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The Pseudopod System for Axon-Glia Interactions: Stimulation and Isolation of Schwann Cell Protrusions that Form in Response to Axonal Membranes

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Abstract

In the peripheral nervous system, axons dictate the differentiation state of Schwann cells. Most of this axonal influence on Schwann cells is due to juxtacrine interactions between axonal transmembrane molecules (e.g., the neuregulin growth factor) and receptors on the Schwann cell (e.g., the ErbB2/ErbB3 receptor). The fleeting nature of this interaction together with the lack of synchronicity in the development of the Schwann cell population limits our capability to study this phenomenon *in vivo*. Here we present a simple Boyden Chamber-based method to study this important cell-cell interaction event. We isolate the early protrusions of Schwann cells that are generated in response to juxtacrine stimulation by sensory neuronal membranes. This method is compatible with a large array of current biochemical analyses and provides an effective approach to study biomolecules that are differentially localized in Schwann cell protrusions and cell bodies in response to axonal signals. A similar approach can be extended to different kinds of cell-cell interactions.

Keywords

Axon-Schwann cell interaction; Boyden chamber; Neuronal membranes; Protrusions; Juxtacrine interaction; Early cell-cell contact; Pseudopod

1 Introduction

The prerequisite to myelination is the recognition of large axons by immature Schwann cells. Schwann cells surround bundles of mixed caliber axons and send cytoplasmic protrusions between them to select and segregate large axons that will be myelinated [1]. The final myelin fate of an axon and the thickness of myelin sheath around it depend on the amount of neuregulin-1-type III present on the axonal membrane, in a strictly contact-dependent signaling event [2, 3]. Upon sensing neurons, Schwann cells probably respond with localized amplification of signaling pathways [2, 4–8] and enrichment of specific

proteins [9–13] at the Schwann cell- axon interface. Although localized signaling is critical for the interaction between axons and Schwann cells, the molecular mechanisms that mediate this response remain poorly defined. To investigate the mechanisms of Schwann cell-axon recognition and interaction, we adapted the pseudopod system to the study of juxtacrine signals [13]. The original pseudopod system physically separates cell bodies, in the top compartment of a Boyden chamber, from protrusions, extending in the bottom chamber in response to soluble signals [14]. The cell body and protrusion compartments can then be isolated and compared by differential biochemical analyses. Our modification consisted of using a suspension of neuronal membranes as a stimulus [13], postulating that neuronal membranes are a source of active molecules that elicit juxtacrine signals. Here, we provide detailed methods for the effective purification of Schwann cell protrusions using these pseudopod chambers with microporous filters and for identification of the RNAs and proteins that are enriched in protrusions. These methods include the preparation of the neuronal membrane suspension, the isolation of primary Schwann cells, and the induction of Schwann cell protrusions in response to neuronal membranes. This method can be applied in principle to any type of biologically active cell membrane.

2 Materials

2.1 Dissections

1. Horizontal laminar airflow hood.
2. Dissecting microscope.
3. Cell incubator, 37 °C with 5% CO₂.
4. Centrifuge, refrigerated benchtop.
5. Dissection tools, including one set for macrodissection (one blunt forceps and one medium-sized dissecting scissors) and a microdissection set (two blunts forceps and one #5 dissecting forceps) (*see* Note 1).
6. Beaker, 50 ml, sterile, filled with 70% ethanol and with gauze in the bottom (*see* Note 2).
7. Petri dishes, 10 cm diameter.

2.2 Isolation of Primary Schwann Cells

1. Eight Sprague-Dawley rat pups, at postnatal day 3 (P3) (*see* Note 3).
2. Hemocytometer.
3. Conical centrifuge tubes (15 ml).
4. Hank's Balanced Salt Solution (HBSS).
5. Dulbecco's phosphate-buffered saline (D-PBS).
6. 1 g/l collagenase solubilized in H₂O (*see* Note 4).
7. 2.5% trypsin.

8. 10,000 U/ml penicillin/streptomycin.
9. Antibody, anti-Thy1.1 (Sigma, M7898).
10. Complement, rabbit serum, filtered.
11. PLL solution: 0.01 g/l poly-L-lysine solubilized in H₂O.
12. Calf serum media: Dulbecco's modified Eagle medium (DMEM) supplemented with 10% calf serum and 2 mM L-glutamine.
13. Fetal calf serum media: DMEM supplemented with 10% fetal calf serum and 2 mM L-glutamine.
14. ARA-C media: DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 10 μ M cytosine-B- arabinofuranoside hydrochloride.
15. MSC (media for Schwann cell): DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 2 μ M forskolin, and 2 ng/ml recombinant human neuregulin.
16. HMEM media: DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 20 mM HEPES (4-(2-hydroxyethyl)- L-piperazineethanesulfonic acid) at pH = 7.2.
17. Freezing media: DMEM supplemented with 20% fetal calf serum and 10% DMSO.

