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TITLE:

Culturing rat sympathetic neurons from embryonic superior cervical ganglia for morphological and proteomic analysis

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KEYWORDS:

Superior Cervical Ganglia, Immunostaining, Proteomics, sympathetic, SCG, neurons, embryonic

SUMMARY:

This paper describes the isolation and culturing of embryonic rat sympathetic neurons from the superior cervical ganglia. It also provides detailed protocols for immunocytochemical staining and for preparing neuronal extracts for mass spectrometric analysis.

ABSTRACT:

Sympathetic neurons from the embryonic rat superior cervical ganglia (SCG) have been used as an in vitro model system for peripheral neurons to study axonal growth, axonal trafficking, synaptogenesis, dendritic growth, dendritic plasticity and nerve-target interactions in co-culture systems. This protocol describes the isolation and dissociation of neurons from the superior cervical ganglia of E21 rat embryos, followed by the preparation and maintenance of pure neuronal cultures in serum-free medium. Since neurons do not adhere to uncoated plastic, neurons will be cultured on either 12 mm glass coverslips or 6-well plates coated with poly-D-lysine. Following treatment with an antimitotic agent (Ara-C, cytosine β-D-arabinofuranoside), this protocol generates healthy neuronal cultures with less than 5% non-neuronal cells, which can be maintained for over a month in vitro. Although embryonic rat SCG neurons are multipolar with 5-8 dendrites in vivo; under serum-free conditions, these neurons extend only a single axon in culture and continue to be unipolar for the duration of the culture. However, these neurons can be induced to extend dendrites in the presence of basement membrane extract, bone morphogenetic proteins (BMPs), or 10% fetal calf serum. These homogenous neuronal cultures can be used for immunocytochemical staining and for biochemical studies. This paper also describes optimized protocol for immunocytochemical staining for microtubule associated protein-2 (MAP-2) in these neurons and for the preparation of neuronal extracts for mass spectrometry.

INTRODUCTION:

Sympathetic neurons derived from embryonic superior cervical ganglia (SCG) have been widely used as a primary neuronal culture system for studying many aspects of neuronal development including growth factor dependence, neuron-target interactions, neurotransmitter signaling, axonal growth, dendrite development and plasticity, synaptogenesis and signaling mechanisms underlying nerve-target/neuron-glia interactions[1–9]. Despite their small size (around 10000 neurons/ganglia), there are three main reasons for the development and extensive use of this culture system are i) being the first ganglia in the sympathetic chain, they are larger, and therefore easier to isolate, than the rest of the sympathetic ganglia[10]; ii) unlike central neurons, the neurons in the SCG are fairly homogeneous with all the neurons being derived from the neural crest, having a similar size, dependence on nerve growth factor and being nor-adrenergic. This makes them a valuable model for morphological and genomic studies[10,11] and iii) these neurons can be maintained in a defined serum-free medium containing nerve growth factor for over a month[10,12]. Perinatal SCG neurons have been extensively used for studying the mechanisms underlying the initiation and maintenance of dendrites[2]. This is mainly because, although SCG neurons have an extensive dendritic arbor in vivo, they do not extend dendrites in vitro in the absence of serum but can be induced to grow dendrites in the presence of certain growth factors such as bone morphogenetic proteins[2,12,13].

This paper describes the protocol for isolating and culturing embryonic rat SCG neurons. Over the past 50 years, primary neuronal cultures from the SCG have been mainly used for morphological studies with a limited number of studies examining the large-scale genomic or proteomic changes. This is mainly due to small tissue size resulting in the isolation of low amounts of DNA or protein, which makes it difficult to perform genomic and proteomic analyses on these neurons. However, in recent years, increased detection sensitivity has enabled development of methods to examine the genome, miRNome and proteome in the SCG neurons during dendritic growth development[14–17]. This paper will also describe the method for morphological analysis of these neurons using immunocytochemistry and a protocol to obtain neuronal protein extracts for mass spectrometric analysis.

PROTOCOL:

All procedures performed in studies involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) at Saint Mary's College of California. The animal care and use guidelines at Saint Mary's College were developed based on the guidelines provided by Office of Laboratory Animal Welfare at the National Institute for Health (https://olaw.nih.gov/sites/default/files/PHSPolicyLabAnimals.pdf and https://olaw.nih.gov/sites/default/files/Guide-for-the-Care-and-Use-of-Laboratory-Animals.pdf).

1. Preparation of culture media (also referred to as control medium)

1.1. Add the following ingredients, in the order listed below, to a sterile 500 mL flask: 190 mL of 1x low-glucose DMEM, 10 mL of 20 mg/mL fatty acid free bovine serum albumin(FAF-BSA) in

DMEM, 2.8 mL of 200 mM L-glutamine, 4 mL of 100x insulin-selenium-transferrin mixture, 0.4 mL of 125 µg/mL nerve growth factor (in 0.2% inert protein stabilizer in DMEM), and 200 mL of Ham's F-12 medium.

1.2. Make sure to coat the pipette with the medium containing FAF-BSA prior to the addition of protein-containing solutions such as insulin-selenium-transferrin and NGF to prevent sticking of proteins to the pipette.

1.3. Swirl the flask to mix the ingredients after each addition. Mix thoroughly with a 25 mL pipette after addition of F-12.

1.4. Aliquot the culture media in 10 mL aliquots and store at -80 °C. The aliquots can be stored for six months without loss of activity.

2. Preparation of plates for culturing neurons

2.1. Dilute a 1 mg/mL stock of poly-D-lysine (made in 0.5 M Tris buffer, pH 8) to 100 µg/mL with sterile distilled water.

2.2. For proteomic or genomic analysis, one to two days before the dissection, coat 6-well plates with approximately 2 mL of sterile 100 g/mL of poly-D-lysine (PDL). This is necessary to ensure cell adhesion to the well. Wrap the plates with cling film and store the plates overnight at 4 °C.

