Chapter 7

Isolation and Purification of Primary Rodent Schwann Cells

Marta Palomo Irigoyen, Miguel Tamayo Caro, Encarnacion Pérez Andrés, Adrián Barreira Manrique, Marta Varela Rey, and Ashwin Woodhoo

Abstract

Schwann cells are the main glial cells of the peripheral nervous system (PNS) and play key roles in peripheral nerve development and function, including providing myelin that is essential for normal movement and sensation in the adult. Schwann cells can be readily destabilized by a wide variety of distinct conditions that range from nerve injury to immune assaults, metabolic disturbances, microbial infections, or genetic defects, leading to the breakdown of myelin (demyelination) and a subsequent switch in phenotypic states. This striking feature of Schwann cells forms the cornerstone of several debilitating and even fatal PNS neurological disorders that include the demyelinating neuropathies Guillain Barré syndrome (GBS) and Charcot-Marie-Tooth disease (CMT), and PNS cancers, including Neurofibromatosis.

Primary Schwann cell cultures have proved a valuable tool to dissect key mechanisms that regulate proliferation, survival, differentiation, and myelination of these glial cell types. In this chapter, we describe the steps involved in the isolation and purification of Schwann cells from rodent peripheral nerves and the use of these cultures to model myelination in vitro.

Key words Schwann cell, Sciatic nerve, Brachial plexus, Cyclic adenosine monophosphate, Myelination, Demyelination

1 Introduction

Mature Schwann cells found in adult spinal nerves are derived from multipotent neural crest cells via three main transitions; the first of these is the formation of Schwann cell precursors (SCPs) from neural crest cells. SCPs occupy mouse limb nerves at embryo day (E) 12/13 (E14/15 in the rat). The second transition is the generation of immature Schwann cells, which occupy nerves from E15/16 (E17/18 in the rat). Axon/Schwann cell numbers are matched at this stage by regulation of Schwann cell proliferation and death. The third stage of Schwann cell development is divergence of immature cells to form myelinating and non-myelinating cells [1].

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The process of myelination has evolved as a means to provide rapid saltatory conduction of nerve impulses along large axons. The physiological rationale for this development of myelination in axons of a minimum caliber is that saltatory conduction in small diameter axons would probably not lead to more rapid impulse transmission [2]. There are a number of acquired or inherited conditions that result in malformation or destruction of myelin, leading to sensory or motor symptoms (peripheral neuropathies). Inherited neuropathies are caused by mutations in a number of critical genes regulating formation and maintenance of myelin [3]. Acquired neuropathies are caused by a number of environmental influences, including nutrition (e.g., vitamin B12 deficiency), bacterial and viral infections (e.g., leprosy, AIDS), and metabolic diseases (e.g., diabetes, liver failure).

The myelination program during development proceeds with profound molecular changes as most of the antigens associated with immature Schwann cells are downregulated (e.g., p75NTR, L1, NCAM) and there is an upregulation of a number of genes associated with control of myelination (e.g., Krox-20, Oct-6) and formation of the myelin sheath (e.g., P0, MBP, Connexin 32) [4]. In demyelinating disorders, there is largely a reversal of the changes associated with myelination. Thus, most of the antigens associated with control of myelination and formation of the myelin sheath are downregulated and there is an upregulation of a number of genes associated with immature Schwann cells. The myelination program is dependent on an axon-associated cell-extrinsic signals, as well as on a group of cell-intrinsic factors in Schwann cells, including the transcription factors Krox-20, Sox-10, Oct-6, and NFkB, which act as positive regulators of myelination. Together these factors coordinate and drive the myelination program, and inactivation of these genes impairs or prevents myelination [4, 5].

The transcriptional regulation of myelination during development also incorporates a negative regulatory component. We recently showed that Notch signaling is inhibitory to myelination and that in normal development, Notch has to be inactivated to correctly time the generation of myelinating Schwann cells [6]. In parallel with this, myelin breakdown and other aspects of Schwann cell dedifferentiation are controlled through activation of a group of transcriptional regulators, including c-Jun [7].

Primary Schwann cell cultures have been very useful for identifying and studying key molecular mechanisms and signaling pathways associated with myelination and demyelination [6–8]. In this chapter, we describe the steps required for culturing highly pure primary Schwann cells, including nerve extraction, and Schwann cell isolation, purification, and expansion from rat/mice peripheral nerves (sciatic nerves or brachial plexus) in order to obtain a Schwann cell monolayer culture. We also describe a simple in vitro model of myelination, based on raising intracellular levels of cyclic adenosine monophosphate (cAMP) by treatment with cAMP analogues.