2.3 Schwann Cell Myelination Assay

1. Epifluorescence microscope.
2. Hemocytometer.
3. Dorsal root ganglia (DRG) neuron culture (*see* Note 5).
4. 4-well plates.
5. Glass coverslips, 18 mm, washed in ethanol and sterilized.
6. Glass microscope slides.
7. Nail polish.
8. 100% methanol, store at -20°C .
9. 4% paraformaldehyde in PBS.
10. Mounting media (*i.e.*, Vectashield).
11. 0.1 $\mu\text{g/ml}$ DAPI (4',6-diamidino-2-phenylindole) solubilized in D-PBS.
12. CL solution: 10% collagen and 1% glacial acetic acid diluted in water, freshly prepared (*see* Note 6).
13. C media: Minimum Essential Media (MEM) supplemented with 10% fetal bovine serum, 4 g/l D-glucose, 2 mM L-glutamine, and 50 ng/ml nerve growth factor.

14. C media PLUS: MEM supplemented with 10% fetal bovine serum, 4 g/l D-glucose, 2 mM L-glutamine, 50 ng/ml nerve growth factor, and 50 µg/ml ascorbic acid.
15. MLF blocking: 10% fetal bovine serum, 2% bovine serum albumin, 0.1% Triton-X, and 0.02% sodium azide diluted in PBS.
16. Anti-myelin basic protein antibody (Covance, SMI-99P), anti-neurofilament M (Covance, PCK-593P), anti-mouse Ig 488, and anti-chicken Ig 549.

2.4 Preparation of Dorsal Root Ganglia Neuron Membrane Suspensions

1. One Sprague-Dawley rat female, pregnant at embryonic day E15.5 (*see* Note 7).
2. Centrifuge and ultracentrifuge.
3. 6-well plates, coated with PLL solution for 15 min.
4. Microcentrifuge tubes, 1.5 ml.
5. Dounce homogenizer, 1 ml, tight.
6. DMEM.
7. Leibovitz's L-15 medium.
8. 10,000 U/ml penicillin/streptomycin.
9. NB-media: Neurobasal media supplemented with 1× B27 supplement, 4 g/l D-glucose, 2 mM L-glutamine, and 50 ng/ml nerve growth factor.
10. NB+ media: Neurobasal media supplemented with 1× B27 supplement, 4 g/l D-glucose, 2 mM L-glutamine and 50 ng/ml nerve growth factor, 10 mM fluorodeoxyuridine, and 10 mM uridine.

2.5 Neuronal Membrane Suspension Activity Assay

1. 4-well plates, coated with PLL solution for 15 min.
2. Antibody, anti-Akt (Cell Signaling, 9272), anti-Phospho-Akt (Cell Signaling, 9271).
3. Starvation media: DMEM supplemented with 2 mM L-glutamine, 2 µM forskolin, and 100 U/ml penicillin/streptomycin.
4. PN1: 100 mM pH 7.4, 5 mM EDTA, 150 mM NaCl, 1% SDS, 1 mM sodium orthovanadate, protease inhibitors.

2.6 Schwann Cell Protrusions Assay

1. Epifluorescence microscope.
2. Hemocytometer.
3. Microscissors.
4. Parafilm.

5. Forceps.
6. Glass microscope slides.
7. Glass coverslips, 22 × 50 mm.
8. Microcentrifuge tubes, 1.5 ml.
9. Petri dishes, 10 cm diameter.
10. Transwell 24 mm with 3 µm pore polycarbonate membrane (Corning, 3414) (*see* Note 8).
11. Cotton swabs.
12. Cell scraper.
13. Pointed cotton swabs.
14. Cell culture tray, containing a humid paper.
15. Nail polish.
16. Vectashield (Vector Labs).
17. D-PBS.
18. Bicinchoninic acid assay (BCA).
19. Trizol.
20. 0.25% trypsin.
21. StarvMed (starvation media): DMEM supplemented with 0.5% fetal calf serum, 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin (*see* Note 9).
22. M&A (migration and adhesion media): DMEM supplemented with 0.5% bovine serum albumin, 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin.
23. Lysis buffer: 125 mM Tris pH 7.4, 2 mM EDTA, 1% SDS 1% Triton, protease inhibitors. Store at 4 °C.
24. Quenching solution: 75 mM NH₄Cl, 20 mM glycine diluted in D-PBS.
25. Permeabilizing solution: 0.7% fish skin gelatin, 0.16% saponin diluted in D-PBS. Store at 4 °C.
26. Antibody, anti-His2B (Abcam, ab1790).
27. Rhodamine-coupled phalloidin.

3 Methods

3.1 Isolation of Primary Schwann Cells

The isolation of primary Schwann cells was adapted from Brockes et al. [15].