2.3. For morphological analysis and microscopy, place 12 mm^2 pre-treated German glass coverslips (which can be cleaned using nitric acid treatment as described previously[18] or purchased) into each of the wells of a 24-well plate.

2.3.1. Coat the coverslips with 0.3 mL of sterile 100 g/mL of poly-D-lysine (PDL). Store the plates overnight at 4 °C.

2.4. On the day of the dissection, before the start of the dissection, remove the poly-D-lysine solution from the wells and rinse the wells five times with sterile distilled water, followed by once with low glucose DMEM.

2.5. During the enzymatic digestion of the ganglia (approximately an hour before plating the cells), aspirate the DMEM from the plates and replace it with 0.3 mL of control media. Store the plates at 35.5 °C under 5% CO_2 in a humidified chamber.

3. Dissection setup

3.1. Preparation of 200 mL of media for dissection (referred to as Dissection media)

3.1.1. Add 8 mL of 20 mg/mL fatty acid-free bovine serum albumin (FAF-BSA) in low glucose DMEM (with 1 mg/mL glucose) and 2 mL of 100x penicillin-streptomycin to 193 mL of sterile Leibovitz's L-15 medium (or any air-buffered medium)

3.2. In the hood, set up the following items for dissection: four 50 mL sterile conical tubes with about 20 mL of dissection media in each tube, four sterile 35 mm dishes with 1.5 mL of dissection media, one sterile 50 mL conical tube with 20 mL of dissection media for centrifugation, one sterile 15 mL conical tube for centrifuging the ganglia, and one sterile 10 mL tube for collecting the dissociated cells.

3.3. Use a dry bead sterilizer to sterilize a pair of fine forceps (no.4 or no. 5 forceps) for at least one minute.

4. Isolation of the superior cervical ganglia from embryonic rat pups

4.1. Removal of E21 embryos from the pregnant rat

NOTE: Removal of the uterine horn can be performed outside of the hood if the surrounding area is thoroughly sterilized.

4.1.1. Euthanize the pregnant rat using CO_2 inhalation. Shear the fur from the abdominal region and wipe the skin in the area with 70% alcohol to sterilize it.

4.1.2. Using a fresh set of sterile scissors and forceps, cut through the skin and then the muscle layer to expose the uterine horns containing the embryos. Remove the uterine horns with the embryos using a new set of scissors and forceps, taking care not to damage the intestines in the process.

4.1.3. Transfer the uterine horns with the embryos into a 150 mm^2 sterile Petri dish and transfer them into the hood.

4.1.4. Using a new set of forceps and scissors, remove the embryos from the uterine horn and separate the embryos from the amniotic membranes and the placenta.

4.1.5. To euthanize the embryos, cut the spinal cord of the embryos along the midline under the right arm. This will also reduce the bleeding from the carotid artery during the removal of the SCG.

4.1.6. Transfer these embryos into the prepared 50 mL conical tubes containing dissection media. Make sure the embryos are submerged in the media. Each tube can hold up to 3 embryos.

4.2. Isolation of the superior cervical ganglion from the embryo

4.2.1. Transfer one pup from the dissection media onto a sterile 150 mm^2 Petri dish half-filled with solid substrate (either paraffin wax or silicone polymer), with its dorsal surface on the substrate. Using three sterile 23 G needles, pin the pup to the dish with one needle under each arm and a third needle through the mouth to carefully hyperextend the neck.

4.2.2. Cut through the skin in the neck region using sterile fine forceps (no. 4 or no. 5 forceps) to expose the salivary glands underneath. Remove these glands using fine forceps.

4.2.3. Locate the sternocleidomastoid and omohyoid muscles near the clavicle and trachea, respectively. First cut the transverse sternocleidomastoid muscles and then carefully cut the thin omohyoid muscle using fine forceps. Once these muscles are removed, the bifurcation in the carotid artery on the anterior end will be visible on either side of the trachea, with the SCG located under this fork in the carotid artery.

4.2.4. Using closed forceps, gently lift the carotid artery to visualize the SCG. Using one forceps on either side of the SCG, pull out the carotid and transfer it to the prepared sterile 35 mm^2 dishes. This tissue will most likely contain the SCG with the carotid artery, the vagus nerve with the nodose ganglia as well as other segments of muscle or fat in the area.

4.2.5. Repeat the dissection process on the other side. Remove SCGs from all embryos before continuing with the remaining dissection steps outlined below. Distribute the isolated tissues between two of the 35 mm^2 dishes to facilitate processing of the tissue samples.

4.3. Post-processing of SCG

4.3.1. To separate the SCG from the dissected tissue, first use fine forceps to remove any extraneous tissue, such as fat or muscle, taking care to avoid the area near the carotid bifurcation.

4.3.2. Once these tissues have been removed, two ganglia are visible. The nodose ganglion is smaller and circular, while the SCG is almond-shaped. Gently pull on the vagus nerve to separate the vagus nerve and nodose ganglia from the carotid and then separate the SCG from the carotid artery.

4.3.3. Use fine forceps to remove the capsule that surrounds the SCG. Transfer the SCG to a new 35 mm culture dish. Repeat the process with all the dissected tissue samples.

4.3.4. Coat a sterilized, cotton plugged glass pipette with dissection media to prevent the tissue from adhering to the pipette walls. Use the pipette to replace the dissection media with sterile 2 mL of Collagenase type II (1 mg/mL)/dispase type II (5 mg/mL) in calcium- and magnesium-free HBSS, and incubate for 50 min at 37 °C to help break down the tissues.

NOTE: The incubation time may need to be optimized with different batches of Collagenase/Dispase and usually ranges from 40 min to an hour.

4.3.5. During the incubation, aspirate the DMEM from the plates and replace it with 0.3 mL of control media. Store the plates at 35.5 °C under 5% CO_2 in a humidified chamber.