2 Materials

All cell culture media and reagents should be prepared under a sterile laminar flow hood (horizontal or vertical). Primary cell cultures are maintained in tissue culture incubators at 37 °C under 5% CO_2/95% air and 90% humidity.

2.1 Preparation of Schwann Cell Cultures

1. Postnatal day 3–7 (P3-7) mice/rat pups.
2. Nerve extraction medium: Add 1% Antibiotic/Antimycotic (A/A) to Leibovitz's L-15 medium.
3. Semken blunt forceps.
4. 2 Small size dissecting scissors straight.
5. 1 Micro-dissecting spring scissors Vannas-Tubingen.
6. 1 Dumont # 7 forceps and 3 Dumont # 5 forceps fine tips.
7. Round Handled Vannas straight sharp spring scissors.
8. Dissecting board.
9. 70% Ethanol.
10. Stereomicroscope.
11. Trypsin solution (0.25%): Make 100 µL aliquots. Snap freeze in liquid nitrogen and store aliquots at −20 °C.
12. Collagenase solution (4 mg/mL): Dissolve 100 mg collagenase Type 2 in 25 mL Hanks' Balanced Salt Solution (HBSS without calcium, without magnesium). Filter sterilize using a 0.2 µm syringe filter. Make 100 µL aliquots and store at −20 °C.

2.2 Preparation of Culture Plates

1. Poly-D-lysine solution (PDL) for rat Schwann cells: Dissolve 5 mg PDL in 11.5 mL sterile ddH_2O. Filter sterilize using a 0.2 µm syringe filter. Make 300 µL aliquots and store at −20 °C. Thaw one aliquot at the time and make 1:100 dilution in ddH_2O before use.
2. Poly-L-lysine solution (PLL) for mice Schwann cells: Dissolve 100 mg PLL in 100 mL sterile ddH_2O. Filter sterilize using a 0.2 µm syringe filter. Make 5 mL aliquots and store at −20 °C. Thaw one aliquot at the time and make 1:10 dilution in ddH_2O before use.
3. Laminin solution: Defrost laminin solution (1 mg/mL) on ice, and make 50 or 100 µL aliquots. Freeze down in liquid nitrogen and store at −80 °C. Thaw one aliquot at the time and make a dilution of 1:100 and 1:50 in DMEM for rat and mice Schwann cell cultures, respectively (*see* **Note 1**).
4. Tissue culture dishes (60 mm) and 12- and 6-well plates.
5. 12–13 mm coverslips.

2.3 Cell Culture Reagents

1. Cytosine Arabinoside (AraC) (1 mM): Dissolve 2.79 mg AraC in 2 mL sterile ddH_2O. Filter using a 0.2 μm filter. Make 100 μL aliquots, snap freeze in liquid nitrogen and store at −20 °C. Use at a dilution of 1:100 (final concentration of 10 μM).
2. Forskolin solution: Dissolve 10 mg forskolin powder in 2.4 mL absolute ethanol to make a 25 mM stock solution and store at −20 °C.
3. Progesterone stock solution: Dissolve 2.5 mg progesterone in 100 μL of absolute ethanol. Make fresh and discard after use.
4. Sodium selenite stock solution: Dissolve 4 mg sodium selenite in 10 μL NaOH and 10 mL Neurobasal medium. Sterilize using a 0.2 μm syringe filter. Make fresh and discard after use.
5. 3′,5-Triiodo-L-thyronine sodium salt (T3) stock solution (0.101 mg/mL): Dissolve 0.202 mg of T3 in 2 mL of absolute ethanol. Store at −20 °C.
6. Insulin solution (1 mM): Use at a dilution of 1:1000 (final concentration of 1 μM).
7. Dibutyryl Cyclic Adenosine Monophosphate sodium salt (db cAMP) solution (50 mM): Dissolve 100 mg db cAMP in 1 mL sterile ddH_2O to make 50 mM stock solution. Filter using a 0.2 μm filter. Make 500 μL aliquots, snap freeze in liquid nitrogen and store at −20 °C. Use at a dilution of 1: 50 for myelination assays (1 mM final concentration).
8. Recombinant human NRG1-beta 1 (NRG-1): To 50 μg of recombinant human NRG1-beta 1, add 1 mL of PBS containing 1% BSA. Make 5 aliquots of 200 μL concentrated stocks (50 μg/mL). Snap freeze in liquid nitrogen and store at −80°C. To 1 aliquot of 200 μL concentrated stock, add 800 μL of PBS containing 1% BSA, and then make aliquots (10–50 μL) of working stock solution (10 μg/mL). Snap freeze in liquid nitrogen and store at −80°C. We use this at a 1:1000 dilution giving a 10 ng/mL final concentration (*see* **Note 2**).
9. SATO supplement: To 50 mL of neurobasal medium, add 500 mg of BSA powder, 500 mg of apo-Transferrin powder, 80 mg of Putrescine dihydrochloride, 12.5 μL of Progesterone stock solution, and 500 μL of Sodium selenite stock solution. Filter sterilize using a 0.2 μm syringe filter. Make 1 mL aliquots, snap freeze in liquid nitrogen, and store aliquots at −20 °C.
10. Schwann cell defined medium: To 100 mL of Dubelcco's Modified Eagle Medium:Nutrient Mixture F-12 (DMEM/F12; 1:1, without glutamine), add 1% Antibiotic/Antimycotic (A/A), 1 mL of 100× Glutamax supplement, 2 mL of 50× B-27 supplement, 1 mL SATO supplement, 10 μL T3 stock solution, and 100 μL of 1 mM Insulin solution (*see* **Note 3**).

