Chapter 2

Preparation of Neonatal Rat Schwann Cells and Embryonic Dorsal Root Ganglia Neurons for In Vitro Myelination Studies

Patrice Maurel

Abstract

The ability to understand in great details, at the molecular level, the process of myelination in the peripheral nervous system (PNS) is, in no minor part, due to the availability of an in vitro culture model of PNS myelination. This culture system is based on the ability to prepare large population of highly purified Schwann cells and dorsal root ganglia neurons that, once co-cultured, can be driven to form in vitro well-defined myelinated axon units. In this chapter, we present our detailed protocols to establish these cell cultures that are derived from modifications of procedures developed 35–40 years ago.

Key words Rat, Rodent, Schwann cell, DRG, Dorsal root ganglia, Neuron, Co-culture, Myelination

1 Introduction

The in vitro culture model of peripheral nerve system (PNS) myelination evolved from studies that were characterizing Schwann cells' responses to contact with axons from dorsal root ganglia (DRG) neurons [1–4]. These experiments benefited from recently developed techniques to isolate and culture purified Schwann cells [5, 6] and DRG neurons [6]. One of the most important findings was that under appropriate conditions, Schwann cells form compact myelin sheaths around the axons of the purified DRG neurons [3, 4]. These myelin sheaths are structurally comparable to those formed in vivo, with the formation of nodes of Ranvier, paranodes, juxtaparanodes, and internodes. Over the ensuing 35+ years of research, the Schwann cell/DRG neuron co-culture system has been central, on its own and as a necessary complement to in vivo mouse models, to most of the major discoveries pertaining to PNS myelination: from the molecular characterization and assembly of these different domains to the characterization of the molecular signals that initiate, promote, and regulate Schwann cell fate and

Paula V. Monje and Haesun A. Kim (eds.), *Schwann Cells: Methods and Protocols*, Methods in Molecular Biology, vol. 1739, https://doi.org/10.1007/978-1-4939-7649-2_2,

behavior. This type of myelinating culture model has been in fact so important that the lack, until recently [7], of an equivalent central nervous system (CNS) in vitro system has been considered *a longtime limitation in studying the molecular basis of CNS myelination* [8]. With the development of molecular biology tools and techniques such as lentiviral transduction (*see* Chapter 12) that allow for the easy manipulation of Schwann cells and DRG neurons, allied to the simplicity of the model (only two cell types), the Schwann cell/DRG neuron myelinating culture system is likely to continue contributing to the comprehensive dissection of the molecular mechanisms that control PNS myelin formation. Of course, the in vitro culture system cannot fully represent what happens in vivo. These molecular mechanisms need to be placed in the context of the complex morphological process that myelination is. That is where the strength of the in vivo model lies.

This chapter is divided in three main sections that provide detailed step-by-step information to prepare purified Schwann cells and DRG neuron cultures and to set up the myelinating co-culture system. Sciatic nerves from postnatal day 2 Sprague-Dawley rats are used as the source of Schwann cells, and NGF-dependent neurons are isolated from embryonic day 15 DRGs. The procedures include three basic steps: (1) dissection (i.e., the harvesting of sciatic nerves or DRGs), (2) dissociation of tissues into individual cells and plating, and (3) purification of Schwann cells or DRG neurons with antimetabolic and antimitotic agents. For Schwann cells, two additional steps, to expand and freeze the cells, are described. These protocols allow generating highly purified (>99%) Schwann cells (upward of 1×10^8 cells at third passage) and DRG neurons (upward of 300 individual cultures in 10 days).

2 Materials

2.1 Chemicals and Biochemicals (See Note 1)

2.1.1 For Schwann Cell Preparation

1. Culture-grade poly-L-lysine (MW range of 70,000–150,000): stock concentration is prepared at 1 m/ml in culture-grade water, filter sterilized, and stored at 4 °C, protected from light. Working concentrations of 100 and 10 μg/ml are made freshly at the time of use in sterile culture-grade water.
2. Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/l of glucose and without L-glutamine.
3. Fetal bovine serum (FBS; Gibco 16000) (*see* **Note 2**), heat-inactivated; store in 50 ml aliquots at −80 °C (*see* **Note 3**).
4. Forskolin: prepare a 2 mM stock solution in 100% ethanol; store at −20 °C.

5. EGF-D: recombinant EGF domain of human neuregulin-1-ß1 (R&D Systems 396-HB): prepare a stock at 100 µg/ml in DMEM and store at −80 °C in 50 µl aliquots.
6. GlutaMAX™-I.
7. [100×] Penicillin/streptomycin solution (respectively, at 10,000 units/ml and 10,000 µg/ml).
8. Hanks' balanced saline solution (HBSS), calcium- and magnesium-free.
9. Leibovitz's L-15 medium.
10. Collagenase, type 1 (Worthington Biochemical Corp. cat # LS004194): prepare a 1% stock solution in culture-grade sterile distilled water; filter sterilize; store in 1 ml aliquots at −80 °C.
11. Cytosine-ß-arabinofuranoside hydrochloride (Ara-C): prepare a 1 mM stock solution in culture-grade sterile distilled water; filter sterilize; store 1 ml aliquots at −80 °C.
12. Anti-Thy-1.1 antibody at 1 mg/ml (AbD Serotec MCA04G).
13. 2.5% trypsin in HBSS.
14. 0.25% trypsin/2 mM EDTA solution in HBSS.
15. Dimethyl sulfoxide (DMSO), suitable for cell culture.
16. 70% ethanol.

2.1.2 For DRG Neuron Preparation

1. Matrigel, Growth Factor Reduced (GRF) (Corning, cat # 356231): upon receiving the Matrigel vial, thaw on ice and then use ice-cold Neurobasal to bring the concentration down to 2.5 mg/ml (*see* **Notes 4** and **5**). Make 1.1 ml aliquots (*see* **Note 25**) in microcentrifuge tubes that are kept on ice (*see* **Note 5**). Immediately store at −80 °C. Matrigel will be used at a final concentration of 250 µg/ml, in Neurobasal (*see* Subheading 4.1 and **Note 5**).
2. Neurobasal medium (Gibco cat # 21103).
3. B27 supplement, 50× (Gibco cat # 17504-044).
4. D(+) glucose, suitable for cell culture.
5. Mouse NGF 2.5S: prepare stock solution at 1 mg/ml in sterile culture-grade water. Keep 50 µl aliquots at −80 °C until use. Use at a final concentration of 50 ng/ml.
6. 5-Fluoro-2′-deoxyuridine (FUDR) + Uridine (U): prepare a stock solution in Neurobasal medium, at a final concentration of 10 mM each. Use at a final concentration of 10 µM each.
7. GlutaMAX™-I.
8. [100×] Penicillin/streptomycin solution (respectively, at 10,000 units/ml and 10,000 µg/ml).

9. 0.25% trypsin solution in HBSS.
10. 70% ethanol.

