Chapter 4

Isolation, Culture, and Cryopreservation of Adult Rodent Schwann Cells Derived from Immediately Dissociated Teased Fibers

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Abstract

Adult Schwann cell (SC) cultures are usually derived from nerves subjected to a lengthy step of predegeneration to facilitate enzymatic digestion and recovery of viable cells. To overcome the need for predegeneration, we developed a method that allows the isolation of adult rat sciatic nerve SCs immediately after tissue harvesting. This method combines the advantages of implementing a rapid enzymatic dissociation of the nerve fibers and a straightforward separation of cells versus myelin that improves both cell yield and viability. Essentially, the method consists of (1) acute dissociation with collagenase and dispase immediately after removal of the epineurium layer and extensive teasing of the nerve fibers, (2) removal of myelin debris by selective attachment of the cells to a highly adhesive poly-L-lysine/laminin substrate, (3) expansion of the initial SC population in medium containing chemical mitogens, and (4) preparation of cryogenic stocks for transfer or delayed experimentation. This protocol allows for the procurement of homogeneous SC cultures deprived of myelin and fibroblast growth as soon as 3–4 days after nerve tissue dissection. SC cultures can be used as such for experimentation or subjected to consecutive rounds of expansion prior to use, purification, or cryopreservation.

Key words Peripheral nerve, Teased fibers, Primary Schwann cell cultures, Myelin, Cryopreservation, Fibroblasts

1 Introduction

Schwann cell (SC) cultures are useful in vitro models to study SC biology associated with normal and abnormal nerve development, myelination, and regeneration [1, 2]. Procurement of large populations of SCs is relevant to therapeutic approaches aimed at promoting regeneration and myelination in the injured peripheral and central nervous systems [3–5].

Large-scale manufacturing of SC cultures was made possible after the discovery of critical soluble growth factors able to expand the cells over several passages in vitro [6–8]. Though SCs can be isolated from essentially any type of nerve at any stage of

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differentiation, the use of postnatal nerve tissues has been preferred due to the ease of isolation and high expansion potential of the SCs. In adult nerves, the large load of myelin and the presence of mature connective tissue layers make the separation of SCs versus myelin difficult without damaging the cells themselves. In addition, the presence of abundant connective tissue represents a substantial source of fibroblasts [6, 9] that can contaminate the SC cultures in the short or long term.

It has been shown that delaying enzymatic dissociation of the nerve explants by introducing an in vitro or in vivo pre-degeneration step results in increased cell yields and viability of the initial SC populations obtained from adult nerve digestion [6, 10–15]. However, a delayed dissociation may not be suitable for all applications, as the time needed for pre-degeneration can be considerable and the risk of fibroblast contamination can be increased. To eliminate the pre-degeneration step, we developed a reliable protocol that allows the rapid isolation of adult rat SCs by enzymatic digestion of teased nerve fibers immediately after tissue harvesting [16]. This method introduces a series of steps aimed at facilitating enzymatic dissociation, removing myelin and increasing cell yields and viability. First, the epineurium is mechanically removed, eliminating the most prevalent source of contaminating fibroblasts. Second, an extensive mechanical teasing of the nerve fibers is applied prior to incubation with proteolytic enzymes, which ensures a more complete digestion of the fibers and the release of highly viable individual cells. Third, the cells are plated in suspension as individual droplets onto a highly adhesive laminin substrate, which permits the fast separation of adherent SCs from floating myelin debris. This protocol yields highly viable populations consisting mainly of SCs with minimal fibroblast and myelin contamination. The SC cultures can be used or analyzed as soon as 3 days after plating or further expanded in medium containing chemical mitogens to increase the number of cells. The storage of the excess of cells as cryogenic stocks is made possible by following a simple protocol that does not cause detrimental effects on viability or biological activity after thawing and replating. With the proper modifications, this protocol is suitable for the isolation of SCs from different species and types of nerves. The minimally manipulated SC cultures obtained through this method can be used as such in experimentation. They can also be purified or cryopreserved at essentially any step of the process.

2 Materials

All materials, reagents, and solutions used for nerve tissue dissection and processing should be sterile and cell culture grade. Use ice-cold buffers and solutions during the steps of dissection,

teasing of the nerve fibers, and maintenance of tissues at all times prior to enzymatic treatment.