4.3.6. Following the incubation, transfer the SCGs in collagenase/dispase to a sterile 15 mL conical tube. Use the dissection media to rinse the plates and transfer the solution to the tube. Add enough dissection media to bring up the volume to approximately 10 mL.

4.3.7. Centrifuge at 200 x *g* (1000 - 1200 rpm) for 5 minutes at room temperature to pellet the sample. Aspirate the supernatant, taking care to not dislodge the pellet. Resuspend the pellet with 10 mL of dissection media. Repeat the centrifugation and discard the supernatant.

4.3.8. Replace with 1 - 2 mL of culture medium. Using a narrow-bore, bent-tip sterile pasteur pipette (pre-coated with culture medium), mechanically dissociate the clumps by gently triturating 5-6 times. Let the larger clumps settle for about one minute. Transfer the supernatant cell suspension to a new 10 mL tube.

4.3.9. Repeat this process 3 more times, with increasing force of trituration each time to ensure almost complete dissociation of the SCGs. Transfer the supernatant after each round of trituration to a 10 mL tube with the supernatant from the first trituration.

4.3.10. Add enough culture media to bring up the volume to 8 - 10 mL. Gently mix the cell suspension and quantify the cell density with a hemocytometer.

4.3.11. Distribute the cell suspension into the wells at the appropriate cell density for the experiments. Mix the cell suspension continually during the plating process to ensure even distribution of cells into the wells.

NOTE: For morphological analysis, plate the cells around 8,000 cells/well in a 24 well plates and for genomic and proteomic protocols, plate the cells as high as 30,000 cells/well.

4.3.12. Transfer the plates to a glass desiccator with sterile water at the bottom to create a humidified chamber. Inject enough CO_2 (around 120 mL) to obtain a 5% CO_2 environment in the desiccator, prior to sealing. Maintain the plates at 35.5 °C. This is referred to as Day 0 in the protocol.

NOTE: These plates can also be maintained in a regular 5% CO2 incubator. The method described above minimizes temperature and pH changes and also helps prevent cross-contamination.

5. Maintenance of the cultured SCG neurons and treatments

5.1. On Day 1 (24 hours after plating), carefully remove half of the culture media, and replace with 2 µM Ara-C (cytosine β-D-arabinofuranoside, an anti-mitotic agent). Leave the treatment on the cells for 48 h. Usually, this length of treatment is sufficient to eliminate non-neuronal cells in the culture.

5.2. On Day 3, gently aspirate half the medium and replace with control medium.

5.3. On Day 4, the cells are ready for experimental treatments. Feed cultures every other day with the appropriate medium, gently replacing half of the medium in the well with fresh medium.

5.4. Cultured SCG neurons in serum-free medium extend only axons. If experiments require the presence of dendrites, treat cells with either 10% fetal calf serum, 75-100 µg/mL of basement membrane matrix extract or 50 ng/mL of bone morphogenetic protein-7. Dendritic growth is observed within 48 hours of treatment with elaborate dendritic arbor observed within a week of treatment.

6. Immunostaining cultured SCG neurons

6.1. Gradually replace the cell culture medium in the well with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2 in a fume hood.

6.1.1. Remove half the culture media in the well and replace it gently with 4% paraformaldehyde. Then, repeat the process at least two more times, removing two-thirds of the media in the well each time and replacing with 4% paraformaldehyde. At the end of this process, the color of media in the well should have changed from pink to colorless.

6.1.2. Once all the medium has been replaced by 4% paraformaldehyde, incubate the wells for 15 - 20 minutes at room temperature.

6.2. With a similar process as described above, gradually replace the 4% paraformaldehyde solution with phosphate-buffered saline (PBS), pH 7.2. Leave for 5 minutes. Rinse the wells two more times for 5 minutes each with PBS.

6.2.1. Once the 4% paraformaldehyde is removed, move the plates to the benchtop for further processing. This step is a good stopping point where plates can be stored at 4 °C overnight.

6.3. Remove all the PBS. Add enough volume of 0.1% Triton X-100 in PBS to cover the cells. Leave it on the cultures for 5 minutes to permeabilize the neurons. The timing of this step is critical to ensure good staining while maintaining the integrity of the cells.

6.4. Carefully remove the Triton X-100 solution and rinse the wells three times for 5 minutes each time with PBS. Remove the PBS solution.

6.5. Add enough 5% BSA in PBS to cover the cells and incubate at room temperature for 20 minutes to reduce non-specific antibody binding.

6.6. Remove the blocking solution and replace it with a mouse monoclonal antibody against MAP-2 protein diluted in 5% BSA in PBS. Leave the primary antibody on the cells overnight at 4 °C.

6.7. Remove and save the primary antibody for reuse. The primary antibody can be reused at least twice without loss of detection intensity.

6.8. Rinse three times with enough PBS to cover the cells. Leave PBS on the cells for 10 minutes per rinse.

6.9. Remove the PBS solution and replace it with fluorescent tag-conjugated Goat anti-Mouse IgG secondary antibody at 1:1000 dilution in 5% BSA in PBS. Incubate for 2 hours in the dark at room temperature.

6.10. Remove the secondary antibody and replace it with PBS. Rinse the wells three times with PBS for 10 minutes per rinse. Rinse the wells one time with water to remove any salts.

6.11. Mount the coverslips onto glass slides containing a drop of aqueous mountant that is appropriate for fluorescently labeled samples. Store the slides at 4 °C, in a slide folder until ready for imaging.

7. Sample preparation for analysis of the proteome using liquid chromatography coupled with mass spectrometry

7.1. Lysis of cultured neurons

7.1.1. Gently remove all the culture media in the wells. Replace it with cold, sterile calcium and magnesium-free phosphate-buffered saline (PBS), pH 7.4. Remove quickly and replace with PBS and let it sit for 5 min. This step is done to remove any proteins present in the culture media.

7.1.2. Carefully remove all the PBS from all the wells for a particular treatment. Repeat the process one more time. Maintain plates over ice when lysing the cells to minimize neuronal damage and proteolysis.