1. Prior to start the dissection, soak the microdissection set in the beaker filled with 70% ethanol. Also, fill three Petri dishes with D-PBS and 100 U/ml penicillin/streptomycin.
2. Cool animals on ice for 10 min before the procedure to minimize pain and distress. Behead one P3 rat, and place the pup in prone position. Spread out the hind limb laterally (such as the body will become an inverted Y) fixing the paw with needles. Wash the skin with ethanol 70%. The nerves lie beneath an imaginary line going diagonally from the toes up to the spine. A bony protuberance, the sciatic notch, can be felt at about one-third proximally in this line. Cut skin, then muscle and fascia at about the height of the sciatic notch through this diagonal line. The nerve, identifiable as a whitish string, can be seen underneath the muscle.
3. Using two sterile forceps, expose the sciatic nerves by running parallel to the nerve up to the fourth lumbar vertebra and down to the gastrocnemius muscle. Grab the sciatic nerve near the L4 extremity being careful not to damage any major blood vessels and without poking through the peritoneal cavity, which would contaminate the preparation, and then gently pull away the nerve toward the gastrocnemius muscle. Using the dissection microscope, remove any excess muscle and connective tissue from the sciatic nerves. Sever the sciatic nerve, and place it in the Petri dish with D-PBS and 1× penicillin/ streptomycin (*see Note 10*).
4. Shred the sciatic nerves into small pieces until there is no structural integrity remaining in the nerve (*see Note 10*).
5. Combine and pool the nerves in a 15 ml Falcon with 8 ml of D-PBS. Add 1 ml of collagenase and 1 ml of 2.5% trypsin. Incubate for 30 min at 37 °C in a water bath. Mix by inversion during the incubation.
6. Spin at $930 \times g$ -force (g) at room temperature for 10 min to pellet the nerves. Pipette and discard the supernatant.
7. Wash the pellet with 5 ml of calf serum media, and spin at $930 \times g$ at room temperature for 5 min. Repeat the washing step a second time.
8. Suspend the pellet in 2 ml of fetal calf serum media by pipetting at least 20 times up and down.
9. Count the cells with the hemocytometer, and plate 1,000,000 cells per poly-L-lysine-coated Petri dishes. Incubate with fetal calf serum media for 24 h to let the cells adhere in the cell incubator.
10. Wash the cells twice with HBSS, add 5 ml of ARA-C media, and incubate for 48 h in the cell incubator.
11. Wash the cells twice with HBSS, add 5 ml of Fetal calf serum media, and incubate for 48 h in the cell incubator.
12. Remove media, and add 5 ml of MSC and incubate for 72 h in the cell incubator.

13. Wash the cells in HBSS, and then wash in HMEM. Add 2 ml of HMEM containing 40 μ l of anti-Thy1.1, and incubate for 15 min at 37 °C. Add 400 μ l of complement, and incubate for 40 min at 37 °C (*see* Note 11).
14. Wash the cells twice with HBSS, add 5 ml of MSC, and incubate for 72 h in the cell incubator.
15. When cells are confluent, remove MSC, and replace with 1 ml of trypsin. Gently swirl to cover the whole plate with trypsin and incubate at 37 °C for 5 min (*see* Note 12).
16. When cells are detached, add 9 ml of fetal calf serum media.
17. Spin at $900 \times g$ for 5 min, suspend the pellet in 1 ml of pre- warmed MSC, and count cells with a hemocytometer.
18. Adjust the concentration to 500,000 cells/ml by adding pre- warmed MSC.
19. Transfer 1 ml of cell suspension (500,000 cells) per poly-L-lysine-coated Petri dishes containing 7 ml of pre-warmed MSC.
20. Gently swirl the petri dishes, and incubate the dishes in the cell incubator. When cells reach confluency, repeat **steps 13–20** once.
21. Remove MSC and replace with 1 ml of trypsin and incubate at 37 °C for 5 min.
22. When cells are detached, add 9 ml of fetal calf serum media.
23. Spin at $900 \times g$ for 5 min and suspend the pellet in 1 ml of pre-warmed MSC.
24. Discard supernatant, suspend 4,000,000 Schwann cells in 1 ml of freezing media, and store at -80 °C (*see* Note 13).

3.2 Schwann Cell Myelination Assay

Isolated Schwann cells can myelinate neurons in vitro. However, after being subcultured more than five times, Schwann cells start to lose this ability [16, 17]. Therefore, to identify relevant proteins for Schwann cell-axon interaction, it is important to ensure first that the batch of Schwann cells used in the protrusion assay retain the capability to myelinate axons.