2.1 Tissue Harvesting, Removal of the Epineurium, and Teasing of the Nerve Fibers

1. Female Sprague-Dawley rat, adult, ~3-month-old.
2. 70% (v/v) ethanol.
3. Surgical fine scissors with straight sharp and blunt tips.
4. Forceps with serrated tips and straight or curved ends.
5. Scalpel handle with a surgical blade.
6. Vannas spring scissors.
7. Dumont number 3, 4, and 5 forceps with straight tips.
8. 60 mm × 15 mm polystyrene cell culture dishes.
9. L-15 medium. Leibovitz's L-15 medium supplemented with 25 μg/ml gentamicin. Maintain the L-15 medium on ice prior to and during the steps of nerve isolation, cleaning and teasing of the nerve fibers.
10. Horizontal laminar flow hood.
11. Dissecting stereo microscope.
12. Fiber-optic illuminator. 150 W dual-gooseneck fiber-optic illuminator for cool light illumination.

2.2 Enzymatic Dissociation of the Nerve Fibers

1. High-glucose Dulbecco's Modified Eagle's Medium (DMEM) with phenol red, pH 7.2.
2. Enzymatic cocktail. Prepare a 10 × stock solution by dissolving 125 mg of dispase II (neutral protease) and 25 mg of type I collagenase in 5 ml of DMEM. Sterilize by filtration through 0.22 μm filters. Store the 10× stock in aliquots at −80 °C. The 10× stock can be stored frozen for at least 1 year without significant loss of enzymatic activity. At the moment of use, thaw the aliquots of the 10 × stock on ice, and prepare a working dilution in DMEM. This solution is referred to as 1× enzymatic cocktail.
3. CO_2 cell incubator. Set at 37 °C with a humid atmosphere containing 9% CO_2 (*see* **Note 1**).
4. Plastic transfer pipettes with fine tip.

2.3 Plating and Growth of SC Cultures

1. 15 ml round-bottom centrifuge tubes with snap cap, polypropylene.
2. Straight end glass Pasteur pipettes, 5 × 3 in., with a flame-narrowed tip.
3. FBS/HBSS medium: 40% (v/v) decomplemented fetal bovine serum (FBS) prepared in HBSS, pH 7.2.
4. Benchtop centrifuge. Low-speed centrifuge with swinging bucket rotor, set at 4 °C prior to use.

5. Disposable 10 ml serological pipets, polystyrene.
6. Pipet-Aid.
7. 100 mm × 20 mm polystyrene cell culture dishes.
8. Distilled water, cell culture grade.
9. Poly-L-lysine (PLL) stock and working solutions. Prepare a 100 × stock solution of PLL by resuspending 500 mg of PLL (powder) in 12.5 ml of 1.9% (w/v) sodium tetraborate and 12.5 ml of 1.2% (w/v) boric acid. Prepare a 1 × PLL working solution by diluting the 100 × stock in distilled water. Aliquots of the 100 × PLL stock solution can be stored at −20 °C for up to 1 year. The 1 × PLL working solution can be prepared in advance and stored at 4 °C for up to 1 month.
10. Plastic paraffin film.
11. Dulbecco's phosphate-buffered saline (DPBS), pH 7.2.
12. Laminin stock and working solutions. The laminin stock solution consists of a commercially available sterile 1 mg/ml laminin from Engelbreth-Holm-Swarm murine sarcoma basement membrane, aliquoted and stored at −80 °C. Thaw the laminin aliquots slowly at 2–8 °C at the moment of use to avoid irreversible gelification. Prepare a working solution of laminin by diluting 55 μg of laminin stock solution in 10 ml of DPBS to coat one 100 mm cell culture dish. Scale the volume of the laminin working solution up or down according to the surface and the number of dishes used. It is recommended to use about 0.7–1 μg of laminin per cm^2 of coated surface.
13. PLL-/laminin-coated dishes. Add 1 × PLL working solution to cover the bottom of a 100 mm cell culture dish, and incubate it for 1 h at room temperature. Wash the plate three times with distilled water, and let it dry under the laminar flow hood. Air-dried PLL-coated dishes properly sealed with plastic paraffin film can be stored for up to 1 month at 4 °C. To proceed with laminin coating, add 10 ml of laminin working solution per PLL-coated dish. Incubate the plate at room temperature for 1 h, or place it at 4 °C until use without removing the laminin solution. Prepare at least five PLL-/laminin-coated dishes for plating the cell material derived from the processing of two sciatic nerves (*see* **Note 2**).
14. HBSS. Hank's balanced salt solution, formulated without calcium or magnesium. Containing phenol red, pH 7.2.
15. Low proliferation medium. DMEM supplemented with 10% FBS, 1% (v/v) 200 mM L-alanyl-L-glutamine dipeptide in 0.85% NaCl, 1% penicillin-streptomycin, and 25 μg/ml gentamicin.

16. High proliferation medium. Low proliferation medium supplemented with 10 nM heregulin-β1177–244 and 2 μM forskolin.
17. CO_2 cell incubator.
18. Inverted phase contrast microscope.