7.1.3. Add 100 - 150 µL of 50 mM ammonium bicarbonate, pH 7.5 (NH_4HCO_3) to one of the wells and scrape cells using a sterile cell scraper. Using a micropipette, transfer the liquid and repeat the scraping process with all the wells for a particular treatment. Examine the wells under the microscope after scraping to make sure that most of the cells have been removed.

7.1.3.1. With the low number of neurons, use a limited volume to lyse the cells to ensure high enough concentration for proteomic analysis.

7.1.4. Freeze the solution at -80 °C overnight to help with cell lysis. The lysates can be stored at this stage until ready for further processing.

7.1.5. Once thawed, squirt the samples through a syringe with a 26 G or 28 G needle to mechanically lyse the cells.

7.1.6. Sonicate the samples in a sonicating water bath two times for 10 minutes. Add ice to the water bath to prevent overheating and denaturation of proteins. Centrifuge for 5 minutes at 4 °C at 12,000 x *g*.

7.1.7. Measure the protein concentration. Typical protein concentrations range from 0.4 - 1 µg/µL

7.2. Sample preparation and trypsin digestion for proteome analysis

7.2.1. Transfer a maximum of 60 µL of the lysate or 50 µg of protein into a DNase-, RNase- and protease-free 1.5 mL tube. If the volume of the lysate is less than 60 µL, add enough 50 mM ammonium bicarbonate to make up the volume.

7.2.2. Add 25 µL of 0.2% acid labile surfactant and vortex. Incubate the tube in a block heater at 80 °C for 15 minutes. Centrifuge the tube at 12,000 x *g* for 30 s.

7.2.3. Add 2.5 µL of 100 mM dithiothreitol and vortex. This makes the protein more accessible for alkylation and digestion. Incubate the tube at 60 °C in a block heater for 30 minutes. Cool to room temperature and centrifuge.

7.2.4. Add 2.5 µL of 300 mM iodoacetamide to the sample and vortex. This step helps to alkylate the cysteines and prevents them from reforming the disulfide bonds. Incubate the tubes at room temperature in the dark for 30 minutes.

7.2.5. Add mass spectrometry grade trypsin (0.5 µg/µL) to the tube at a trypsin: protein ratio of 1:10. Digest the samples at 37 °C overnight.

7.2.6. Add 10 µL of 5% trifluoroacetic acid (TFA) and vortex. Incubate the samples at 37 °C for 90 minutes. This step is necessary to hydrolyze the acid labile surfactant to prevent interference during mass spectrometry.

7.2.7. Centrifuge the samples at 12,000 x *g* at 4 °C for 30 minutes and transfer supernatant to a chromatography certified clear glass vial with pre slit Teflon/Silicone septum caps. Samples can be stored at -20 °C prior to mass spectrometry analysis.

7.2.8. Subject the sample vials to liquid chromatography coupled with high definition mass spectrometry.

REPRESENTATIVE RESULTS:

Isolating and maintaining neuronal cultures of embryonic SCG neurons

Dissociated cells from the rat embryonic SCG were plated in a poly-D-lysine coated plate or coverslip and maintained in serum free culture media containing b-nerve growth factor. The dissociated cells containing a mixture of neurons and glial cells look circular upon plating (**Figure 1A**). Within 24 hours of plating, the neurons extend small axonal processes with glial cells flattening and appearing phase-dark under phase contrast microscopy (**Figure 1B**). Following the treatment with Ara-C, 99% of the glial cells are eliminated and the cultures contain predominantly neuronal cells with an oval cell body, extensive axonal growth and no dendrites (**Figure 1C**).

Immunocytochemical staining of cultured embryonic rat superior cervical ganglia neurons

Previous studies have shown that cultured sympathetic neurons grown in serum-free medium extend only axons and only extend an elaborate dendritic arbor in the presence of basement membrane extract, 10% serum or 50 ng/mL bone morphogenetic protein-7 (BMP-7)[2,12]. In agreement with these observations, phase contrast microscopy shows that neurons grown in the presence of BMP-7 (50 ng/mL) for 5 days have a flattened cell body with multiple thick tapered processes when compared to neurons grown in serum-free control media (**Figure 2**). The identity of these processes as dendrites is confirmed by the presence of MAP-2, a cytoskeletal protein predominantly found in the cell body and dendrites[19,20]. Under control conditions, fluorescent staining for MAP-2 is observed within the cytoplasm and proximal axons and excluded from the nucleus (as evidenced by co-labeling with a nuclear stain to visualize the nucleus (**Figure 3A-3D**). Following treatment with BMP-7 at 50 ng/mL for 5 days, immunoreactivity for MAP-2 is observed not only in the cytoplasm but also in dendrites (**Figure 3E-3H**).

Sample proteomic analysis of cultured SCG neurons grown in control media

E21 rat sympathetic neurons were cultured in control media for 6 days in vitro, lysed and subjected to LC-MS analysis. The sample was run in three replicates on the mass spectrometer and the proteins were identified based on the number of fragmented peptides observed for each sample. The abundance of the proteins (ranging from 0 to over 300000 arbitrary units) and confidence score for protein ID based on the number of fragmented peptides observed for each protein (ranging from 5 - 500) was calculated. A sample data set from the mass spectrometric analysis of the proteome of 30 µg protein from SCG neurons cultured in serum-free control media is shown in **Figure 4**.

There were 13, 134 peptides detected in the sample which corresponded to 1287 proteins. Of these, 1100 of which were present in all three technical replicates with normalized abundance values greater than 500. Of these 1100 proteins, 90 proteins were identified by the presence of a single peptide hit, which only covered a small portion of the protein. Therefore, it was difficult to determine if the identification was correct and these proteins had a low protein confidence score (score <10).These proteins were eliminated from the analysis and the remaining 1010 proteins were further analyzed using Gene ontology to determine whether the protocol was successful in detecting proteins with different localization and function.