1. Thaw Schwann cells by resuspending them in 9 ml of DMEM. Spin at $900 \times g$ for 5 min.
2. Plate Schwann cells on poly-L-lysine-coated Petri dishes, and incubate with MSC for 6 days in the cell incubator at 37 °C to let the cells reach confluence.
3. Wash cells with D-PBS, and incubate for 5 min at 37 °C with 1 ml of 0.25% trypsin.
4. When cells are detached, inactivate the trypsin by adding 9 ml of fetal calf serum media, and then spin at $900 \times g$ for 5 min.
5. Suspend cells in 1 ml of C media.
6. Count the Schwann cells, and adjust the concentration to 500,000 cells/ml.

7. Plate 400 μ l of Schwann cells per coverslip of DRG neuron culture (*see* Note 5).
8. 24 h later, replace the C media by C media PLUS.
9. Change the media every 2 days for 14 days.
10. Remove C media PLUS, and rinse with D-PBS.
11. Fix co-culture for 15 min in 4% paraformaldehyde, and then wash three times with D-PBS.
12. Permeabilize cells by incubation in cold methanol for 10 min, and then rinse twice with D-PBS.
13. Incubate 1 h in MLF blocking then incubate overnight with anti-myelin basic protein (1:1000) and anti-neurofilament M (1:700) diluted in MLF blocking.
14. Wash three times in D-PBS, and then incubate 1 h with anti- mouse Ig 488 (1:1000) and anti-chicken Ig 549 (1:1000) diluted in MLF Blocking.
15. Wash three times in D-PBS and incubate 10 min in DAPI solution.
16. Wash three times in D-PBS, and then mount the coverslip with Vectashield on a glass slide.
17. Seal the coverslip with nail polish, and analyze myelination under the microscope (*see* Note 14).

3.3 Isolation of Dorsal Root Ganglia Neurons

The isolation of primary dorsal root ganglia neurons was adapted from Wood et al. [18].

1. Coat two 6-well plates by adding 500 μ l of CL solution in each well, and incubate for 15 min. Then, aspirate the CL solution back, and leave behind a thin film of collagen. Let dry, and then add 1 ml of C media.
2. Prior to start the dissection, soak the microdissection set in the beaker filled with 70% ethanol. Also, fill three Petri dishes with L-15 medium.
3. After euthanasia, place the rat in supine position, and cut the lower abdominal skin and muscles. Gently remove the uterus by dissecting away connective tissues.
4. Rinse the uterus with L-15 to remove as much blood as possible, transfer it to a sterile Petri dish with L-15, and place the dish in the dissection hood.
5. Remove all embryos from the uterus by opening the amniotic sac with blunt forceps.
6. Gently grab the embryos by the head, and transfer them to a fresh Petri dish with L-15.
7. For each embryo, under the dissecting microscope, cut off the head at the cervical flexure and the tail just caudal to the hind limbs using micro-dissecting scissors.

8. With the embryo lying on the side, remove all the ventral portion of the embryo to isolate the dorsal structures containing the spinal cord. Flip the embryo, and proceed similarly on the other side.
9. Remove all viscera.
10. Place one blade of micro-dissecting scissors between the vertebral column and spinal canal at the rostral end, and very carefully cut through vertebral column proceeding caudally, and then tease apart the right and left halves of vertebral column to expose the spinal cord and DRGs.
11. Gently attempt to lift the spinal cord from dorsal structures by grasping the cord at rostral end and pulling perpendicularly.
12. Once the entire spinal cord with attached DRGs is isolated and can be peeled off from the underlying tissues, transfer it to a clean Petri dish containing L-15.
13. After isolating all spinal cords, pluck off DRGs using the #5 forceps, and transfer them to 6-well plates filled with C media. Place 30 DRGs per well.
14. Once all wells are filled with DRGs, remove the C media for 15 min to let DRGs adhere to the bottom of the well (*see* Note 15).
15. Add 1 ml of C media with 100 U/ml penicillin/streptomycin in each well, and then gently transfer 6-well plates to the cell incubator.
16. Twenty-four hours after dissection, replace C media by 1.5 ml of NB+ media per well, and then every 2 days, alternate NB+ and NB- media for three cycles (*see* Note 16).

3.4 Preparation of Neuronal Membrane Suspension

The preparation of neuronal membranes that maintain biological activity and can behave for a short time like live neurons was first described in 1980 by the Bunge lab [19]. In this study, authors showed that DRG neurons express on their surfaces pro-mitogenic signals for Schwann cells. Using neuronal membranes, it was then showed that neuregulin 1 type III and the PI3-kinase pathway mediate these signals [2, 4, 20]. The protocol below recapitulates the critical steps for the preparation of neuronal membrane suspension.

1. After eliminating the population of glial cells as described in Subheading 3.3, rinse DRG neurons and their network of axons once with ice-cold D-PBS (*see* Note 17).
2. With a blunt forceps, push the network of neurons to the center of the well. All the neurons can be collected in one pinch (*see* Note 18).
3. For each well of the 6-well plate, transfer the DRG neurons in a 1 ml Dounce homogenizer, and proceed to 30 tight strokes in 200 μ l of ice-cold D-PBS.
4. Transfer the homogenized solution in a 1.5 ml vial on ice, and then rinse the pestle with 200 μ l of ice-cold D-PBS at the end.
5. Proceed similarly for the second 6-well plate.