2.4 Expansion and Passaging of SC Cultures

1. Low and high proliferation media.
2. Disposable 5 and 10 ml serological pipettes, polystyrene.
3. HBSS.
4. Trypsin/EDTA solution. At the moment of use, prepare a working solution of trypsin/EDTA in ice-cold HBSS from a 10× stock consisting of 0.5% trypsin/EDTA. Maintain the stock and working solutions of trypsin/EDTA on ice at all times.
5. Inverted phase contrast microscope.
6. 50 ml polypropylene conical-bottom centrifuge tubes.
7. Benchtop centrifuge.
8. Digital cell counter or hemocytometer.
9. Trypan blue solution: 0.4% (w/v) trypan blue in 0.81% sodium chloride and 0.06% potassium phosphate dibasic solution (optional).
10. PLL-/laminin-coated 100 mm cell culture dishes.
11. CO_2 cell incubator.

2.5 Cryopreservation Thawing, and Plating of SC Stocks

1. Low and high proliferation media.
2. Trypsin/EDTA working solution and HBSS.
3. 15- and 50-ml polypropylene conical-bottom centrifuge tubes.
4. Inverted phase contrast microscope.
5. Benchtop centrifuge.
6. Cell counter.
7. Trypan blue solution (optional).
8. Freezing medium: 10% (v/v) dimethyl sulfoxide (DMSO) prepared in FBS (*see* **Note 3**).
9. Cryogenic vials, polypropylene, 2 ml, round bottom, self-standing.
10. Freezing container. Polycarbonate container with a tube holder, filled with 100% (v/v) isopropyl alcohol (*see* **Note 4**).
11. −80 °C laboratory freezer.
12. Liquid nitrogen tank.
13. Containers with wet ice and dry ice.
14. PLL-/laminin-coated dishes.
15. CO_2 cell incubator.

3 Methods

The protocol described below has been optimized for the processing of two sciatic nerve biopsies obtained from an adult female Sprague-Dawley rat. The schematic diagram and representative images presented in Figs. 1 and 2, respectively, are provided to illustrate the methodological steps in sequential order. Figure 3 contains representative images of typical SC cultures obtained via the drop-plating method. Proper scaling up or down of this protocol may be necessary according to the experimental needs and the availability of resources. The use of tissues from rats of a different gender or strain should render similar results. Optimization of this protocol for other types of nerves and species may be needed.

3.1 Tissue Harvesting, Removal of the Epineurium, and Teasing of the Nerve Fibers

Work inside a laminar flow, and implement good practices in the use of sterile technique for the procurement, handling, and use of cells and tissues. This is imperative to reduce the time of operation and avoid contamination in this and all subsequent steps. Prepare an excess of sterile forceps and other dissection tools for the fast replacement of instrumentation while performing the steps of tissue dissection and teasing of the nerve fibers. The risk of contamination can be minimized by the use of antibiotics in all media formulations, but pertinent precautions should be taken at all times to minimize exposure to potential sources of contamination. The use of antifungal reagents such as amphotericin B is not recommended for SC cultures, as this drug impairs cell survival. Prepare all reagents, materials, and equipment beforehand, and start the procedure in the early afternoon of day 0 so as to adhere to the digestion times recommended in Subheading 3.2 (*see* Fig. 1).

1. Euthanize an adult rat according to the regulations set forth by institutional policies for animal care and use.
2. Immediately after, place the rat body with its dorsal side facing upward on a clean surface. Thoroughly douse the hind limb area with 70% ethanol to disinfect the skin giving at least 1 min of skin contact so as to minimize the risk of contaminating the cell preparations. Next, make an incision in the skin located at the end of the abdominal zone (in the posterolateral hind limb area) using surgical fine scissors.
3. Lift and grasp the skin using serrated forceps. Extend the cut forward from the site of incision parallel to the femur and toward the knee by using surgical scissors (*see* **Note 5**).
4. Using a scalpel handle with a surgical blade, make a superficial cut to divide the muscle parallel to and just inferior to the femur. This procedure will expose the sciatic nerve.
5. Place the tip of the Vannas spring scissors right below the sciatic nerve, and proceed to open both ends to detach the nerve