2.1.3 For Myelinating Schwann Cell/DRG Neuron Co-cultures

1. Minimum Essential Medium Eagle (MEM) with Earle's salts, without glutamine.
2. L-Ascorbic acid, suitable for cell culture.
3. Fetal bovine serum (FBS; Gibco 16000) (*see* **Note 1**), heat-inactivated; store in 50 ml aliquots at −80 °C (*see* **Note 2**).
4. D(+) glucose, suitable for cell culture.
5. Hanks' balanced saline solution (HBSS), calcium- and magnesium-free.
6. GlutaMAX™-I.
7. 0.25% trypsin/2 mM EDTA solution in HBSS.

2.2 Culture Dishes, Plates, and Coverslips (See Notes 6 and 7)

1. ø60 mm tissue culture dish (for Schwann cell preparation).
2. ø100 mm tissue culture dish (for Schwann cell and DRG neuron preparations).
3. ø35 mm tissue culture dish (for DRG neuron preparation).
4. 24-well plates (for DRG neuron preparation).
5. ø12 mm glass coverslips, 0.13–0.17 mm thick (for DRG neuron preparation).

2.3 Culture Media and Solutions (See Note 8)

1. *SC-BM*: culture medium to maintain Schwann cells: DMEM, 10% FBS, 2 mM GlutaMAX™-I.
2. *SC-EM*: culture medium to expand Schwann cells: SC-BM supplemented with 2 μM forskolin and 10 ng/ml EGF-D.
3. *DRG-SM*: culture medium to maintain DRG neurons: Neurobasal, 2% B27 supplement, 1% GlutaMAX™-I, 0.08% (w/v) glucose, and 50 ng/ml 2.5S NGF.
4. *DRG-FU*: culture medium to purify DRG neurons: DRG-SM supplemented with 10 μM of FUDR and 10 μM Uridine.
5. *CCM*: Schwann cell/DRG neuron co-culture medium: MEM, 10% FBS, 1% GlutaMAX™-I, 0.4% (w/v) glucose, and 50 ng/ml 2.5S NGF.
6. *MM*: myelinating medium: CCM supplemented with 50 μg/ml of ascorbic acid.
7. *Freezing media*: SC-BM supplemented with 10% DMSO.

2.4 Animals

1. *Schwann cell preparation*: postnatal day 2 (p2) Sprague-Dawley rat pups; two litters (about 20–25 pups) (*see* **Note 9**).
2. *DRG neuron preparation*: embryonic day 15 (E15) Sprague-Dawley rat embryos; 1 pregnant female (about 10–16 embryos) (*see* **Note 9**).

2.5 Dissecting Tools and Equipment

1. Stereoscopic dissecting microscope.
2. Large scissors (3), 13 cm, straight, blunt/blunt tips.
3. Extra-fine Bonn scissors (1), 8.5 cm, straight, sharp/sharp tips.
4. Spring scissors (1), 10 cm, straight, sharp/sharp tips.
5. Moria fine scissors (1), 10.5 cm, curved, sharp/sharp tips.
6. Graefe forceps (1), 10 cm, curved, serrated tips.
7. Tissue forceps (3), 12 cm, straight, 1 × 2 teeth.
8. Dumont forceps #5 mirror, finished forceps, straight, standard tips (0.1 × 0.06 mm) (*see* **Note 10**).
9. Dumont forceps #5 mirror, finished forceps, straight, biology tips (0.05 × 0.02 mm) (*see* **Note 10**).
10. Dumont #5/45 coverslips forceps.
11. Needles 27 $G^{1/2}$ and 23 $G^{1/2}$.
12. 15 ml conical tubes, sterile.
13. 50 ml conical tubes, sterile.
14. Hemocytometer.
15. 2 ml serological pipets.
16. Wide orifice 200 µl pipet tips (*see* **Note 11**).
17. 200 µl regular pipet tips.
18. 200 µl Pipetman or equivalent.
19. 9 in. glass Pasteur pipets, sterile.
20. 2 ml cryotubes.
21. Humidified tissue culture incubator set at 37 °C and 10% CO_2 for Schwann cell cultures.
22. Humidified tissue culture incubator set at 37 °C and 5% CO_2 for DRG neurons and myelinating co-cultures.
23. Centrifuge with swing bucket and adaptors that accommodate 15 and 50 ml conical tubes.
24. Liquid nitrogen tank, vapor phase.
25. −80 °C freezer.
26. 0.22 µm filtering devices, low-protein-binding membrane.
27. Dissecting board (*see* **Note 12**).
28. Water bath set at 37 °C.
29. Heat block set at 37 °C.
30. Microcentrifuge tubes.
31. Vacuum line.

3 Preparation of Primary Schwann Cell Cultures from Neonatal Rat Sciatic Nerves

3.1 Day 0: Preparing Culture Plates

1. Coat ø60 mm plates with 2 ml of poly-L-lysine (10 μg/ml). Incubate overnight at 37 °C in a humidified cell culture incubator (% of CO_2 is not important at this time).

   Prepare one ø60 mm plate per four pups (eight sciatic nerves collected).

3.2 Day 1: Harvesting Sciatic Nerves, Tissue Dissociation, and Cell Plating

3.2.1 Preparation

1. Sterilize the dissection tools by immersing them into 70% ethanol for 30 min (*see* **Note 13**). Then, air-dry.

   The following tools will be needed: one large (13 cm) scissors, curved Moria fine scissors, curve Graefe forceps, Dumont forceps #5 (standard tips), and spring scissors.
2. Prepare one 15 ml conical tube with 13 ml of L-15 medium; keep chilled on ice.
3. To a 15 ml conical tube, add 1 ml of 2.5% trypsin solution and 8 ml of L-15 medium. Thaw quickly (37 °C, water bath) a 1 ml aliquot of 1% collagenase and transfer to the trypsin solution. Invert the tube a few times to mix. Keep warm at 37 °C (water bath).
4. Finish preparing the ø60 mm plates. Using a Pasteur pipet connected to a vacuum line, aspirate the poly-L-lysine solution and rinse quickly once with culture-grade sterile water. Discard the water and replace with 3 ml of SC-BM media, supplemented with Penicillin/Streptomycin ([1×] final concentration). Keep the plates at 37 °C in the 10% CO_2 humidified cell culture incubator (*see* **Note 14**).
5. Wrap the dissecting board with absorbent paper towel. Spray with 70% ethanol.