6. Centrifuge at $300 \times g$ for 20 min at 4 °C to remove debris and collagen.
7. Ultracentrifuge at $35,000 \times g$ for 1 h at 4 °C.
8. Resuspend the pellet in 800 μ l DMEM, vortex, and store at –80 °C (*see* Note 19).

3.5 Neuronal Membrane Suspension Activity Assay

Maurel et al. showed that contact with neuronal membranes was sufficient to activate Akt in Schwann cells [4]. Thus, to determine if new batches of neuronal membrane preparations contain active growth factors, it has to be assessed if isolated neuronal membranes have the ability to elicit phosphorylation of Akt in Schwann cells.

1. Plate 400,000 Schwann cells in a well of a 4-well plate coated with poly-L-lysine.
2. Maintain Schwann cells in MSC for 48 h.
3. 12 h prior to the experiment, starve Schwann cells by changing MSC to Starvation media.
4. Thaw neuronal membrane suspension, and suspend them with a vortex.
5. Put Schwann cells on ice, and keep them always on ice (*see* Note 20).
6. Remove media from the well, and then add 33 μ l of neuronal membrane suspension and 167 μ l of DMEM.
7. Spin the 4-well plate at $200 \times g$ for 10 min at 4 °C, and then incubate at 37 °C for 20 min.
8. When the incubation is over, put the 4-well plate on ice, and wash the Schwann cells with ice-cold D-PBS.
9. Lysate the cells with PN1, boil for 5 min, and spin at $16,000 \times g$ for 8 min at 16 °C.
10. Proceed to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-Page) and detect immunoreactive bands with anti-Akt and anti-phospho-Akt antibodies (*see* Notes 21 and 22).

3.6 Schwann Cell Protrusions Assay

The Schwann cell protrusions assay is adapted from the method developed by the Klemke lab [21]. The assay uses a Boyden chamber which is separated into lower and upper chambers by a 3- μ m porous filter. First, cells are seeded in the top chamber and attached to the microporous filter. Second, a stimulus is added in the bottom chamber. Third, cells respond to the stimulus by becoming polarized and extending protrusions through the 3 μ m pores into the bottom chamber. Because cell bodies cannot pass through the 3 μ m pores, they remain on the top. Protrusions passing in the bottom chamber can be separated and their content analyzed. In the original system, cells were stimulated by a chemotactic or haptotactic stimulus on the bottom chamber. This method has been used to isolate novel proteins and RNA in protrusions of migrating cells [22–26]. The following protocol is

innovative as it uses cell membranes as stimuli instead of soluble or extracellular matrix molecules (Fig. 1).

3.6.1 Induction of Schwann Cell Protrusions

1. Plate 500,000 Schwann cells in a 10 cm Petri dish coated with poly-L-lysine. Add 7 ml of MSC and incubate at 37 °C for 7 days (*see* Notes 23 and 24).
2. Replace the media by StarvMed, 16 h before the protrusion assay (*see* Note 25).
3. Coat the transwell with poly-L-lysine for 30 min and rinse once with D-PBS (*see* Note 26).
4. Wash Schwann cells twice with D-PBS.
5. Add 1 ml of 2.5% trypsin onto Schwann cells, and incubate for 5 min at 37 °C to detach the cells from the plate.
6. Stop the reaction with 9 ml of MSC. Collect Schwann cells, and spin at $900 \times g$ for 5 min.
7. Suspend the pellet in 1 ml of M&A. Count the cells, and adjust the concentration to 1,500,000 cells/ml.
8. Add 1 ml of the cell suspension in the top chamber of the transwell and 2 ml of DMEM in the bottom chamber of the transwell. Let the Schwann cells attach for 4 h (*see* Notes 27 and 28).
9. Thaw the neuronal membrane suspension. Replace the DMEM in the bottom chamber by the neuronal membrane suspension (*see* Notes 29 and 30), and let the protrusions grow for 2 h (*see* Note 31).
10. Once the protrusions have been induced, wash the top and bottom chambers with D-PBS.
11. Proceed to immunohistochemistry of Schwann cell protrusions (*see* Subheading 3.6.2), RNA extraction (*see* Subheading 3.6.3), or protein extraction (*see* Subheading 3.6.4).

3.6.2 Immunohistochemistry of Schwann Cell Protrusions

1. To test the extension of Schwann cell protrusion in the bottom chamber, use a cotton swab to remove half of the Schwann cell bodies in the top chamber of the transwell (*see* Note 32) (Fig. 2).
2. Place the transwell back in the 6-well. Wash the inside the top chamber of the transwell twice by adding 2 ml and 1 ml of D-PBS in the top and bottom chamber, respectively. Gently swirl the transwell, and remove the D-PBS by pipetting.
3. Add 2 ml and 1 ml of quenching solution in the bottom and top chamber, respectively. Incubate for 10 min at room temperature, and then wash once with D-PBS as in **step 2** (*see* Note 33).