Gene ontology analysis of this dataset was performed using the Panther classification database (http://www.pantherdb.org/) to determine if this data set included proteins with different

cellular localization or molecular and biological functions and to examine whether there were the connections between the identified proteins and known pathways[21]. The analysis showed that although over 700 of the proteins were cytoplasmic, that data set contained proteins that were localized to other regions of the cell including the nucleus, the membrane, several cellular organelles, and cell junctions (**Figure 4A**). The analysis also revealed that over 400 proteins had catalytic activity, and around 300 proteins were involved in signaling pathways (**Figure 4B** and **Figure 4C**). In addition, the dataset contained proteins involved in various signaling pathways. **Table 1** shows five of the proteins in G-protein signaling, cell adhesion, growth factor signaling pathway, and synaptic vesicle trafficking pathways, respectively, that were identified in the dataset.

FIGURE AND TABLE LEGENDS:

Figure 1: Changes in the morphology of cultured E21 rat embryonic SCG neurons over time.
Representative phase contrast microscopy images at 10x magnification of dissociated cells from E21 rat SCG 20 min after plating (**A**), one day after plating (**B**) and after 48 hours of Ara-C treatment (5 days after plating) (**C**). Note the presence of glial cells (arrows) and axonal extension (arrow heads) in (**B**), and absence of glial cells and neurons showing extensive axonal growth in (**C**).

Figure 2: Changes in neuronal morphology of E21 rat SCG neurons following treatment with BMP-7
Following the elimination of non-neuronal cells, cultured SCG neurons were treated with control media (A) or BMP-7 at 50 ng/mL (B) for 5 days. Representative phase contrast micrographs (10x magnification) show the circular neuronal cell body with axons in control neurons (**A**) and neurons with multiple short dendrite-like processes when treated with BMP-7 (arrows, **B**).

Figure 3: Immunostaining for MAP-2 protein in cultured embryonic rat sympathetic neurons.
Following the elimination of non-neuronal cells, cultured SCG neurons were treated with control media (**A-D**) or BMP-7 at 50 ng/mL (**E-H**) for 5 days and immunostained using a mouse monoclonal antibody against MAP-2 and co-labeled with a nuclear marker. Representative micrographs showing immunocytochemical staining of the neurons with microtubule associated protein-2 (MAP-2) (**C,G**), a nuclear stain (**D,H**) with phase contrast micrographs (**B,F**) and a merge of the three channels (**A,E**). Immunocytochemical staining for microtubule associated protein-2 is present in the cell body and proximal axons of control neurons (**C**) and staining for MAP-2 protein in the cell body and dendrites in BMP-7 treated neurons (**G**).

Figure 4: Classification and distribution of proteins identified in the proteome analysis of cultured E21 rat SCG neurons
Protein IDs for the 1100 proteins were subjected to gene ontology analysis on Panther classification system to examine the distribution of proteins in the data set with respect to cellular localization (**A**), cellular function and biological processes they were involved in (**B**) and molecular function class based on protein structure (**C**).

Table 1: Representative proteins identified in the proteome data from cultured rat embryonic SCG neurons, classified by their biological functions

The proteome data with the 1100 proteins was subjected to gene ontology analysis using the Panther classification system. Listed below are the top five proteins identified for four biological pathways.

DISCUSSION:

This paper describes the protocols for culturing sympathetic neurons from superior cervical ganglia of embryonic rat pups. The advantages of using this model system are that it is possible to obtain a homogeneous population of neurons providing a similar response to growth factors, and since the growth factor requirements for these neurons has been well -characterized, it is possible to grow these neurons in vitro in defined media, under serum-free conditions[10]. Although the protocol describes the process for isolating SCG from E21 rat pups, this protocol can be used for dissection of SCG from rat pups from E17 to E21 and for dissection of embryonic mouse SCG[10, 22, 23]. This dissection protocol can also be used for isolating SCG from postnatal rats with minor modification to the initial steps. These modifications include elimination of need for C-section, euthanasia of the postnatal animals using carbon dioxide, and sterilization of the postnatal pups following euthanasia by immersion of the pups in 70% alcohol, prior to transferring them to dissection media[10]. This neuronal culturing protocol can also be adapted for studying axonal guidance, axonal transport and synapse formation using Campenot chambers and microfluidic chambers[24,25]. These neurons can also be co-cultured with neurons from the dorsal root ganglia or spinal motor neurons or with cardiomyocytes to study the neuronal interactions and neuron – target interactions in the peripheral nervous system[26–29]

The use of cultured SCG neurons for immunocytochemical studies is well documented. The protocol described in the paper provides a good starting point for working with most antibodies. However, there are antibodies, such as those that detect nuclear proteins which may require a modification to the fixation protocol and permeabilization protocol. In the case of phospho-SMAD antibody, treatment with 100% methanol at -20 °C has been used for fixing and permeabilizing the neurons[30, 31].

The main limitations of working in this model system stem from the small size of SCGs in embryonic rat pups, which results in small number of neurons. This results in a limited amount of nucleic acids or protein that can be obtained from the dissection of one litter of pups. This can be problematic for genomic and proteomic analysis, especially when comparing between multiple treatments in one dissection. Also, it is not possible to obtain SCG neuronal cultures from a single embryo. All the experiments are therefore performed on pooled samples that are obtained from SCG of all rat embryos in a litter. Another limitation of the system is that SCG neurons have lower DNA transfection efficiency (10 - 20%, unpublished observations) and higher toxicity following transfection compared to central neurons[32]. Although this transfection efficiency is fine for morphological studies with individual neurons, it is difficult to perform molecular or biochemical studies to detect/confirm gene expression changes.