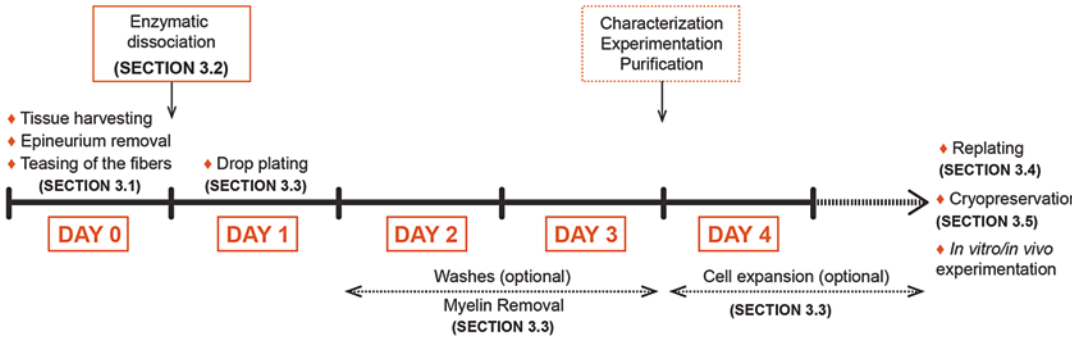

Fig. 1 Schematic diagram depicting the isolation of SCs from immediately dissociated teased nerve fibers. The timeline summarizes the sequence of events in the isolation protocol highlighting each section described in the chapter. Dotted lines represent undetermined or optional steps. This method allows the preparation of viable SC cultures with minimal myelin and fibroblast contamination. The cultures can be used for experimentation, purification, or cryopreservation prior to or after being subjected to expansion

from the surrounding muscular and adipose tissue. Next, cut the most proximal end of the nerve while holding it with a Dumont number 5 forceps. Then, cut the distal end of the nerve at the level of the knee.

6. Place the nerve segment in a 60 mm dish containing ice-cold L-15 medium immediately after dissection.
7. Proceed to dissect the sciatic nerve from the contralateral side as described above.
8. Prepare the laminar flow hood by placing a dissecting stereo microscope and fiber-optic illuminator in a conveniently located spot.
9. (Optional) While working under the laminar flow hood, rinse the nerve segments by transferring them into new 60 mm dishes containing ice-cold L-15 medium to remove blood and non-peripheral nerve tissues loosely attached to the nerve explant.
10. Remove any excess tissue from the sciatic nerves by using Dumont number 3 or 4 forceps while observing under the dissecting stereo microscope (*see* **Note 6**).
11. Transfer the clean nerve tissue into a new 60 mm dish containing ice-cold L-15 medium.
12. Mechanically remove the epineurium layer as one single sheath. Use Dumont number 4 or 5 forceps to grasp the nerve fibers at the proximal end, and subsequently pull the epineurium layer toward the distal end (*see* **Note 7**). Repeat this procedure with the contralateral nerve.

13. Discard the epineural layers, and collect the fibers from both nerves in a new 60 mm dish containing ice-cold L-15 medium.
14. Tease the nerve fibers with the aid of Dumont number 5 forceps by pulling off individual nerve fascicles prior to separating them into fibers of decreased caliber. The teasing step is repeated until all fascicles are separated as finely as possible into individual fibers regardless of the initial caliber of the fascicles (*see* Fig. 2a). This procedure is carried out while observing the tissue at the highest possible magnification under the dissection stereo microscope (*see* **Note 8**).
15. Carefully transfer the teased nerve fibers into a new 60 mm dish containing ice-cold L-15 medium using Dumont number 5 forceps for final inspection. At this stage, visually monitor the fiber preparation at high magnification under the dissecting