4. Add 2 ml and 1 ml of permeabilizing solution in the bottom and top chamber, respectively. Incubate for 15 min at 37 °C, and then wash once with D-PBS as in **step 2**.
5. Place a piece of Parafilm in a humid cell culture tray. Prepare 150 µl of phalloidin diluted 1:400 in permeabilizing solution. Add a 40 µl drop of the phalloidin solution on the Parafilm. Place the transwell on top of it, and then add the remaining 110 µl in the top chamber. Incubate for 1 h at 37 °C (*see Note 34*).
6. Wash twice with D-PBS as in **step 2**.
7. Add 2 ml and 1 ml of permeabilizing solution in the bottom and top chamber, respectively. Incubate for 5 min at room temperature, then twice with D-PBS as in **step 2**.
8. Cut out the membrane of the transwell with micro-dissecting scissors.
9. On a glass microscope slide, put a drop of Vectashield. Grab the membrane with forceps, and place it on the Vectashield drop. Add another drop of Vectashield on the top of the membrane.
10. Cover with a glass coverslip, and seal with nail polish.
11. The production of pseudopods can be evaluated and imaged with an epifluorescence microscope as displayed in Fig. 2 (where half of the top chamber cell bodies were removed). Alternatively, cell bodies and protrusions can be observed in confocal microscopy without swab of the cell bodies from the top chamber, as displayed in Fig. 3.

3.6.3 Extraction of RNA

1. Fix the cell bodies and protrusions with cold methanol for 30 min, by adding 2 ml and 1 ml of methanol in the top and bottom chamber, respectively, to reduce most of the protease and phosphatase activity (*see Note 35*).
2. Remove the methanol by pipetting, and wash the inside of the top chamber of the transwell twice by adding 2 ml and 1 ml of D-PBS in the top and bottom chamber, respectively. Gently swirl the transwell. Do not remove the PBS after the second wash.
3. To collect the protrusion fraction, remove the Schwann cell bodies with a cotton swab. Apply a gentle pressure inside the transwell to detach the Schwann cell bodies. Rinse the inside of the transwell with D-PBS, and remove the D-PBS by pipetting. Repeat the procedure until all cell debris floating in the upper chamber are removed.
4. Proceed similarly by using a pointed cotton swab to detach the cell bodies at the edge of the transwell (*see Note 36*).
5. Alternatively, to collect the cell body fraction, flip over the transwell, and remove the Schwann cell protrusions with a cotton swab.
6. Cut the membrane of the transwell with micro-dissecting scissors (*see Note 37*).

7. Place the membrane on a piece of Parafilm with the protrusion side up.
8. Add 100 μ l of Trizol on the membrane.
9. Hold the membrane with a forceps, and gently use a cell scraper to free the Schwann cell protrusions (*see* Notes 38 and 39).
10. Collect carefully the Trizol in a 1.5 ml vial, and proceed to RNA isolation (*see* Note 40).

3.6.4 Extraction of Proteins

1. Perform **steps 1–6** as described above for extraction of RNA (Subheading 3.6.3).
2. Transfer the membrane to the bottom of a 1.5 ml vial with 100 μ l of lysis buffer.
3. Chop the membrane with microdissection scissors (*see* Note 41).
4. Sonicate for four cycles of 20 s, and place it on ice for 30 s between each cycle.
5. Place the vial on ice for 30 min, and then spin at $16,000 \times g$ for 30 min at 4 °C.
6. Transfer carefully the supernatant to a new 1.5 ml vial, and measure protein concentration by BCA (*see* Note 42).
7. Proceed to SDS page analysis for nuclear marker (i.e., His2b) to assess the purity of the Schwann cell protrusion fraction (*see* Note 43).

4 Notes

1. Wash all instruments with sterile water, followed by ethanol 70% then autoclave.
2. The gauze at the bottom of the beaker protects the tools from being damaged. Alternatively, a plastic tip can be used as a protecting sleeve for the tools.
3. The average litter size of the Sprague-Dawley rat is ten pups.
4. The collagenase can be stored in 1 ml aliquots at –20 °C for up to 6 months.
5. To prepare coverslips of dorsal root ganglia neurons, proceed as indicated in Subheading 3.3, but instead of plating ganglia in 6-well plates, seed 1 ganglion per 18 mm coverslip in a 4-well plate. As for 6-well plates, coverslips have to be coated in CL solution for 15 min.
6. The CL solution has to be fresh. If the CL solution is too old, it can affect the capability of neurons to adhere to the cover-slips. Use a 10% solution for the first month after opening the collagen bottle and a 25% solution between the second and third months. Discard the collagen if it is older than 3 months.
7. To obtain E15.5 embryos, mate a male and female rat on the evening. The next morning, check for a vaginal plug, and if the female is pregnant, the pups will be at E0.5. Proceed to the dissection exactly 15 days later, in the morning. Dissection can also be performed on the afternoon of the same day (~E16). At E15, the embryo is too soft, which complicates the pulling of the spinal cord from the rest of the tissues. At E16.5, DRGs have started to send axons in the

peripheral tissues, and the DRGs will detach from the spinal roots when pulling the spinal cord out.