3.2.2 Dissection (One Pup at a Time)

1. Euthanize one rat pup by decapitation using the large scissors (13 cm). Place the animal on the dissecting board, dorsal side up, and pin it into place with 27 $G^{1/2}$ (legs) and 23 $G^{1/2}$ (body) needles, splaying the hind legs into an inverted V shape (*see* Fig. 1a). Spray the body with 70% ethanol.
2. Using the small Graefe forceps, pull up the skin. Then using the small curved scissors, cut the skin away from the hind limbs and the lower back (*see* Fig. 1b).
3. With the #5 Dumont forceps, gently tease apart the *quadriceps femoris* (QF), medial hamstring (MH), and *biceps femoris* (BF) muscles, from the knee to the hip bone (*see* Fig. 1c).
4. Locate the sciatic nerve, which runs posterior and parallel to the femoral bone (*see* Fig. 1c). Gently grasp the sciatic nerve with one the Dumont forceps and cut the nerve at each end

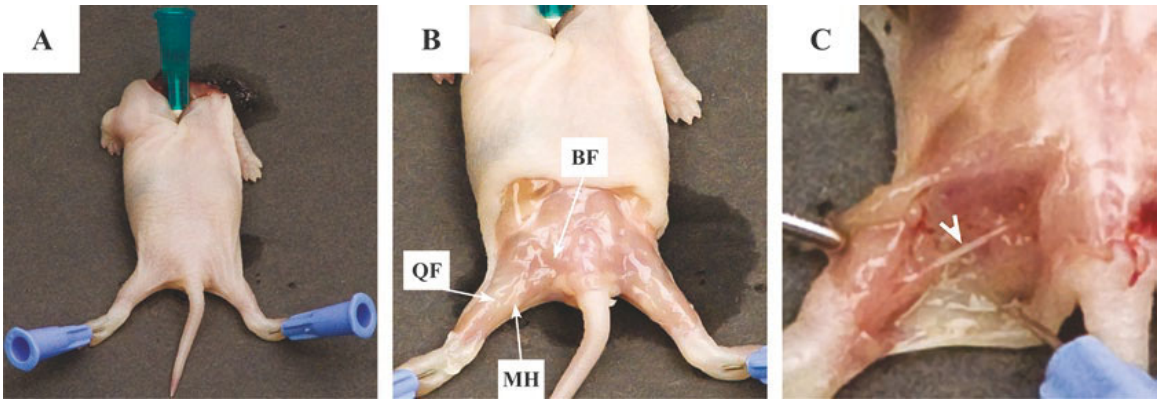

Fig. 1 Harvesting sciatic nerves from p2 neonatal rats. (**a**) A euthanized pup on the dissecting board, dorsal side up, pinned down in place with the hind legs splayed in an inverted V shape. (**b**) The skin has been removed from the lower back and hind legs. *QF* quadriceps femoris, *BF* biceps femoris, *MH* medial hamstring. (**c**) The sciatic nerve (*arrowhead*) after teasing the muscles apart

with the spring scissors. The dissected nerves will be about 1 cm (0.5 in.) in length.

5. Transfer the sciatic nerves immediately into the 15 ml conical tube that contains the ice-cold L-15 medium.
6. Repeat **steps 1** through **5** for all the remaining pups.

3.2.3 *Tissue Dissociation*

1. Once all the sciatic nerves have been harvested, centrifuge the 15 ml tube for 5 min at 50 × *g*, at room temperature.
2. Gently decant the supernatant and resuspend the sciatic nerves with the 10 ml of the pre-warmed trypsin-collagenase solution. Incubate at 37 °C in the water bath for 30 min, inverting the tube a couple of times every 5 min.
3. Centrifuge for 5 min at 50 × *g*, at room temperature. Decant the supernatant and gently resuspend the pelleted sciatic nerves with 10 ml of SC-BM. Repeat the centrifugation.
4. Decant the supernatant and add 2 ml of SC-BM supplemented with Penicillin/Streptomycin ([1×] final concentration) to the sciatic nerves. Using a 2 ml serological pipet, triturate the sciatic nerves until complete dissociation of the tissue; it should take about 10–20 pipettings.
5. Add SC-BM supplemented with Penicillin/Streptomycin so that you have 1 ml of dissociated tissue for every eight sciatic nerves that were collected.
6. Plate 1 ml of the cell suspension per ø60 mm plate. Incubate for 24 h at 37 °C in the 10% CO_2 humidified cell culture incubator. The cultures should consist of Schwann cells and fibroblasts (*see* Fig. 2a).

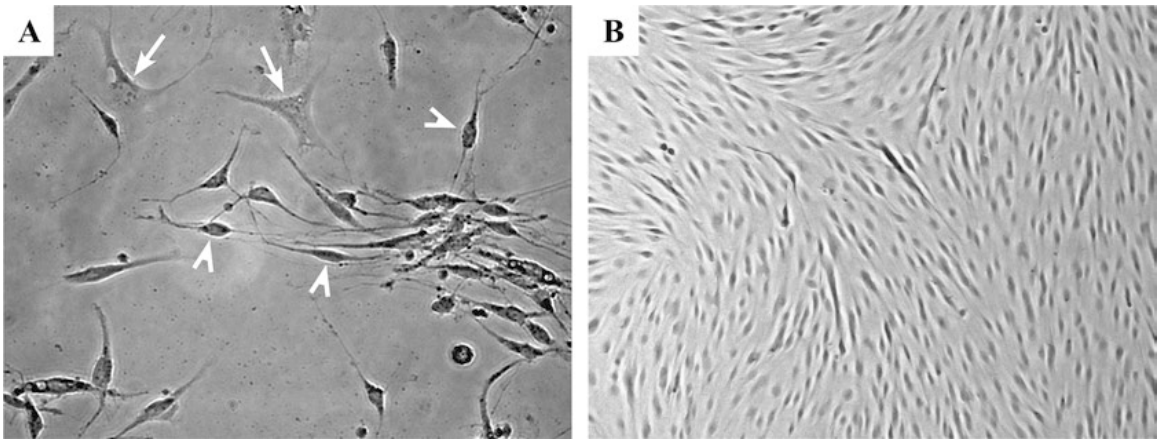

Fig. 2 Purification of rat Schwann cells. (**a**) Cells 24 h after dissection. Schwann cells have an elongated, spindle-shaped morphology (arrowheads), whereas fibroblasts are very flat and have a spread-out morphology (arrows). (**b**) Monolayer of purified Schwann cells at confluence. Note the elongated shape of the cells and swirling pattern of the monolayer

3.3 Day 2–8: Removal of Fibroblasts by Antimetabolic Treatment (See Note 15)

1. After 24 h (day 2), use a glass Pasteur pipet to aspirate the culture media, and wash the cells twice with 3 ml of HBSS.
2. After the last wash, add 3 ml of SC-BM containing Ara-C at 10 μM (1:100 dilution from stock), and incubate the cultures in the humidified cell culture incubator (37 °C, 10% CO_2) for 3 days (day 5; *see* **Note 16**).
3. Remove the culture media. Wash the cells twice with 3 ml of HBSS and replace with 3 ml of SC-EM (no antibiotics should be necessary from now on) to initiate Schwann cell proliferation (*see* **Note 17**). Place the cultures in the incubator for another 3 days (day 8).