11. Rat Schwann cell purification medium: DMEM containing 10% Fetal Bovine Serum (FBS), 1% A/A, and 10 μM AraC.
12. Mouse Schwann cell purification medium: DMEM with 5% horse serum (HS), 1% A/A, and 10 μM AraC.
13. Rat Schwann cell expansion medium: Schwann cell defined medium containing 0.5% FBS, 2 μM Forskolin, and 10 ng/mL NRG-1 (*see* **Note 4**).
14. Mouse Schwann cell expansion medium: Schwann cell defined medium containing 0.5% HS, 2 μM Forskolin, and 10 ng/mL NRG-1 (*see* **Note 4**).
15. Rat Low Serum medium: Schwann cell defined medium containing 0.5% FBS (*see* **Note 4**).
16. Mouse Low Serum medium: Schwann cell defined medium containing 0.5% HS (*see* **Note 4**).
17. Rat Schwann cell myelination medium: Schwann cell defined medium containing 0.5% FBS and 1 mM db cAMP (*see* **Note 5**).
18. Mouse Schwann cell myelination medium: Schwann cell defined medium containing 0.5% HS, 1 mM db cAMP and NRG-1 (10 ng/mL) (*see* **Note 6**).
19. Freezing medium: 90% FBS and 10% dimethyl sulfoxide (DMSO).

2.4 Purification of Schwann Cells by Immunopanning

1. Hybridoma medium: 10% FBS, 1% A/A, 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol in RPMI medium. Filter sterilize using a 0.2 μm filter and store medium at 4 °C.
2. Ox-7 hybridoma cell line (rat Schwann cells) (*see* **Note 7**) or T24 hybridoma cell line (mouse Schwann cells) (*see* **Note 8**).
3. Rabbit/Goat anti-mouse/rat IgG.
4. Blocking solution (0. 2% BSA in PBS): Add 285 μL of 35% bovine serum albumin solution to 50 mL PBS.
5. Tris–HCl (50 mM, pH 9): Dissolve 12.1 g Tris in 200 mL ddH_2O. Adjust pH to 9.5 with HCl. Filter sterilize, and keep at 4°C.
6. Immunopanning medium: 10% FBS and 1% A/A in DMEM/F12 medium. Filter sterilize using a 0.2 μm filter and store at 4°C.
7. 10% Sodium azide: Add 5 g of sodium azide to 50 mL of water. Filter sterilize using a 0.2 μm filter and store at 4 °C.
8. Non-tissue culture treated plastic dishes (90 mm).
9. Centrifuge tubes: 15 and 50 mL.
10. Phase contrast microscope.

3 Methods

Cell culture experiments subsequent to tissue dissociation should be performed under sterile conditions and all procedures should be carried out at room temperature unless otherwise specified.