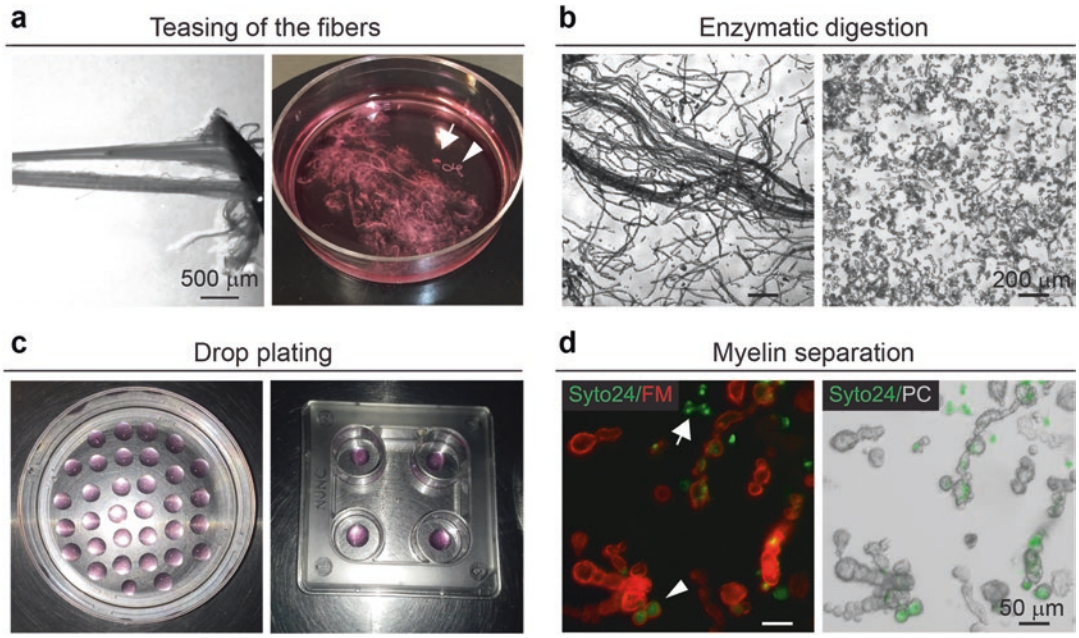

Fig. 2 Nerve teasing, dissociation, and plating of SCs obtained from immediately dissociated nerves. (**a**) Low-magnification view of the nerve teasing step (left). Macroscopic visualization of the teased fibers (*right*). An incomplete teased fiber (*arrowhead*) and segment of non-peripheral nerve tissue (arrow) were separated towards the side of the dish for further teasing and removal, respectively. (**b**) Phase contrast images showing dissociated fibers at 3 (left) and 18 h (right) after addition of the enzymatic cocktail. (**c**) Drop plating of the cell suspension onto PLL-/laminin-coated dishes. A variety of cell culture dishes can be used according to the experimental needs, such as 100 mm plates (left) for cell expansion and multiwell plates (right) for analysis. (**d**) End product obtained immediately after dissociation and plating. The fluorescence microscopy and phase contrast (PC) images show abundant cell nuclei stained with Syto24 (green) and floating myelin labeled with FluoroMyelin™ (FM, red). These preparations contain myelin-free cells (arrow) and myelin-associated cells (arrowhead)

stereo microscope to ensure the absence of non-teased fibers or excess tissue. Continue to mechanically tease and wash the fibers by transferring them to new L-15-containing dishes as many times as needed. Frequent addition or replacement of the L-15 medium is recommended to avoid warming of the media and to reduce the risk of contamination.

3.2 Enzymatic Dissociation of the Nerve Fibers

The enzymatic dissociation of the nerve fibers is the most important step that affects cell yields and viability. The temporal course of dissociation needs to be controlled and optimized for each enzymatic preparation particularly if mature nerves with fully developed connective tissue layers are used. We recommend the use of an enzymatic cocktail solution composed of type I collagenase and dispase II, as reported previously [6]. Increasing the digestion time or the concentration of the enzymes can increase cell recovery but may compromise the viability of the isolated cells. For best results, we suggest the experimenter to optimize this step by monitoring the progression of the digestion (*see* Fig. 2b) and the viability of the cells throughout the time course of digestion [16].

1. Remove the L-15 medium from the 60 mm dish containing the teased fibers by using a plastic transfer pipette being careful not to disturb the fibers.
2. Add 3 ml of 1X enzymatic cocktail to the teased fibers derived from both nerves ensuring that the fibers are completely immersed in this solution.
3. Incubate the nerve fibers with the enzymatic cocktail overnight (on average 15–18 h) in a CO_2 cell incubator (*see* **Note 9**).
4. (Optional) Monitor the progression of the enzymatic treatment and the isolation of viable cells by phase contrast (*see* Fig. 2b) and fluorescence microscopy intermittently during the digestion step (*see* **Note 9**).

3.3 Plating and Growth of SC Cultures

The drop plating of the cell suspensions onto a laminin-coated surface in the presence of chemical mitogens enhances the viability and growth rate of the cells. Plating the suspensions as small droplets allows SCs to rapidly attach to the surface and thus separate from the floating myelin. The combination of soluble heregulin and forskolin synergistically enhances SC proliferation and reduces fibroblast growth [8]. This section describes the protocol used for cell plating immediately after enzymatic dissociation of the teased nerve fibers. Proliferating SC cultures containing minimal myelin and fibroblast contamination can be obtained within 3 days post-plating.

1. Before cell plating, remove the laminin solution from each dish, wash the dish with distilled water, and let it dry in the laminar flow hood up until no traces of liquid are observed by visual inspection. The plates typically dry within 30 min.