3.4 Day 8 to About Day 12: Removal of Contaminating Fibroblasts by Complement-Mediated Killing (See Note 18)

1. On day 8, wash the cells once with 3 ml of HBSS.
2. Add 3 ml of SC-EM, supplemented with 60 μl of the anti-Thy-1.1 antibody, and incubate for 30 min in the cell culture incubator.
3. Add 400 μl of rabbit complement; mix by gently swirling the dishes.
4. Incubate for 30 min to 3 h in the cell culture incubator. Monitor the cells under a phase contrast microscope every 20 min, to assess the death of the fibroblasts as well as the health of the Schwann cells (*see* **Note 19**).
5. Remove the culture medium and wash the cells twice with 3 ml of HBSS.
6. Add SC-EM, and incubate cells in the humidified cell culture incubator (37 °C and 10% CO_2) for 2 days (day 10).

7. Observe the cultures for fibroblasts contamination and Schwann cell viability. Schwann cells should have a bipolar, spindle-shaped morphology (arrowheads in Fig. 2a), distinguishable from the fibroblasts flattened and spread-out morphology (arrows in Fig. 2a). At this stage, Schwann cell purity should be greater than 99%. If it is not, the complement-mediated killing (**steps 1** through **6**) should be repeated before expanding the cells further.
8. Otherwise, keep the cultures until they form a 100% confluent monolayer (about 3–4 more days; day 11 to day 12) (*see* **Notes 20** and **21**).
9. When Schwann cells reach confluence (Fig. 2b), coat ø100 mm plates with 5 ml of poly-L-lysine (10 μg/ml). Incubate overnight at 37 °C in the humidified cell culture incubator (% of CO_2 is not important at this time).

Prepare one ø100 mm plate per ø60 mm plate.

3.5 Day 12 to About Day 17: Expanding Schwann Cells to Passage 1

1. Finish preparing the ø100 mm plates. Remove the poly-L-lysine solution and rinse quickly once with culture-grade sterile water. Discard the water and replace with 5 ml of SC-EM media. Keep the plates at 37 °C in the humidified cell culture incubator (10% CO_2).
2. Wash the Schwann cells twice with 3 ml of HBSS. Discard HBSS washes and add 2 ml of 0.25 trypsin-EDTA solution; incubate at 37 °C for about 5 min. Observe the cells under phase contrast microscopy while gently shaking the culture dish.
3. When the cells start to come off the dish, add 2 ml of SC-BM, and collect the cells in 15 ml tubes, one tube per ø60 mm plate.
4. Centrifuge for 5 min, at 200 × *g*, at room temperature.
5. Discard the supernatants and gently resuspend the cells in 5 ml of SC-EM medium.
6. Plate cells onto the ø100 mm tissue culture plates, cells from one ø60 mm plate to one ø100 mm plate. Incubate cells in the humidified cell culture incubator (37 °C, 10% CO_2) until cells are confluent in about 5 days. This split will correspond to passage 1 (*see* **Note 22**).
7. These are the cells that we use for lentiviral transduction experiments (*see* Chapter 12) once they have reached 100% confluence (*see* **Note 23**).

3.6 Day 17 to About Day 22: Expanding Schwann Cells to Passage 2

1. When 100% confluent, the number of Schwann cells per ø100 mm plate should be about 4–5 × 10^6. Cells will be split 1:4. The day before splitting, prepare four ø100 mm plates for every ø100 mm plate to be split. Follow **step 9** (Subheading 3.4) and **step 1** (Subheading 3.5). In **step 1**, prepare the plates with 9 ml of SC-EM medium.

2. Then follow **steps 2** through **6** from Subheading 3.5, with the following modifications:
   - **Step 2**: wash cells with 5 ml of HBSS, and use 5 ml of 0.25 trypsin-EDTA solution.
   - **Steps 3** and **4** are not changed; collect cells in one tube per ø100 mm plate.
   - **Step 5**: resuspend cells in 4 ml of SC-EM.
   - **Step 6**: plate 1 ml of cell suspension per ø100 mm plate.

3.7 Further Expansion

1. Follow steps in Subheading 3.6 to expand Schwann cells further (*see* **Note 24**).
2. Before using Schwann cells in any experiments, replace the culture medium (SC-EM) with Schwann cell medium that does not contain forskolin and EGF-D (SC-BM) for 2–3 days.

3.8 Freezing Schwann Cells for Storage

1. Wash cells twice with 5 ml of HBSS.
2. Add 5 ml of 0.25 trypsin solution; incubate at 37 °C for 5 min.
3. Observe the cells under light microscopy while gently shaking the culture dish.
4. When cells start to come off the dish, add 2 ml of SC-BM and pool the cells in one or two 50 ml conical tubes.
5. Centrifuge for 10 min, at 200 × *g*, at room temperature.
6. Discard supernatant and gently resuspend the cells in 5–10 ml of SC-BM (adjust the volume based on the number of plates being used). Count cells using a hemocytometer.
7. Centrifuge for 10 min, at 200 × *g*, at room temperature.
8. Discard supernatant and gently resuspend the cells in ice-cold freezing medium, 1 ml per 2 × 10^6 Schwann cells.
9. Make 1 ml aliquots in cryotubes, and freeze overnight at −80 °C.
10. The following day, transfer frozen aliquots to a vapor phase liquid nitrogen tank for long-term storage.

3.9 Thawing Schwann Cells

1. Quickly thaw a frozen aliquot at 37 °C in a water bath.
2. Transfer the cell suspension to a 15 ml tube containing 9 ml of pre-warmed (37 °C) SC-BM.
3. Centrifuge for 10 min, at 200 × *g*, at room temperature.
4. Discard supernatant and gently resuspend the cells in 8 ml of SC-EM medium.
5. Plate cells onto two ø100 mm tissue culture plates, 4 ml per plate (prepared the day before; *see* Subheadings 3.4 **step 9** and 3.5 **step 1**).

6. Incubate cells at 37 °C and 10% CO_2 in a humidified cell culture incubator until cells are 100% confluent (about 5–7 days). Change culture medium with fresh 10 ml of SC-EM at day 3.
7. When cells are confluent, replace medium with SC-BM for 3 days before use in experiments. Alternatively, cells may also be passaged (*see* **Note 24**) following the steps in Subheading 3.6.