However, in recent years, cultured SCG neurons have been used for studies examining the mechanisms underlying dendritic growth and for transcriptome and miRNome analyses[14, 15, 33]. As indicated earlier, due to low protein amounts, cultured neurons from embryonic SCG have not been used previously for proteomic analysis. This protocol and representative results provide evidence that even with low concentration of samples, current high definition mass spectrometry instruments could be used for proteomic studies on these neurons. Although the sample preparation protocol described here is for SCG neurons, this protocol can be used for preparation of any sample with low protein concentration for LC-MS analysis. One of the limitations is that small variations in protein concentration or efficiency of trypsin digestion can result in incomplete fragmentation and a dramatic decrease in the number of proteins detected by LC-MS. Therefore, it is critical to ensure that starting protein concentrations are as close to 50 µg with a 10:1 ratio of protein: trypsin. Also, it is a good practice to aliquot the mass spectrometry-grade trypsin to prevent multiple freeze-thaw cycles.

Another limitation of the protocol is that there were only a limited number of transmembrane proteins detected in the proteome. While the acid labile surfactant has been shown to be an effective surfactant for mass spectrometry, a previous study on cultured cells found that these surfactants can slightly increase the number of cytoplasmic proteins and decrease the membrane proteins in the proteomic profile[34] . Also, it has been suggested that pre-digestion using a bacterial endopeptidase such as Lys-C, prior to trypsin digestion may improve the efficiency of trypsin digestion of membrane bound proteins and prevent the membrane protein fragments from being retained on the LC columns[35].

In summary, this paper provides detailed protocols for growing cultured rat embryonic sympathetic neurons and for performing morphological and proteomic studies on these neurons.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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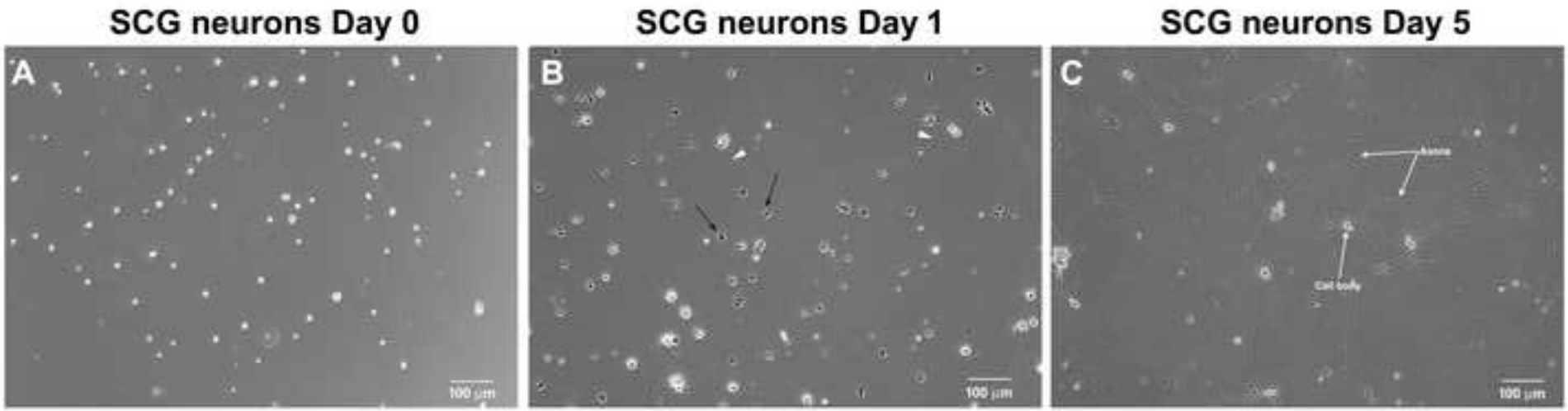