3.1 Substrate Coating

3.1.1 PDL-Coated Coverslips (for Rat Schwann Cells).

1. Autoclave 12–13 mm glass coverslips (100–200).
2. Dissolve 1 aliquot (300 μL) PDL solution in 30 mL ddH_2O.
3. Add PDL solution to coverslips in 150 mm culture dish.
4. Keep at RT for 2 h on shaker.
5. Remove PDL.
6. Wash with three changes of ddH_2O on shaker for 15 min each.
7. Leave to air-dry thoroughly by standing coverslips in racks in hood overnight.
8. Store desiccated at room temperature (*see* **Note 9**).

3.1.2 PDL-Coated Cell Culture Dishes (for Rat Schwann Cells)

1. Dissolve 1 aliquot (300 μL) PDL solution in 30 mL ddH_2O.
2. Add enough volume of PDL solution to completely cover dishes (e.g., 1.5 mL in 60 mm dishes). Leave at RT for 2 h.
3. Remove the solution and allow to air-dry.
4. Store desiccated at RT.

3.1.3 PLL-Coated Coverslips (for Mouse Schwann Cells)

1. Autoclave 12–13 mm glass coverslips (100–200).
2. Add 25 mL of 1 mg/mL PLL solution to coverslips in 150 mm culture dish.
3. Leave for 24 h at RT on shaker.
4. Remove PLL solution (*see* **Note 10**).
5. Wash with six changes of ddH_2O on shaker over 3 days.
6. Leave to air-dry thoroughly by standing coverslips in racks in hood overnight.
7. Store desiccated at room temperature (*see* **Note 9**).

3.1.4 PLL-Coated Dishes (for Mouse Schwann Cells)

1. Dilute 5 mL of 1 mg/mL PLL solution 1:10 to give 100 μg/mL solution.
2. Add enough volume of diluted PLL solution to completely cover dishes (e.g., 1.5 mL in 60 mm dishes). Leave at RT for 2 h.
3. Remove the solution and wash three times with ddH_2O.
4. Store desiccated at room temperature.

3.1.5 Laminin Coating

PLL- and PDL-coated dishes must be coated with laminin at least 1 h prior to plating cells.

1. Dilute the stock solution of laminin in DMEM to a final concentration of 20 μg/mL (1:50 dilution) for PDL- and PLL-coated coverslips, and PLL-coated dishes, and to a final concentration of 10 μg/mL (1:100 dilution) for PDL-coated dishes.
2. Add laminin solution to plates and coverslips, and leave the solution on for at least 1 h (*see* **Note 11**).
3. Remove immediately prior to plating cells (*see* **Note 1**).

3.2 Preparation of Schwann Cell Cultures

3.2.1 Peripheral Nerves Dissection

1. Sterilize dissecting area and dissecting equipment with 70% ethanol.
2. Sacrifice mice/rat pups (P3–7 days old) by decapitation with scissors (*see* **Note 12**).
3. Pin the body to a dissecting board with the dorsal side up.
4. Spray bodies with 70% ethanol (*see* **Note 13**).
5. Remove skin from lower part of the body (Fig. 1a).
6. Dissect out sciatic nerves, and place in 60 mm dish containing 5 mL nerve extraction medium (Keep dishes on ice) (Fig. 1c) (*see* **Note 14**).
7. Pin the body of the same animal this time with the ventral side up.
8. Remove skin from upper part of the body (Fig. 1b).
9. Dissect out brachial plexus, and place in 60 mm dish above containing 5 mL nerve extraction medium (Keep dishes on ice).
10. Once nerves have been extracted from all animals, proceed to clean the nerves from muscle and fat, and then remove epineurium from nerve (the outermost layer of connective tissue surrounding a peripheral nerve) using forceps under a stereomicroscope (*see* **Note 15**).

Fig. 1 Dissection of sciatic nerves and brachial plexus. (**a**) Sciatic nerves removal. Rat P5 pup body pinned with the dorsal face up, and skin removed from lower part of the body. A small cut in the muscle reveals the underlying sciatic nerve (red arrow). (**b**) Brachial plexus removal. Rat P5 pup body pinned with the ventral face up, and skin removed from upper part of the body. Arrow shows the brachial plexus underneath the muscle layer. (**c**) Sciatic nerves isolated after dissection