4 Preparation of Dorsal Root Ganglia Neurons from Rat Embryos (*See* Note 25)

4.1 Day 0: Preparing Culture Plates

1. Using the coverslips forceps, place one glass coverslip in each well of 24-well plates (*see* **Note 26**).
2. Thaw, on ice (*see* **Note 5**), one, two, or three aliquots of Matrigel (1 aliquot per 4½ 24-well plates).
3. Depending on the number of Matrigel aliquots to be thawed, prepare one 15 ml conical tube with 9.9 ml of ice-cold Neurobasal (1 aliquot) or one 50 ml conical tube with either 19.8 or 29.7 ml of ice-cold Neurobasal (2 or 3 aliquots). Keep tube on ice.
4. Transfer the Matrigel to the tube containing the Neurobasal medium, using a prechilled 2 ml serological pipet (*see* **Note 5**). Ensure thorough mixing by immediately pipetting up and down several times, keeping the tube on ice. Matrigel will be at a final concentration of 250 μg/ml.
5. Using a 200 μl wide orifice pipet tip, gently transfer 100 μl of Matrigel solution directly onto each glass coverslip. The solution must stay on the coverslip (*see* **Note 27**).
6. Slowly and without any abrupt moves, transfer the plates at 37 °C into the humidified cell culture incubator set at 5% CO_2. Incubate overnight.

4.2 Day 1: Harvesting DRGs, Tissue Dissociation, and Plating

4.2.1 Preparation

1. Sterilize the dissection tools by immersing them into 70% ethanol for 30 min (*see* **Note 13**). Then, air-dry.

   The following tools will be needed: three large scissors, three tissue forceps, curved Graefe forceps, extra-fine Bonn scissors, Dumont forceps #5 (standard tips), Dumont forceps #5 (biology tips), and spring scissors.
2. Prepare one 1.5 ml microcentrifuge tube with 1 ml of L-15 medium; keep warm in the heat block at 37 °C.
3. Prepare two ø100 plates with 30 ml of L-15 at room temperature.
4. Prepare one ø35 mm plate with 4 ml of L-15 at room temperature.
5. Warm 5 ml of DRG-BM in a 15 ml conical tube at 37 °C in water bath.
6. Warm 20–40 ml of DRG-BM in a 50 ml conical tube at 37 °C in water bath (*see* **Note 28**).

4.2.2 Collecting the Uterine Horns of a Pregnant Rat

1. Euthanize the pregnant rat by CO_2 inhalation.
2. Place the rat ventral side up, on absorbent paper towels. Sterilize by spraying with 70% ethanol until the fur is thoroughly soaked (*see* **Note 29**).
3. Using one tissue forceps, lift the belly skin up and cut a buttonhole with one of the large scissors. Insert the blunt-ended tips of the scissors in between the skin and the abdominal muscles to separate both apart. Cut the skin up to the sternum and down to the pubic symphysis, then left and right. Move the skin flaps away.
4. With a clean pair of tissue forceps and large scissors, cut the abdominal muscle wall as was done for the skin and move the flaps away (*see* **Note 30**).
5. With the last clean tissue forceps, grab the cervix and lift up gently. Using the last clean large scissors, cut the cervix close to the vaginal orifice and continue lifting. The uterine horns should follow easily. Cut away any tissue that comes along (uterine artery and some fat). Transfer the horns to a sterile empty (no medium) ø100 mm plate (*see* **Note 30**).

4.2.3 Extraction of Embryos from the Uterine Horns

1. Using the Graefe forceps, pinch the outside wall of the uterine horns at the level of an embryo. Use the Bonn scissors to cut a small hole through the wall of the uterus. When you see the amniotic sac, punch a whole. The embryo will naturally come out due to the internal pressure from the amniotic fluid. Cut the umbilical cord. Use the curved end of the Graefe forceps to lift (do not squeeze) the embryo and transfer to one of the ø100 mm plate with L-15 medium.
2. Repeat **step 1** until all embryos have all been collected in the same dish.
3. Swirl the dish to wash the embryos. Then transfer the embryos to a clean ø100 mm plate with L-15 medium.

4.2.4 Harvesting of the Spinal Cords and Collection of the DRGs

1. Under magnification of the dissecting scope, hold an embryo in place with a #5 Dumont forceps (standard tips) and euthanize by cutting off the head with the spring scissors (line 1 in Fig. 3a). Repeat for all embryos.
2. Place an embryo to its side and pin it down through the belly with one of the #5 Dumont forceps (standard tips). Insert the tips of spring scissors vertically toward the center of the embryo (center along the anteroposterior axis) and ventral to the vertebral column.
3. Make a couple of sequential cuts toward the anterior end of the embryo and then a couple of sequential cuts toward the posterior end of the embryo, along line 2 shown in Fig. 3a.

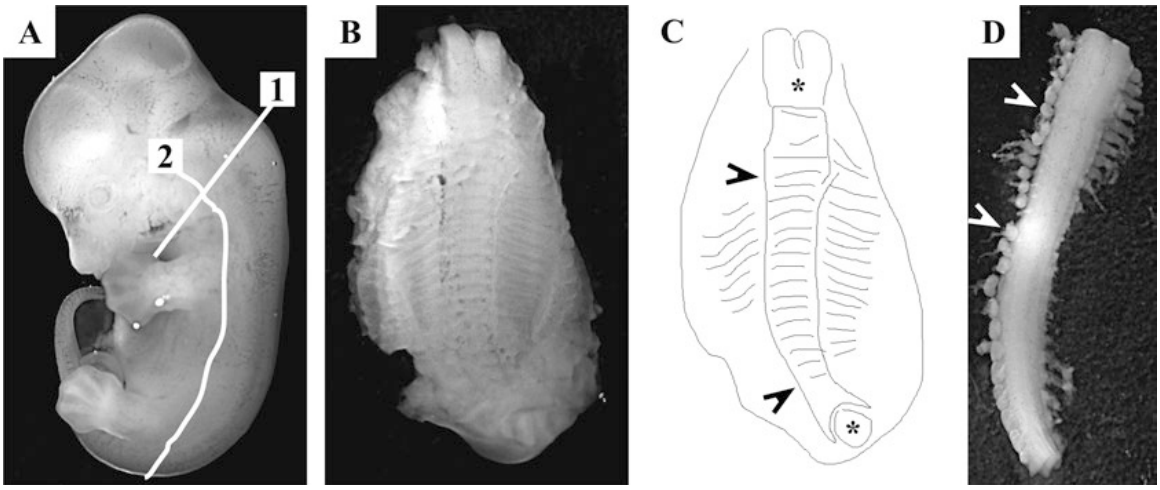

Fig. 3 Representative steps of the DRG dissection. (**a**) An E15 rat embryo. Lines 1 and 2 represent cuts that are to be made as described in Subheading 4.2.4. (**b**) The dorsal part of the embryo, ventral side up, after cutting along lines 1 and 2. (**c**) A schematic representation of panel B. The arrowheads point to the vertebral body, and the asterisks indicate visible portions of the spinal cord. (**d**) An isolated spinal cord with DRGs (arrowheads) attached

4. Remove the ventral half portion of the embryo including all the limbs. Continue until all embryos are similarly processed. Transfer the ventral portions (*see* Fig. 3b) into a clean ø100 mm plate with L-15 medium.
5. Lay the remaining dorsal tissue on its back so that the ventral side is facing up. Hold the tissue down with one #5 Dumont forceps (standard tips) and remove any remaining organs (mostly lungs, kidneys, and aorta at this stage) from the ventral surface with the other one. The vertebral body should be visible in the middle of the dorsal plate (*see* Fig. 3b, c).
6. While gently holding the dorsal plate in the central region (in reference to the anteroposterior axis) with the tips of one of the #5 forceps (standard tips) on either side of the vertebral column, use the other #5 forceps (standard tips) to cut the vertebrae along the anterior to posterior axis. To do so, insert one branch of the forceps in between the vertebral body and the spinal cord, the other branch remaining outside. Close and pull out to cut open the vertebral body. This will expose the underlying spinal cord.
7. Gently lift up the anterior end of the spinal cord and pull it up, freeing it from the vertebral canal. Dorsal root ganglia attached along the sides of the cord will follow (*see* **Note 31**). Transfer the spines with the attached DRG to the ø35 mm dish with L-15 medium (*see* Fig. 3d). A good dissection should have about 35–40 DRGs per spine.

