
petri dish	94 x 16 mm; lined with clear silicone	Greiner bio-one, Germany	633180	used to pin the isolated chain of ganglia
ring scissors	ThoughCut, cutting edge: sharp/blunt, straight: 13cm	Fine Science Tools (FST), Germany	14054-13	for gross dissection (steps 2.1 - 2.11)
saline dispenser with a 16 gauge needle (outer ϕ 1.6mm) attached via a flexible tube.	Volume ~ 60ml,			used for exsanguination
spring scissors or alternative: Vannas spring scissors	cutting edge: 8 mm, tip diameter: 0.2mm, straight: 10cm or cutting edge 2.5 mm, tip diameter 0.075 mm, straight: 8cm	Fine Science Tools (FST), Germany	15024-10 or 15000-08	for desheathing
stainless steel wire ϕ 0.125 mm	to cut pins of 4-7 mm length	Goodfellow GmbH, Bad Nauheim, Germany		for pinning of the nerve cord

student Vannas spring scissors or alternative: Moria Spring Scissors	cutting edge: 5mm, tip diameter: 0.35mm, straight: 9cm or cutting edge: 5mm, tip diameter 0,1 mm, straight: 8 cm	Fine Science Tools (FST), Germany	91500-09 or 15396-00	for gross and fine dissection (steps 2.11 - 3.14)
sylgard	184 Silicone Elastomer Base and Curing Agent; for black sylgard add activated carbon	Dow Corning, Midland, MI, USA		
syringe filled with petroleum jelly and equipped with a 20 gauge needle with rounded tip				for extracellular recording



1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:

The swimmeret system of crayfish: A practical guide...

Author(s):

H. Seichter, F. Blumenthal, C. Smarandache-Wellmann

Item 1 (check one box): The Author elects to have the Materials be made available (as described at <http://www.jove.com/publish>) via: ☒ Standard Access ☐ Open Access

Item 2 (check one box):

- ☒ The Author is NOT a United States government employee.
- ☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.
- ☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: **"Agreement"** means this Article and Video License Agreement; **"Article"** means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; **"Author"** means the author who is a signatory to this Agreement; **"Collective Work"** means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; **"CRC License"** means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: <http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode>; **"Derivative Work"** means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; **"Institution"** means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; **"JoVE"** means MyJoVE Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments*; **"Materials"** means the Article and / or the Video; **"Parties"** means the Author and JoVE; **"Video"** means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4 and 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such

statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's

expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US\$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to be one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name: CARMEN SMARANDACHE-WELLMANN

Department: Emmy-Noether-Group / Zoological Institute

Institution: University of Cologne

Article Title: The swimmeret system of crayfish: A practical guide for the...

Signature: [Signature] Date: 03/13/2014

Please submit a signed and dated copy of this license by one of the following three methods:

- 1) Upload a scanned copy of the document as a pdf on the JoVE submission site;
- 2) Fax the document to +1.866.381.2236;
- 3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

Dear Dr. Nguyen,

Thank you for supporting our manuscript “The swimmeret system of crayfish: A practical guide for the dissection of the nerve cord and extracellular recordings of the motor pattern” (JoVE52109R2).

We revised the manuscript carefully and also reconsidered the highlighted sections. The highlighted Protocol reads now as a cohesive story.

Sincerely,

Carmen Smarandache-Wellmann Henriette Seichter and Felix Blumenthal.

Editorial comments:

The manuscript has been modified by the Science Editor to comply with the JoVE formatting standard. Please maintain the current formatting throughout the manuscript. The updated manuscript (52109_R2_051414.docx) is located in your Editorial Manager account under “File Inventory.” Please download the .docx file and use this updated version for any future revisions.

Changes made by the Science Editor:

1. There have been edits made to the manuscript.

Thank you for the helpful revisions.

Changes to be made by the Author(s):

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

We revised the manuscript carefully

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

We highlighted 2.75 pages of the Protocol with headings and spacing, see also attached document of the highlighted protocol.

3. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences).

The highlighted Protocol forms a cohesive narrative with logical flow. There are only complete sentences highlighted.