3.2.2 Nerve Digestion and Plating

1. Place all nerves together in a 15 mL centrifugation tube containing enzymatic digestion solution (100 μL of trypsin solution and 100 μL collagenase per animal).
2. Incubate nerves at 37 °C for 30 min.
3. Triturate gently using 1 mL pipette tip (four to five times) and continue with a 200 μL pipette tip (ten times) until tissue starts to dissociate (*see* **Note 16**).
4. Incubate the digested nerves at 37 °C for a further 10 min, and triturate nerves (as above) until tissue is fully dissociated.
5. Add an equal volume of L-15 medium containing 10% FBS to stop the enzyme reaction.
6. Centrifuge for 10 min at 200 *g* and at 4 °C.
7. Remove supernatant carefully and discard.
8. Resuspend pellet in Schwann cell purification medium, and plate onto PDL- and laminin-coated dishes (rat) or PLL- and laminin-coated dishes (mice).
9. Culture at 37 °C and 5% CO_2 for 3 days (*see* **Note 17**). Proceed to immunopanning.

3.3 Purification of Schwann Cells by Immunopanning

1. Defrost 1 vial of OX-7 or T24-31 hybridoma cells.
2. Add 1 mL of hybridoma medium and centrifuge at 200 *g* for 5 min.

3.3.1 Production of Hybridoma Supernatant (see **Note 18***)*

3. Discard supernatant and resuspend pellet in 7 mL of hybridoma medium. Plate onto 100 mm tissue culture dishes.
4. When the plate is about 70–80% confluent (after about 3–4 days), replace medium with 5 mL of fresh hybridoma medium.
5. Collect medium 2 days later. Filter sterilize and add sodium azide solution to a final concentration of 0.01% (1: 1000 dilution of 10% stock solution). Keep at 4 °C.
6. Split confluent hybridoma cells into four dishes, and repeat **steps 4** and **5**.

3.3.2 Preparation of Immunopanning Plates

1. Add 40 μL rabbit anti-mouse/rat IgG to 7 mL of 50 mM Tris–HCl solution and coat a 90 mm non-tissue culture plastic Petri dish with the secondary antibody mix. Leave overnight at 4 °C (*see* **Note 18**).
2. Remove the secondary antibody mix solution from the plate and wash with PBS three times.
3. Block nonspecific binding sites by adding a solution of 0.2% BSA in PBS and incubate at RT for 30 min.
4. Prepare primary antibody mix: Add 3 mL mouse OX-7 supernatant (for rat Schwann cells) or with 3 mL T24-31 supernatant

(for mouse Schwann cells) with 3 mL 0.2% BSA solution (*see* **Note 19**).

5. Remove the blocking solution from plates, and add primary antibody mix. Swirl the plates so that surface of each plate is completely covered. Leave for a minimum of 2 h at RT.
6. Wash three times with PBS, leaving the last lot on.

3.3.3 Immunopanning Process

1. Trypsinize AraC-purified rat Schwann cell cultures, and resuspend cells in 7 mL of immunopanning medium.
2. Remove PBS from immunopanning plate and transfer the Schwann cell suspension to this plate.
3. Shake plates gently and incubate at 37 °C for 10 min.
4. Shake plates again and incubate for a further 10 min (*see* **Note 20**).
5. Collect suspension and spin at 200 *g* for 10 min at 4°C.
6. The pellet is then resuspended in the Schwann cell expansion medium, and cultured for Schwann cell monolayer expansion (*see* **Note 21**).

3.4 Schwann Cell Plating and Expansion

1. Add laminin solution to PLL-coated or PDL-coated dishes (enough to completely cover plates, e.g., 1.5 mL onto 60 mm dishes) and leave at RT for at least 1 h prior to plating cells.
2. Remove laminin solution from the plates (*see* **Note 1**).
3. Plate Schwann cells onto laminin-coated dishes, and expand until 80% confluent.
4. Split cells at a ratio of 1:4 for experimentation (e.g., myelination assays) (*see* **Note 22**).
5. Cells can be frozen in cryovials in freezing medium, and stored in liquid nitrogen.

3.5 In Vitro Schwann Cell Myelination Assays

Treatment with db cAMP induces upregulation of myelin differentiation markers, which can be examined by immunocytochemistry, qPCR or western blotting. Below, we present the steps for in vitro myelination assays on coverslips (for immunohistochemical analyses) or plates (for Western blotting or RNA analysis) (Fig. 2).