8. Switch to using the #5 Dumont Forceps biology tips. While gently holding the spinal cord between one set of forceps, use the other one to pinch off the ganglia from the dorsal root. Be careful not to carry over any meninges from the spinal cord. Collect all the ganglia and discard the cord.
9. Repeat **step 8** until the DRGs from all the spines have been collected. All the collection is done in the ø35 mm dish, setting the DRGs aside in a pile.

4.2.5 Tissue Dissociation and Plating of the Cells

1. Using the 200 µl Pipetman setup with a wide orifice tip (*see* **Note 32**), collect all DRGs in one go and transfer to the microcentrifuge tube containing the L-15 in the heat block.
2. Most of the DRGs will settle down quickly by gravity. There is no need to spin. If a few DRGs cling to the sidewall, gently tap with you finger to dislodge them, and they will settle down.
3. Use the 200 µl Pipetman to gently remove the L-15 medium (*see* **Note 33**), and replace with 1 ml of 0.25% trypsin (*see* **Note 34**). Incubate at 37 °C in the heat block for 15 min. Gently flick the tube to resuspend the DRGs (*see* **Note 35**) and incubate for an additional 15 min.
4. Use the 200 µl Pipetman to gently collect the DRGs and transfer to 5 ml of DRG-SM in a 15 ml conical tube. Do not aspirate up and down to dissociate the DRGs just yet (*see* **Note 35**). Close the tube, gently invert a couple of time, and centrifuge at 200 × *g* for 10 min at room temperature.
5. While **step 4** is ongoing, finish preparing the 24-well plates. To do so, place a 200 µl pipet tip (standard yellow tip) to the end of a glass Pasteur pipet connected to vacuum. This will prevent the glass coverslip to become vacuum-stuck to the end of the Pasteur pipet. Aspirate the excess of Matrigel off the coverslips.
6. Once the DRGs have been pelleted (**step 4**), use a Pasteur pipet connected to a vacuum line to aspirate the supernatant, leaving about 50 µl behind. Using a wide bore 200 µl pipet tip, resuspend the cells in 200 µl of DRG-SM. It should take no more than about 10 up-and-down pipettings. Transfer the dissociated cells into the DRG-SM prepared in the 50 ml tube (*see* note in **step 8**). Mix by inverting and swirling.
7. Seed 100 µl of the cell suspension directly on each coverslip using the wide bore 200 µl pipet tips. The drop should stay on the coverslip. Gently mix the cell suspension in the 50 ml tube after every 24-well plate to ensure an even suspension and density homogeneity across all cultures. Transfer the plates gently to a humidified cell culture incubator at 5% CO_2 and 37 °C, and incubate for 24 h.

8. *Note*: the seeding is done at a density of 1.35 DRG per coverslip. Therefore, as an example, for 100 cultures, one would need 135 dissociated DRGs in 10 ml of medium. Since we prepare an extra of 15% (*see* **Note 28**), the actual number of DRGs is 155 whose cells are resuspended in 11.5 ml of medium.
9. For transduction experiments (*see* Chapter 12), DRG cultures are used 24 h after plating.

4.2.6 DRG Neuron Purification

1. Gently shake the plates so that the 100 μl of culture media drop off to the side. Aspirate the drop with a Pasteur pipet connected to a vacuum line. Do not aspirate directly on top of the cultures.
2. Feed the cultures with 200 μl of DRG-FU, and incubate at 5% CO_2 and 37 °C, for 72 h.
3. Replace media with 200 μl of DRG-BM, and incubate at 5% CO_2 and 37 °C, for 48 h.
4. Cultures should be >99% clean. Fibroblasts do not grow in B-27-based Neurobasal media.
5. Change media two more times with fresh DRG-BM, every other day. DRG neurons will develop an extensive neurite network (Fig. 4). Cultures will be ready to use 10 days after dissection and can be kept for about a month for myelination studies if media is changed every 2–3 days (weekday/weekend schedule) with fresh DRG-BM.

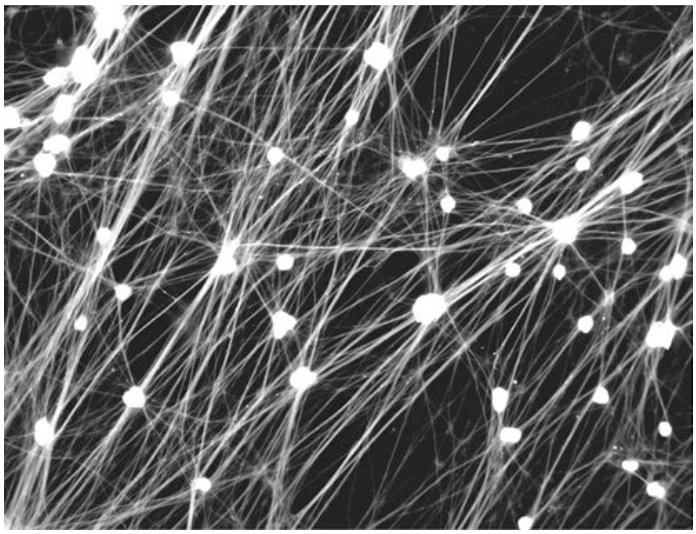

Fig. 4 Representative image of a culture of purified DRG neurons. Phase contrast image. The bright spheres are the neuron bodies; the meshing extensions are the neurite network

6. If an additional round of cleanup with antimitotic agents is necessary after **step 4**, repeat **steps 2** and **3** one more time (*see* **Note 36**).

5 Setting Up the Myelinating Schwann Cell/DRG Neuron Co-culture System

Schwann cells are seeded at a density of 1×10^5 cells per DRG neuron culture. One ø100 mm plate of Schwann cell culture should have about $4–5 \times 10^6$ cells, i.e., enough for about 40–50 DRG neuron cultures. Adapt the following steps (given for one plate) based on the specifics of your experiment.