Figure 2

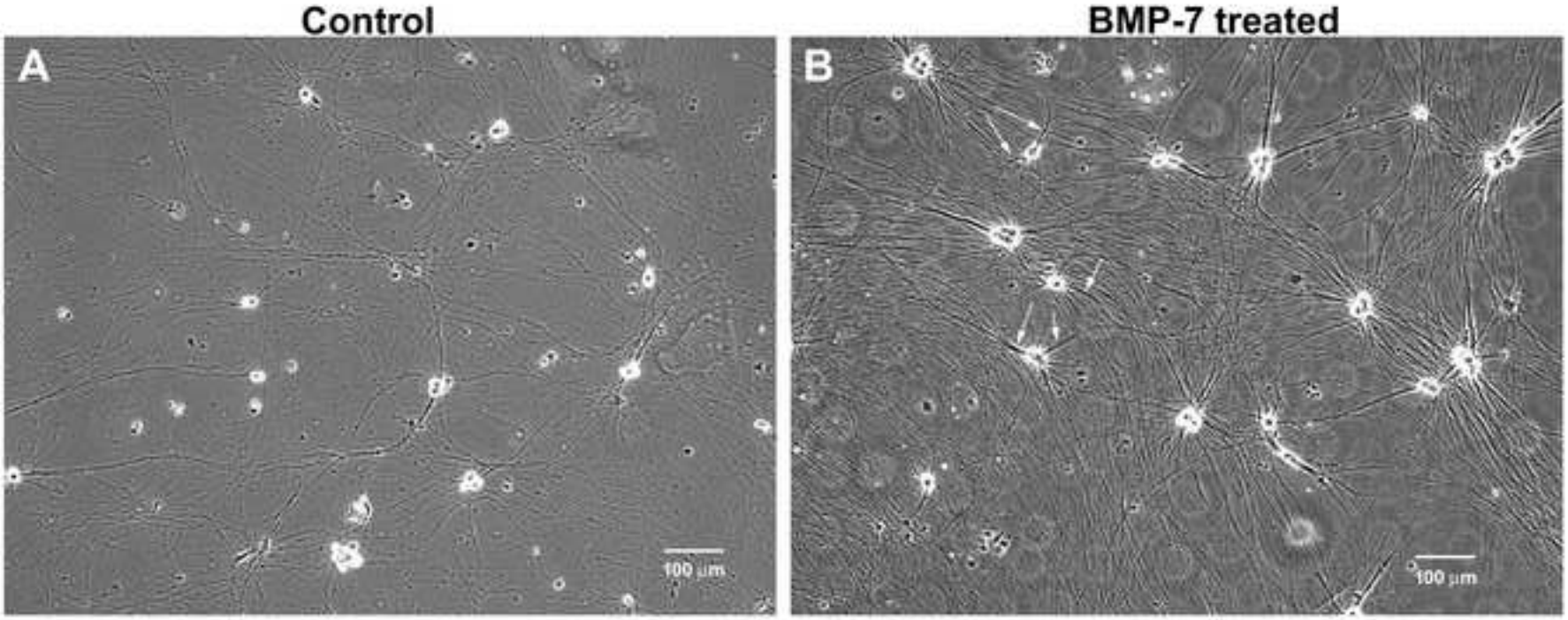

Figure 3

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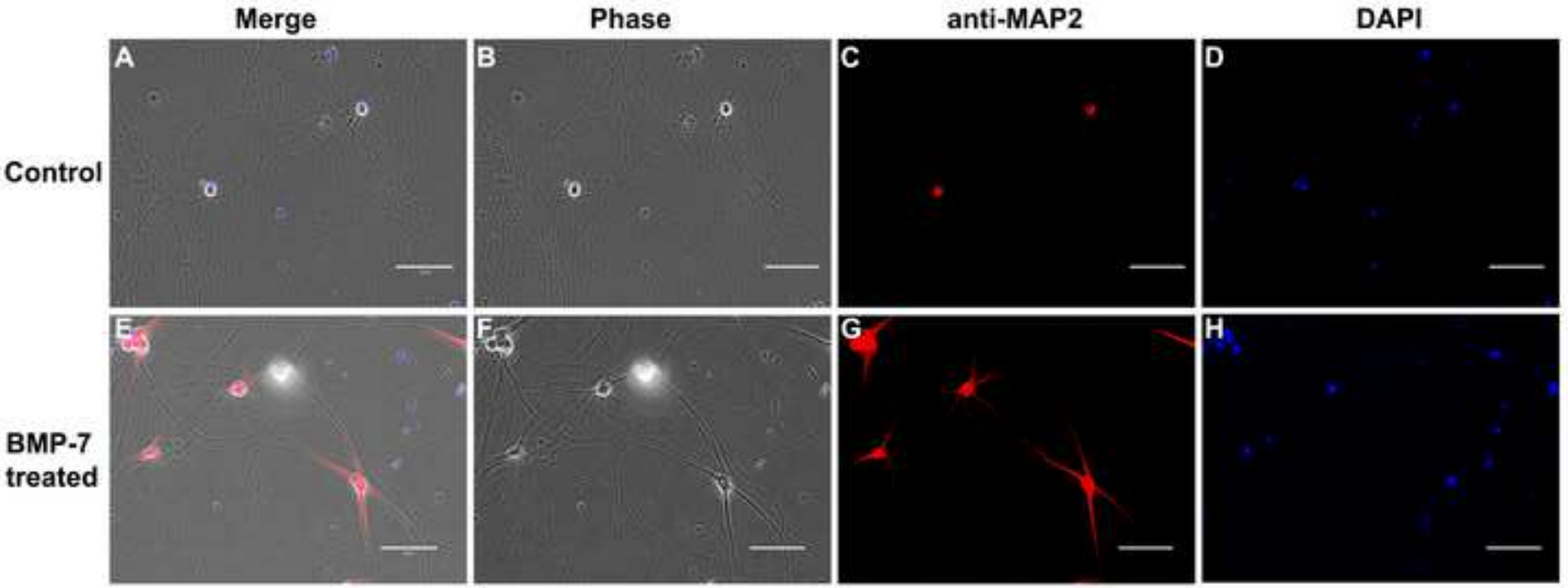

Table 1 : Examples of proteins identified in proteome analysis of lysates of cultured E21 rat SCG neurons

A) Proteins involved in biological adhesion		
UniProt Accession ID	**Gene name**	**Gene symbol**
Q9Z1Y3	Cadherin-2	Cdh2
O35112	CD166 antigen	Alcam
P49134	Integrin beta-1	Itgb1
D3ZES7	Plexin A4	Plxna4
F1LP44	Integrin subunit alpha L	Itgal
B) Proteins involved in growth factor signaling		
P35213	14-3-3 protein beta/alpha	Ywhab
Q5BJ92	Serine/Threonine-protein phosphatase 4 catalytic subunit	Ppp4c
P47196	Rac – alpha serine/threonine protein kinase	Akt1
P62994	Growth factor receptor bound protein 2	Grb2
P21708	Mitogen-activated protein kinase 3	Mapk3
C) Proteins involved in G-protein coupled signaling pathway		
P08753	Guanine nucleotide-binding protein G(k) subunit alpha	Gnai3
D4ABV5	Calmodulin-2	Calm2
P18266	Glycogen synthase kinase-3 beta	Gsk3b
P09456	cAMP-dependent protein kinase type I-alpha regulatory subunit	Prkar1a

P53534	Glycogen phosphorylase	Pygb (brain form)
D) Proteins involved in synaptic vesicle trafficking		
P47861	Synaptotagmin-5	Syt5
P63045	Vesicle-associated membrane protein 2	Vamp2
P61265	Syntaxin-1B	Stx1b
Q63537	Synapsin-2	Syn2
P60881	Synaptosomal-associated protein 25	Snap25