2. Transfer the digested nerve fibers into a 15 ml round-bottom tube by using a glass Pasteur pipette. Gently rinse the dish twice by using 5 ml of FBS/HBSS medium to stop the enzymatic digestion and increase the recovery of cells.
3. Collect the cells by low-speed centrifugation at 200 × *g* for 10 min at 4 °C.
4. Remove the supernatant by using a glass Pasteur pipette, and mechanically resuspend the cell pellet by adding 5–10 ml of low proliferation medium. Gently and smoothly pipet the cell suspension up and down using a glass Pasteur pipette until it becomes homogeneous and no clumps are visible (*see* **Note 10**).
5. Centrifuge the cell suspension at 200 × *g* for 5 min at 4 °C (*see* **Note 11**).
6. Remove the supernatant, and resuspend the cells in 4–5 ml of high proliferation medium for plating onto PLL-laminin-coated dishes.
7. Plate the cell suspension as discrete 30 μl drops regularly distributed throughout the surface of a PLL-/laminin-coated dish. Usually, 20–30 drops are plated per 100 mm dish as shown in Fig. 2c.
8. Carefully transfer the culture dishes to a CO_2 cell incubator, and incubate the cells overnight for at least 15–18 h without disturbing the drops (*see* **Note 12**).
9. The following day, use a phase contrast microscope to confirm that the cells have attached properly. Gently and smoothly fill each 100 mm dish with 8–10 ml of high proliferation medium (*see* **Note 13**).
10. Feed the cultures with high proliferation medium three times per week. Monitor the progression of the culture on a daily basis with a phase contrast microscope. The increase in cell density should become apparent as soon as 2 days after plating.
11. (Optional) Determine the viability (*see* Fig. 3a), identity, and purity (*see* Fig. 3c–e) of the cultures by means of immunostaining using antibodies against SC-specific markers such as $p75^{NGFR}$, O4, S100, and glial fibrillary acidic protein (GFAP), alone or together with Thy-1, which is a fibroblast-specific marker (*see* **Note 14**). Representative images of SC cultures immunostained with antibodies for the abovementioned markers are presented in Fig. 3c–e. Myelin contamination can be determined by staining with FluoroMyelin™ (Fig. 3b).
12. Use the SC cultures for experimentation, or further expand the cells in high proliferation medium as described in Subheading 3.4. The use of an intermediate cell purification step is optional, as the percentage of contaminating Thy-1-positive cells usually does not exceed 10% of the population at this stage.