1. Three days before use, change Schwann cell media from SC-EM to SC-BM.
2. Then wash the cells twice with 5 ml of HBSS at room temperature.
3. Discard HBSS and add 5 ml of 0.25 trypsin-EDTA solution; incubate at 37 °C for about 5 min.
4. Observe the cells under light microscopy while gently shaking the culture dish.
5. When the cells come off the dish, add 2 ml of CCM, and collect the cells in a 15 ml conical tube.
6. Centrifuge for 10 min, at 200 × *g*, at room temperature.
7. Discard supernatant and gently resuspend the cells in 5 ml of CCM.
8. Count the cells using the hemocytometer.
9. Use CCM to adjust cell density to 1×10^5 cells per 500 µl of media.
10. Gently aspirate DRG neuron culture media and add 500 µl of Schwann cell suspension per DRG culture. Incubate at 37 °C, 5% CO_2, for 24 h (*see* **Note 37**).
11. The following day, change media to remove any dead cells, replacing with fresh 200 µl of CCM. Incubate for 6 days, replacing with 200 µl of fresh CCM at day 3.
12. On the sixth day, initiate myelination by changing the CCM culture medium to MM medium, 200 µl. Change every other day during the weekdays, 3 days over the weekend. A few myelinated segments should be visible by phase contrast within 4 days (*see* **Note 38**). Figure 5 shows a representative image of a myelinated culture at day 10.

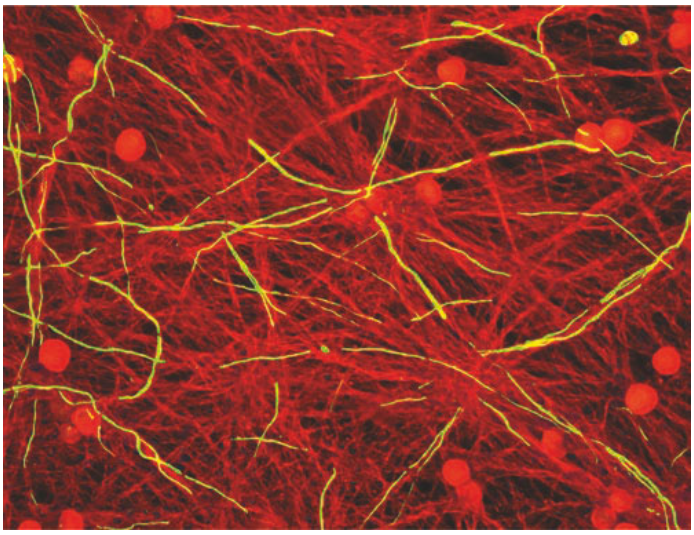

Fig. 5 Immunofluorescent visualization of a myelinated co-culture. Ten-day-old culture. The neurite network is detected by staining for neurofilaments (red). Compact myelinated segments (green) are detected by staining for myelin basic protein, a major component of the compact myelin sheath

6 Notes

1. Whenever possible, select chemical reagents that have been certified for cell culture or biology work.
2. Serum quality is critical for Schwann cell preparation and myelination. Some serum lots will induce substantial Schwann cell proliferation even in the absence of added mitogenic factor such as EGF-D. Also not all lots of serum will support myelination. Screen several lots to identify a serum that is adequate for both Schwann cell preparation and myelination. We usually test for proliferation by EdU incorporation assays (Invitrogen C10337). Basal level of incorporation in purified Schwann cells cultured in SC-BM for 48 h, with EdU added during the last 4 h should be less than 5%. Test for myelination following the protocol in Subheading 5. The Gibco catalog # 16000 for FBS is provided as an indication as to the quality of serum to look for.
3. Heat inactivate for 30 min in a water bath set at 56 °C. Have a bottle with an equivalent volume of water with a thermometer. Begin timing when the thermometer reaches 56 °C.
4. Matrigel concentration is usually around 9–13 mg/ml. Pay attention to this lot-to-lot variability to prepare the 2.5 mg/ml stock solution.
5. Keeping Matrigel on ice using ice-cold Neurobasal and pre-chilled pipets (by running ice-cold media up and down several

times) is absolutely necessary. Matrigel at high concentrations (1 mg and above) will start polymerizing at about +10 °C and will gel very quickly if brought up to room temperature. It will not gel at the final concentration of 250 μg/ml and can then be used like any other culture substrate. Do not freeze/thaw aliquots; we found that the Matrigel substrate peeled off easily when we used aliquots that went through a couple of freeze/thaw cycles.

6. Use culture plates sterilized by gamma ray irradiation. Ethylene oxide sterilization leaves residuals to which many primary cells are sensitive to, particularly Schwann cells and DRG neurons.
7. There are different types of glass material (borosilicate, soda lime), surface treated or not, to which many cell types are sensitive to. We exclusively use coverslips made from the highest-quality borosilicate glass that can be found from suppliers such as Bellco Glass, Inc. (cat # 1943-10012A) and Carolina (cat # 633029). We have used these coverslips for close to 10 years without any issues.
8. All media and solutions are sterilized using 0.22 μm, low-protein-binding membrane, filtering devices.
9. The average litter size for Sprague-Dawley rats is 10–12 pups.
10. The mirror finish of the tips will prevent adhesive tissues, particularly neural tissue, from sticking to the tips. It will facilitate the dissection but it is not an absolute requirement.
11. They will facilitate the collection and rapid transfer of intact DRGs from plate to tubes. They will also be used to dissociate DRGs by up-and-down pipetting. The shearing forces generated by the narrow orifice of regular tips will damage the neurons. While a 2 ml serological pipet can be used, the small length of the 200 μl tips also minimizes the loss of neurons that would stick to the inside wall of the serological pipet.
12. A thin Styrofoam platform (about ½ in. thick), covered with towel paper and sprayed with 70% ethanol, is adequate. The thickness is more important: sufficient to provide hold to the syringe needles, but not too thick to create focusing problems once under the dissecting microscope.
13. Most materials used in the fabrication of dissecting tools develop brownish, rusty-looking spots upon repeated autoclaving. Whether these may have some toxic effects on cells we do not know. We prefer to err toward the side of caution.
14. The 3 ml per ø60 mm plate may seem a lot, and once the cell suspension derived from the dissociation of the sciatic nerves is added, the final volume will be 4 ml. This will ensure an even distribution of the cells across the plates, which will facilitate the next step of fibroblasts elimination.