8. The 3 μ m pore of the transwell membrane allows Schwann cells to extend protrusions while blocking their 5 μ m nucleus from passing on the lower side of the membrane.
9. StarvMed is different in composition from starvation media in Subheading 2.5.
10. Proceed to these steps as quickly and efficiently as possible. The less time used, the greater the yield of Schwann cells.
11. This procedure selectively removes fibroblasts from the Petri dishes. Isolated fibroblasts, or patches of fibroblasts rounding up and then detaching from the plates, should be observable. Healthy Schwann cells have a slender, elongated, bipolar morphology, exhibit contact-dependent inhibition of proliferation, and, when they are in a confluent monolayer, present swirling patterns.
12. Monitor the detachment of SC from the plate using a light microscope. A gentle tap of the side of the flask can help to determine the proper detachment of the cells from the plate.
13. One litter of rat will give about 60 millions of Schwann cells.
14. An efficient myelination should be reflected by at least 1000 segments of myelin per coverslip. Several factors can affect the efficiency of myelination, i.e., the composition of the fetal calf serum and the presence of mycoplasma. Therefore, it is recommended when trying to myelinate axons with Schwann cells for the first time to test several batch of serum and to test for the presence of mycoplasma.
15. Close the plate with the cap to avoid DRGs to dry out. Drying out DRGs increase cell death.
16. NB+ contains fluorodeoxyuridine, an inhibitor of cellular proliferation that blocks DNA synthesis. Schwann cell and fibro-blast proliferation is blocked, and fibroblasts and Schwann cells are eliminated from the culture. Cycling NB+ and NB media to obtain a pure neuronal culture takes 12 days.
17. Membrane suspensions can be prepared from non-neuronal cells (i.e., CHO, COS-7) to be used as a negative control. Non-neuronal membranes do not activate Akt in Schwann cells (*see* Subheading 3.5) [13].
18. The collection of neurons can be done macroscopically. However, it is convenient for the first time to do it under a microscope to appreciate how the network of neurons is moved by the forceps.
19. Neuronal membrane suspension can be stored up to 6 months at -80°C , and efficiently activate Akt in Schwann cell (*see* Subheading 3.5). A longer storage has not been tested. It is also recommended to pool several neuronal membrane suspension preparations to reach a volume of 2 ml or above. This ensures to have enough membrane suspension to test its activity (*see* Subheading 3.5) and to

perform several protrusion assays (Subheading 3.6). Finally, do not freeze/thaw the neuron membrane suspension more than one time.

20. It is critical to keep Schwann cells on ice, as this limits the activation of Akt.
21. Non-starved Schwann cells and Schwann cell in contact with MSC for 20 min can be used as a positive control for the activation of Akt.
22. As neuronal membranes are not quantified after preparation, it is critical to test that neuronal membranes have the potency to activate Akt.
23. For protein or RNA extraction (*see* Subheadings 3.6.3 and 3.6.4), 12 Petri dishes of Schwann cells are necessary.
24. After 7 days, Schwann cells should have reached confluence, and a Petri dish should contain around 4,000,000 Schwann cells.
25. The starvation of Schwann cells causes 30–40% of cell death.
26. The coating of the transwell with poly-L-lysine is done the day prior to the protrusion assay.
27. As the polycarbonate membrane is opaque, it is not possible to appreciate the health and adhesion of the Schwann cells until the end of the immunocytochemistry assay (Subheading 3.6.2).
28. For protein or RNA extraction (*see* Subheadings 3.6.3 and 3.6.4), two 6-well plates are necessary (6 wells will be induced with neuronal membranes in the bottom chamber, and 6 wells will be used as control with DMEM in the bottom chamber).
29. The neuronal membranes are the limiting reagent in this experiment. It is possible to reduce the quantity of neuronal membrane suspension to use in the bottom chamber by diluting the neuronal membrane suspension. However, the dilution has to activate efficiently Akt in Schwann cells (*see* Subheading 3.5). Another approach is to reduce the volume of the bottom chamber by lowering the distance between the microporous membrane and the floor of the bottom chamber. This can be done by using a laser cutting machine to reduce the height of the walls supporting the transwell (Fig. 1, vertical structures in gray). However, a minimal distance of 200 μm between the microporous membrane and the floor of the bottom chamber is necessary to prevent Schwann cells protrusions, 25 μm on average, to contact the bottom of the well.
30. When putting the neuronal membrane suspension in the bottom chamber, remove the transwell, add the suspension, and replace the transwell. Avoid creating air bubble between the transwell and the bottom of the well, as they can disrupt the juxtacrine interaction between neuronal membranes and Schwann cells. Several conditions can be used in parallel to neuronal membrane suspension. DMEM and non-neuronal membranes are good negative controls. MSC and lysophosphatidic acid (100 ng/ml) are efficient positive controls for the induction of Schwann cell protrusions.