Table of Materials

Name of Material/Equipment	Company	Catalog Number	Comments/Description
2D nanoACQUITY	Waters Corporation		
Ammonium bicarbonate	Sigma-Aldrich	9830	
BMP-7	R&D Systems	354-BP	
Bovine Serum Alumin	Sigma-Aldrich	5470	
Cell scraper	Corning	CLS-3010	
Collagenase	Worthington Biochemical	4176	
Corning Costar or Nunc Flat bottomed Cell culture plates	Fisher Scientific	07-200, 140675, 142475	
Cytosine- β- D-arabinofuranoside	Sigma-Aldrich	C1768	
D-phosphate buffered saline (Calcium and magnesium free)	ATCC	30-2200	
Dispase II	Roche	4942078001	
Distilled Water	Thermo Fisher Scientific	15230	
Dithiothreitol	Sigma-Aldrich	D0632	
DMEM - Low glucose + Glutamine, + sodium pyruvate	Thermo Fisher Scientific	11885	
Fatty Acid Free BSA	Calbiochem	126609	20 mg/mL stock in low glucose DMEM
Fine forceps Dumont no.4 and no.5	Ted Pella Inc	5621, 5622	
Forceps and Scissors for Dissection	Ted Pella Inc	1328, 1329, 5002	
Glass coverlips - 12mm	Neuvitro Corporation	GG-12	
Goat-Anti Mouse IgG Alexa 488 conjugated	Thermo Fisher Scientific	A32723	
Ham's F-12 Nutrient Mix	Thermo Fisher Scientific	11765	
Hank's balanced salt soltion (Calcium and Magnesium free)	Thermo Fisher Scientific	14185	
Insulin-Selenium-Transferrin (100X)	Thermo Fisher Scientific	41400-045	

Iodoacetamide	Sigma-Aldrich	A3221	
L-Glutamine	Thermo Fisher Scientific	25030	
Leibovitz L-15 medium	Thermo Fisher Scientific	11415064	
Mounting media for glass coverslips	Thermo Fisher Scientific	P36931, P36934	
Mouse-anti- MAP2 antibody (SMI-52)	BioLegend	SMI 52	
Nerve growth factor	Envigo Bioproducts (formerly Harlan Bioproducts)	BT5017	Stock 125 μg/mL in 0.2% Prionex in DMEM
Paraformaldehye	Spectrum Chemicals	P1010	
Penicillin-Streptomycin (100X)	Thermo Fisher Scientific	15140	
Poly-D-Lysine	Sigma-Aldrich	P0899	
Prionex	Millipore	529600	10% solution, 100 mL
RapiGest SF	Waters Corporation	186001861	5 X 1 mg
Synapt G2 High Definition Mass Spectrometry	Waters Corporation		
Trifluoro acetic acid - Sequencing grade	Thermo Fisher Scientific	28904	10 X 1 mL
Triton X-100	Sigma-Aldrich	X100	
Trypsin	Promega or NEB	V511A, P8101S	100 μg or 5 X 20 μg
Waters Total recovery vials	Waters Corporation	186000385c	

Response to Reviewer Comments – JoVE61283R1

We would like to thank the reviewers for their helpful suggestions, which have improved the manuscript. Below are our detailed responses to their comments and suggestions.

Editorial comments:

Changes to be made by the Author(s) regarding the written manuscript:
1. Figure 1: Please provide scale bars.

Response: Figure 1 has been updated with scale bars.

Changes to be made by the Author(s) regarding the video:
1. Video Delivery:
• The duration of the video should be 15 minutes or less.
2. Editing & Narration:
• General note: This is a long protocol. There are sections that are well covered with dissection or pipetting footage and the rest are lacking in visual content. Additionally, for this video to pass our video production quality control and ultimately be published, the video must be less than fifteen minutes. Filming and editing in more video is not going to shorten the video, so I would suggest this video be examined in a broad sense by the authors to determine the real "heart of the protocol" and to address the essential questions of: "What is the most important thing to show the viewers, and what can be compressed, omitted, or referred to in the manuscript?" Some notable areas lacking in visual content and places that may benefit from omission from the video are given below:
• 00:08-01:25 This first protocol section has verbal instructions for preparation but no visuals other than a reagent list. This section would benefit greatly from two changes: 1. It's probably not necessary to read all of the reagents out loud, just make sure you emphasize any important or novel ones and leave the text on-screen long enough for it to be read. 2. Add shots of the specific instructions you're issuing here. It would help greatly in us visualizing what is being said.
• 01:31-02:57 This time range consists of two shots of the talent placing a substance into the wells, but there are many more instructions that follow that no longer correspond to what we're seeing on screen.. All of these steps that are mentioned in the verbal protocol need visuals to correspond to them- there's too much information being given here without enough visual correspondence, and the shot of coating the plates becomes irrelevant a few moments after the narration moves on to the next instructions. We strongly suggest additional shooting and editing in this section to better illustrate what is being said.
• 02:57-04:10 In this time range, the reagents and tools for dissection are introduced on a text card and on still images of the hood setup. Consider cutting down or eliminating this section since it will be somewhat redundant when following along in the manuscript and also since the tools and materials will be mentioned when they are used in the protocol. You can compress sections like this down to narrations along the lines of: "Refer to the manuscript for instructions on setting up the hood workspace and preparing the dissection media." (Something like that.) This narration would then go at the beginning of the next section, which will show the use of the materials and media.
• 13:26-18:03 This time range consists of written on-screen instructions accompanied by an audible narration of the text. Having these sections in the video without any real visuals is not ideal

and questionable in terms of necessity. Consider filming and editing these steps into the video if they are important or even eliminating these sections by pointing the viewers to the manuscript.

3. Please ensure that a protocol is presented in the protocol section of the video at all times. A powerpoint

Response:
Thank you for your suggestions regarding video edits.

We have edited the video to ensure that the focus of the video is on the isolation and culturing of sympathetic neurons. Therefore, we have eliminated the details pertaining to media preparation (00:08-01:25) and pertaining to methods for immunocytochemistry and proteomics (13:26 – 18:03) and included a statement referring to the manuscript for detailed protocols. In addition, we have added visuals to the plate treatment (1:31 – 2:57). We have also included additional arrows during the dissection to identify the different tissues being removed during the process. The updated video has been uploaded onto Dropbox.