3.5.1 Immunocytochemical Analyses (Coverslips)

1. Add a drop of laminin (15 μL volume) in the center of PLL- or PDL-coated coverslips and leave in incubator for 1 h.
2. In the meantime, trypsinize AraC-purified or immunopanned Schwann cell cultures, wash, and resuspend in Schwann cell purification medium (without AraC), and count number of cells.
3. Remove the drop of laminin solution from the coverslips, and plate the cells at a density of 5000 cells/coverslip in that exact

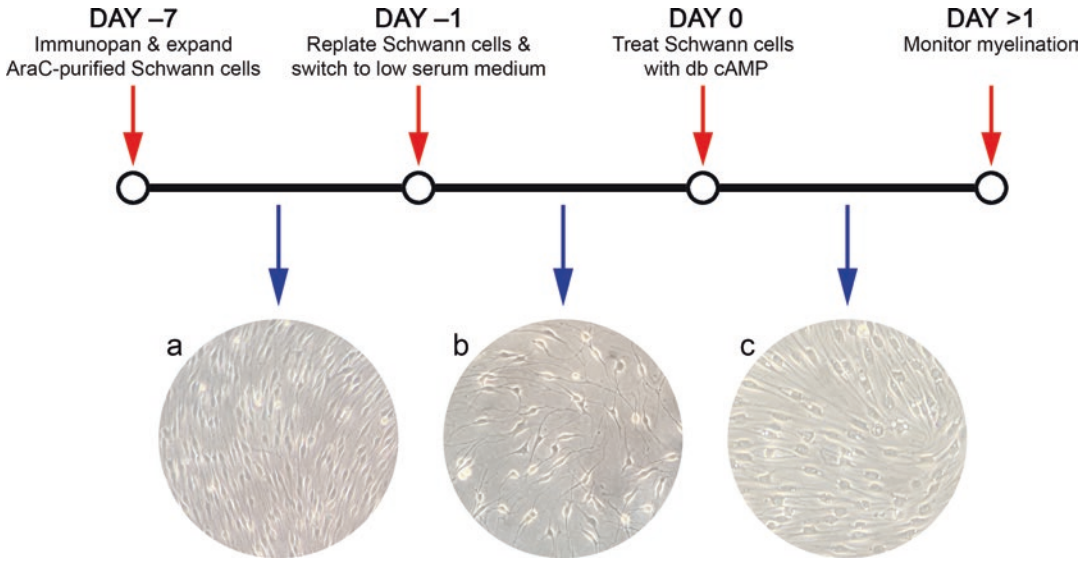

Fig. 2 Timeline of cAMP myelination assay. AraC-purified Schwann cells are immunopanned and expanded in expansion medium for several days, after which the cells are replated at an appropriate density and cultured overnight in low serum medium. The next day, the cells are treated with db cAMP, and myelination monitored afterwards using different techniques. Pictures show (**a**) confluent Schwann cells cultures in expansion medium, (**b**) Schwann cells cultures after replating and overnight culture in low serum medium, and (**c**) Schwann cells at 2 days after db cAMP treatment. Note the characteristic vesicular structures present in these db cAMP-treated Schwann cells

position as the laminin. Leave the cells to attach for 3 h in the incubator.

4. Add 485 μL of medium to each well (for rat Schwann cell: Schwann cell defined medium containing 1% A/A and 0.2% FBS; for mouse Schwann cells: Schwann cell defined medium containing 1% A/A and 0.35% HS). This brings that final concentration of serum per well to 0.5%.
5. Culture cells overnight at 37 °C and 5% CO_2.
6. The next day, medium change cells to myelination medium, and culture for 1–3 days (*see* **Notes 5** and **6**).
7. Fix coverslips and perform immunocytochemistry for myelination markers, e.g., Egr2, MPZ, and Periaxin [8].

3.5.2 Western Blot Analyses (Plates)

1. Trypsinize immunopanned Schwann cell cultures, wash, and resuspend in expansion medium (*see* **Note 23**).
2. Count cell numbers, and plate onto 12-well plates (100,000 cells per well) or 6-well plates (200,000 cells per well). Culture until at least 80–90% confluent.
3. Medium change cells to low serum medium and culture overnight at 37 °C and 5% CO_2.