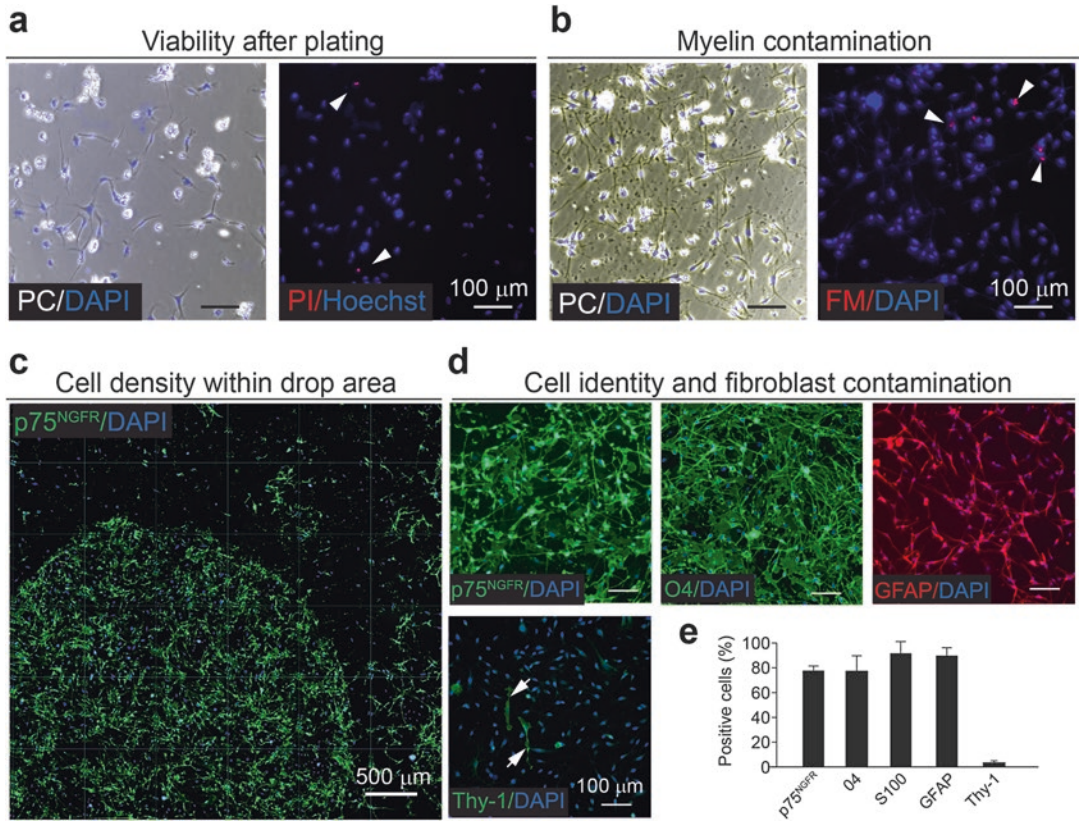

Fig. 3 Viability and phenotypic characterization of adherent SC cultures. (**a**) Viability of adherent SCs 24 h after plating. In the phase contrast (PC) image (left), note the numerous cells exhibiting a typical SC morphology. In the fluorescence microscopy image (right), note the low percentage of dead cells (arrowheads) labeled with propidium iodide (PI, red). All nuclei are counterstained with Hoechst (blue). (**b**) Myelin contamination 72 h post-plating. Staining with FluoroMyelin™ (FM, red) and DAPI (blue) reveals a high proportion of cells versus myelin debris (arrowheads). (**c**–**d**) Confirmation of SC phenotype through immunostaining 72 h post-plating. The low-magnification image (**c**) reveals the distribution and density of $p75^{NFGR}$-positive SCs within the area initially delimited by the drop. The high-magnification images (**d**) reveal the high purity and typical morphology of individual cells, as evidenced by the expression of markers specific to SCs ($p75^{NGFR}$, O4, and GFAP) and fibroblasts (Thy-1), respectively. (**e**) Fibroblast contamination 72 h post-plating. Quantitative results of a typical immunofluorescence analysis after staining the cultures with SC- and fibroblast-specific antibodies. Note the low percentage of fibroblasts as judged by the proportion of Thy-1-positive cells (arrows)

3.4 Expansion and Passaging of SC Cultures

The primary SC cultures obtained by this method can be passaged and further expanded to obtain sufficient numbers of cells for experimentation (*see* **Note 15**). Monitor the cultures daily by phase contrast microscopy to prevent overgrowth of the populations within the area originally restricted by the individual droplets (*see* Fig. 3c). Given the fact that the available surface area is limited and the cells actively divide in proliferation medium, the cultures can reach confluence within 4–5 days after plating. At this time the cultures need to be used for experimentation or passaged to

prevent cell loss due to substratum detachment. A standard protocol for cell expansion and subculture is provided below:

1. Once the cultures reach confluence within local areas, remove the culture medium and rinse the cultures with 10 ml of HBSS.
2. Add 5 ml of trypsin/EDTA working solution to the cells. Incubate the plates for 2–5 min at room temperature while monitoring the cells under the phase contrast microscope for signs of cell detachment (*see* **Note 16**).
3. Stop the action of the trypsin once the cells begin to detach by adding 10 ml of low proliferation medium directly onto the cultures.
4. Collect the cell suspension and transfer it to a 50 ml centrifuge tube. Rinse the dish with 5 ml of low proliferation medium to help detach and collect remnant cells. Use a 10 ml plastic pipette to transfer the cells while pipetting up and down several times (not more than 6–8 times) during the collection and transfer steps. This helps to both detach the cells from the dish and dissociate the small clumps.
5. Confirm the effectiveness of trypsinization and collection by inspection of the culture plate by phase contrast microscopy.
6. Collect the cells by centrifugation at 200 × *g* for 10 min at 4 °C.
7. Remove the supernatant, and resuspend the cell pellet in high proliferation medium for replating onto PLL-/laminin-coated dishes.
8. (Optional) Count the cells and estimate the percentage of cell viability according to the method of choice (*see* **Note 17**).
9. Plate the cells as homogeneously dispersed cells (*see* **Note 18**).
10. Place the dishes inside a CO_2 cell incubator immediately after seeding for improved attachment and growth.
11. Replace the medium two to three times per week with high proliferation medium until the cultures reach confluency.
12. Repeat **steps 1–11** in case further expansion is desired.

3.5 Cryopreservation Thawing, and Platingof SC Cultures

Primary SC cultures can be cryopreserved without losing their distinctive biochemical and functional properties [16]. In this section, the steps necessary to prepare cells for cryogenic storage and their subsequent replating after thawing are described in detail. It is recommended to use healthy SC cultures harvested in the logarithmic or exponential phase of growth for optimal results. Standard safety practices in cell cryopreservation should be used to minimize the risk of contamination and exposure of the SCs to the toxic effects of the DMSO.