31. Two hours of induction is optimal for the production of Schwann cell protrusions [13], similar to other cell types [21, 25, 27]. After this time, pseudopods retract. It is critical to not move the plate nor open the incubator for these 2 h as any movement might disrupt the contact that is forming between Schwann cells and neuronal membranes.
32. Removing only half of the cell bodies allows the visualization of cell bodies on one half (non-swabbed region) and the visualization of protrusions by epifluorescence microscopy on the other half (swabbed region), because the cell bodies in the upper chamber were removed. A similar approach can be adapted for immunolocalization of proteins.
33. The quenching solution is used to reduce autofluorescence.
34. The piece of Parafilm is essential as it will retain the drop of phalloidin solution by surface tension, and avoid any leakage.
35. For RNA and protein extraction, 6 transwells need to be prepared. One will be used for the biochemistry assay on the cell body fraction, four for biochemistry assay on the protrusion fraction, and one for the immunohistochemistry of Schwann cell protrusions.
36. Be very careful. If the integrity of the microporous membrane is affected, it could contaminate the protrusion fraction with the Schwann cell body fraction.
37. Removing the cell bodies properly is critical for the purity of the protrusion fraction. The cell bodies on the edge are often the hardest to remove. When cutting out the membrane, leave a 1 mm edge behind to reduce possible contamination.
38. Because cell bodies are swabbed before cutting the membrane, one transwell cannot be used to isolate both cell body and protrusion fractions. Similarly when cell bodies need to be isolated, pseudopods need to be swabbed before and cannot be isolated.
39. While scraping, avoid the formation of bubbles. Bubbles will reduce the amount of Trizol that can be recovered.
40. From one 24 mm transwell, the RNA concentration will be around 100 ng. It is possible to increase this concentration by scraping several transwell membranes with the same Trizol suspension, which will increase the final yield of RNA.
41. An approach similar to the RNA isolation with a cell scraper can also be done for the proteins. However, as the SDS increases the formation of bubbles, it can limit the amount of Lysis buffer recovered after scrapping. Also the sonication method allows to collect the protrusions proteins inside the transwell membrane (10 μ m thick) and therefore to increase the final yield of proteins.
42. From one 24 mm transwell, the protein concentration is around 0.15 g/l. It is possible to increase this concentration by pooling multiple transwells.

43. There is no good loading control for proteins that are equally distributed between the cell body and the pseudopod fraction. Therefore, Amido Black, Coomassie, or Silver staining are recommended to visualize equal loading of proteins. Histones, present at around 15 kDa, are enriched in the cell body fraction, as shown in Fig. 4. The presence of Histones in the protrusion fraction indicates an incomplete removal of the cell bodies in the top chamber.

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Fig. 1.
Schematic illustration of the Boyden chamber used for juxtacrine stimulation of Schwann cells with neuronal membranes

Fig. 2.

Immunohistochemistry of Schwann cell protrusions. DAPI and phalloidin staining of Schwann cell protrusions and cell bodies in response to neuronal membrane suspension (**a**, neuronal membrane) or a negative control (**b**, DMEM). In both cases the left side of the membrane is intact, while the cell bodies of the right side on the top chamber were swabbed away (swab). The pseudopods can be seen below the microporous membrane in the swabbed region, because the cell bodies in the upper chamber were removed. The polycarbonate microporous membrane does not block the phalloidin signal in epifluorescent microscopy. The staining with phalloidin can be used to verify that the pseudopods were formed in higher quantity in the membrane-stimulated condition when compared to the DMEM control (no stimulus)

Fig. 3.

Confocal imaging of Schwann cell protrusions in response to neuronal membranes. Cells were stained with DAPI (blue) and phalloidin (red). Progressive Z-sections were collected with a step size of 0.15 μm from the whole top or bottom chamber and stacked in Z-projection. No nuclei are seen in the bottom chamber. If using confocal microscopy, there is no need to swap the upper chamber. Bar = 25 μm

Fig. 4. Silver staining of proteins in the Schwann cell body and protrusion fractions. The silver staining shows lysates from Schwann cell bodies or protrusions separated on SDS-PAGE. Histones are observed at 15 kDa in the cell body fraction (arrow)