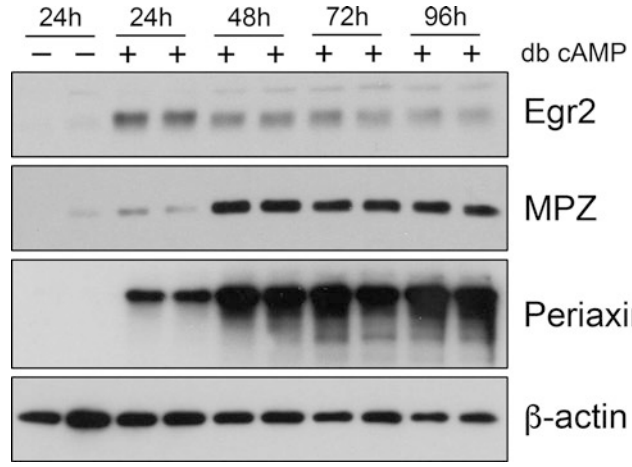

Fig. 3 Western blot showing upregulation of the myelin proteins Egr2, MPZ, and Periaxin in Schwann cell cultures treated with db cAMP at different time-points

4. The next day, medium change to myelination medium and culture for at least 48 h (*see* **Notes 5** and **6**).
5. Proteins and RNA can be extracted from these cultures for analysis of upregulation of myelin markers by Western blot (Fig. 3) and qPCR, respectively.

4 Notes

1. Laminin solution can be reused for up to four times. After use, keep at 4 °C. Reuse within 1 month after first use.
2. Discard working stock aliquots after use.
3. Schwann cell defined medium should not be stored for more than 1 month after the addition of all reagents.
4. Medium should be made fresh just before use.
5. NRG-1 can be added to rat myelination medium. It can greatly increase upregulation of myelin proteins [8].
6. In contrast to rat Schwann cells, both NRG-1 and db cAMP are required for the induction of myelin proteins in mouse Schwann cells [8].
7. The Ox-7 hybridoma produces a mouse IgG1 antibody that reacts with rat CD90 (Thy-1) and mouse CD90.1 (Thy-1.1), the latter being an allelic form of CD90 expressed by mouse strains AKR/J, PL, and FVB/N. The OX-7 antibody does not react with CD90.2, which is expressed by many mouse strains, including CBA and BALB/c.

8. The T24-31 hybridoma produces a rat IgG2a antibody that recognizes a non-polymorphic determinant on the Thy1.1 and Thy1.2 alloantigens [9].
9. Best not to use within 24 h. Can be kept for many months.
10. PLL solution can be re-frozen and used for up to three times.
11. Add enough volume of laminin solution to completely cover dishes (e.g., 1.5 mL in 60 mm dishes). For coverslips, we typically put a drop of laminin (15 mL volume) in the center of coverslip and leave in incubator for 1 h. We them remove the drop of laminin solution, and plate the cells in that drop. We leave the cells to attach for 3 h in the incubator before adding more medium (485 mL) per well.
12. All experiments using mice must be approved by the respective institutional animal ethics committee. The criteria may change between institutions and countries, and also over time, so please refer to the respective guidelines.
13. It is important to maintain sterile conditions to avoid contamination of cultures.
14. Use different sets of materials to remove skin, and to remove nerves.
15. It is important to remove as much as possible the epineural sheaths, since this is the greatest source of contaminating fibroblasts in Schwann cell cultures.
16. Do this gently to avoid making bubbles.
17. Cytosine arabinoside (AraC) is a pyrimidine antimetabolite that kills proliferating cells by inhibiting DNA synthesis. Contaminating fibroblasts found in these cultures are killed over this period of 3 days by AraC, since they are the only proliferative cells (Schwann cells do not proliferate in this medium). These cultures can be used straight away for experiments, especially immunocytochemical analyses, but would require further purification by immunopanning if expansion of these cultures is required. Purity of the Schwann cell cultures can be determined by performing immunocytochemical analyses using Thy1 antibodies to label fibroblasts and/or $p75^{NTR}$ antibodies to label Schwann cells.
18. This can also be done for 2 h at RT.
19. Commercial antibodies can also be used instead of hybridoma supernatant.
20. When looking down the microscope, you will see fibroblasts stuck to the surface of the dish and flattening, whereas the rest of the cells (Schwann cells) will be floating. Do not leave for longer than 20 minutes, since there will be nonspecific binding of Schwann cells to surface of dish.

21. Purity of these cells is typically >99% if immunopanning is performed properly.
22. We normally use expand Schwann cells for five passages only.
23. We use only immunopanned Schwann cells and not serum-purified Schwann cells to prevent expansion of contaminating fibroblasts in these long-term cultures.

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