15. Schwann cells proliferate very little in medium containing only 10% FBS and lacking growth factors such as Neuregulin-1-ß1 (*see* **Note 2**). However fibroblasts do proliferate. Ara-C is an antimetabolic agent that, after conversion into cytosine arabinoside triphosphate, impairs DNA synthesis of rapidly dividing cells, such as fibroblasts.
16. After 3 days of treatment with Ara-C, there should be many dead cells, mainly fibroblasts, floating in the medium. Schwann cells, spindle-shaped, should remain attached to the plates.
17. Schwann cells will respond to forskolin and EGF-D by proliferating and will start to crowd out the remaining fibroblasts
18. Thy 1.1 is a murine alloantigen that is displayed at the surface of rat fibroblasts, but not of rat Schwann cells [9]. Using an anti-Thy 1.1 antibody in conjunction with serum complement, it is therefore possible to selectively kill the remaining contaminating fibroblasts by complement-mediated cytolysis.
19. The length of treatment with complement is variable from one dissection to another, even for the same combination of Thy1.1 and complement lot numbers. Fibroblasts should visibly shrivel under phase microscope observation.
20. The time it will take to reach confluence depends on many factors, such as FBS quality and lot # of EGF-D. For this particular step, it will also depend on the efficiency of the dissociation of the sciatic nerves to recover Schwann cells, and of their quality after the Ara-C treatment.
21. Note the elongated shape of the Schwann cells, and the swirly pattern of the culture, typical of confluent, non-differentiated Schwann cells. The cells should not look spread out in every direction like fibroblasts.
22. We usually have enough sciatic nerves to prepare 5–6 ø60 mm plates, i.e., 5–6 ø100 mm plates once at passage 1. When at 100% confluence, there will be about 4–5 × 10^6 Schwann cells per ø100 mm plates.
23. Lentiviral transduction efficiency decreases with the number of passages. While it is still possible to use Schwann cells from a later passage if the lentiviral construct is small (about 5 kb between 5′ and 3′ LTRs), it will become a problem when using larger constructs.
24. Schwann cells expanded up to passage 5 will myelinate quite well. It is not recommended to expand further as they will gradually start losing their characteristics. Their response to growth factors diminishes, and they tend to enter senescence and start to myelinate poorly. Cells from passages 1–4 can be frozen for expansion up to the fifth passage in total, i.e., if starting from a frozen passage 3 aliquot, expand only twice. Fifth passage Schwann cells are to be used and unused cells

are to be discarded. We routinely use passage 1 for lentiviral transduction experiments, expand the remaining plates up to passage 3 to use cells in experiments that do not require transduction, and freeze the remaining cells.

25. The whole dissection, from rat euthanasia to the plating of the cells from dissociated DRGs should take no more than 3 h. The longer it takes and the lower the viability of the neurons will be.
26. An average pregnancy yields about 12 embryos. A good dissection will provide spinal cords with about 35–40 DRGs, i.e., a minimum of about 420 DRGs. At 1.35 DRG per culture, one can set up 310 cultures with one dissection, i.e., 13 24-well plates. The 1.1 ml size of the Matrigel aliquots allow for maximizing the use of Matrigel with regard to the uncertainty as to the number of embryos that will be available, since the plates must be prepared the day before dissection. We usually prepare nine 24-well plates, enough in most cases for the bi-weekly needs of a team of three students.
27. Surface tension, the slight viscosity on the diluted Matrigel, and the asperities of the glass coverslip edge ensure that the 100 µl of Matrigel solution will remain on the coverslip. Make sure that the coverslips are not touching the side of the wells. Usually less than 0.5% of the coverslips will have the drop of Matrigel fall off.
28. Prepare volume amounts based on the number of cultures to set up: 100 µl per culture. Due to volume discrepancies when using serological pipet and Pipetmans, we always prepare 15% more than is needed. The number of DRGs collected is for that volume of media to ensure that all coverslips will receive cells at the proper density.
29. It is not necessary to shave the fur off.
30. The use of a succession of scissors/forceps pairs to remove the skin, muscles, and then the uterine horns decreases the chances of contamination. Make sure not to cut through the intestines, which would lead to a massive contamination of the uterine horns and jeopardize the sterility of the embryo dissection.
31. Spinal cord and DRGs are on opposite side of the vertebrae. At this embryonic stage (E15), the vertebrae are barely starting to be cartilaginous and very soft. The DRGs should come along easily. If the embryos are slightly miss-timed (E16–E16.5), it may be necessary to gently score the vertebrae with one of the forceps or too many DRGs will be left imbedded in the dorsal tissue.
32. The narrow opening of a standard tip will damage the DRGs. You can use a Pasteur pipet whose opening has been smoothed by heat. However DRGs do stick quite well to glass.

33. Do not aspirate with a Pasteur pipet connected to a vacuum line. The DRGs are loose and will be sucked up.
34. Do not use trypsin that contains EDTA. The chelation of calcium will kill the neurons.
35. Do not flick hard. The DRGs, although dissociating, remain structurally coherent as a small ball of cells. Keep it that way until it is actually needed to separate the cells away from each other.
36. In our 10-year long experience with this protocol, it has not proven necessary.
37. Do not use DRG cultures immediately after using the DRG-FU media. Always change the media at least twice within a course of 4 days. Trace amounts of FU will either kill the Schwann cells or at the very least will render then unhealthy and myelination will be quantitatively and qualitatively very poor.
38. How long to keep the cultures to myelinate will depend on one's research questions. 10–15 days provide well-myelinated cultures with thousands of segments suitable for quantitative Western blot analyses as well as immunofluorescence qualitative and quantitative analyses of the cultures.

References

1. Bunge MB, Williams AK, Wood PM (1982) Neuron-Schwann cell interaction in basal lamina formation. Dev Biol 92(2):449–460
2. Cornbrooks CJ, Carey DJ, McDonald JA, Timpl R, Bunge RP (1983) In vivo and in vitro observations on laminin production by Schwann cells. Proc Natl Acad Sci U S A 80(12):3850–3854
3. Eldridge CF, Bunge MB, Bunge RP, Wood PM (1987) Differentiation of axon-related Schwann cells in vitro. I. Ascorbic acid regulates basal lamina assembly and myelin formation. J Cell Biol 105(2):1023–1034
4. Moya F, Bunge MB, Bunge RP (1980) Schwann cells proliferate but fail to differentiate in defined medium. Proc Natl Acad Sci U S A 77(11):6902–6906
5. Brockes JP, Fields KL, Raff MC (1979) Studies on cultured rat Schwann cells. I. Establishment of purified populations from cultures of peripheral nerve. Brain Res 165(1):105–118
6. Wood PM (1976) Separation of functional Schwann cells and neurons from normal peripheral nerve tissue. Brain Res 115(3): 361–375
7. Watkins TA, Emery B, Mulinyawe S, Barres BA (2008) Distinct stages of myelination regulated by gamma-secretase and astrocytes in a rapidly myelinating CNS coculture system. Neuron 60(4):555–569. https://doi.org/10.1016/j.neuron.2008.09.011
8. Barres BA (2008) The mystery and magic of glia: a perspective on their roles in health and disease. Neuron 60(3):430–440. https://doi.org/10.1016/j.neuron.2008.10.013
9. Brockes JP, Fields KL, Raff MC (1977) A surface antigenic marker for rat Schwann cells. Nature 266(5600):364–366