3.5.1 Preparation and Storage of Cryogenic Stocks

1. Prepare a single cell suspension of SCs by harvesting the SC cultures via trypsinization and collection by centrifugation, essentially as described in Subheading 3.4 (**steps 1**–6).
2. Resuspend the cell pellet in low proliferation medium, count the cells, and determine their viability by the method of choice.
3. Spin the cells at 200 × *g* for 10 min at 4 °C.
4. Discard the supernatant, and gently resuspend the cell pellet in ice-cold freezing medium at a density of 1–2 × 10^6 cells/ml. Gently resuspend the cells up and down using a 10 ml pipette once or twice to obtain a homogeneous cell suspension prior to aliquoting.
5. Aliquot 1.5 ml of the cold cell suspension directly into properly labeled cryogenic vials placed on ice. Work fast as this is a time- and temperature-sensitive step that can seriously affect the survival and recovery of the cells.
6. Immediately transfer the cryovials to an isopropanol-filled freezing container to be placed in a −80 °C freezer.
7. Twenty-four hours after, transfer the cryovials to a liquid nitrogen tank for long-term storage.

3.5.2 Plating of Cells from Cryogenic Stocks

1. For thawing frozen stocks, transfer the cryogenic vials directly from the liquid nitrogen to a safe container filled with dry ice for transportation to the laminar flow hood.
2. Thaw the cells quickly by placing the vials in a 37 °C water bath up until 70% of the volume is melted.
3. Transfer the cell suspension (1.5 ml) to a 50 ml centrifuge tube containing at least 15 ml of ice-cold low proliferation medium. Diluting the freezing medium at 1:10–1:20 with low proliferation medium is recommended to reduce exposure of the SCs to the DMSO (*see* **Note 19**).
4. Collect the cells by centrifugation at 200 × *g* for 10 min at 4 °C.
5. Remove the supernatant, and immediately add 10 ml of high proliferation medium to resuspend the cell pellet. Gently pipette the cells up and down 2–3 times using a 10 ml pipette up until no cell clumps are observed.
6. Plate the cell suspension in a 100 mm PLL-/laminin-coated dish, incubate inside a CO_2 cell incubator, and allow for expansion as described in Subheading 3.4 (*see* **Note 20**).

4 Notes

1. SCs are highly sensitive to alkalization of the culture medium with a reduction of cell viability. Incubation of the cells in an 8–9% CO_2 atmosphere is preferred to prevent pH changes

when bicarbonate-buffered media formulations such as DMEM are used. It is of utmost relevance to work fast and avoid excessive exposure to the airflow, as this can lead to media alkalization. The use of phenol red-containing media and buffers is recommended to easily monitor pH levels based on color changes.

2. Sequential coating of plates with PLL and laminin provides a better substrate for adhesion and survival of the initial populations when compared to plates coated with PLL alone. Laminin coating may not be required, but it is preferable to maximize the rate of cell proliferation.
3. Commercially available freezing media for mammalian cell cultures such as Recovery™ (Gibco) can be used with nearly identical results in cell viability after thawing.
4. Commercially available freezing containers such as Mr. Frosty™ (Nalgene) placed in a −80 °C standard freezer are useful to deliver the appropriate cooling rate for optimal cryopreservation of SCs.
5. Removing the skin in an inverted V shape is useful to expose the posterior hind limb area up until reaching the knee. This area of the knee, also known as the popliteal fossa, is where the sciatic nerve branches into the fibular or peroneal, tibial, and sural nerves.
6. Remove any muscle, fat, or blood vessels that remain attached to the nerves with Dumont number 3 or 4 forceps. These tissues can increase the load of contaminating cells into the SC cultures if they are not efficiently removed prior to enzymatic dissociation.
7. The epineurium is the outermost layer of connective tissue that surrounds the nerve and constitutes the main source of contaminant fibroblasts. The percentage of fibroblasts in the final cell preparations can be strongly decreased by mechanical removal of this layer simply by pulling it from one extreme while holding the fascicles from the opposite side. Once separated from the fibers, this layer can be recognized on the basis of its whitish color and elastic properties conferred by the high content of collagen. The epineurium layer can also be collected and placed individually into a dish containing ice-cold L-15 for independent digestion and isolation of the constituent cells, as described in [16].
8. The teasing step consists of repeated pulling off the individual nerve fascicles up until fibers of the least possible caliber are obtained. Collectively, the steps of dissection, epineurium removal, and teasing of one nerve biopsy can be completed within a time frame of 30–45 min. The processing time is determined mostly by the technical skills of the operator.

9. The enzymatic digestion can be regularly monitored under the phase contrast microscope to visually follow the progression of tissue digestion. Enzymatic treatment is typically performed overnight to maximize the effectiveness of digestion and ensure cell release without the need for mechanical dissociation. However, the action of the enzymes may be halted at an earlier stage (e.g., 3–4 h after treatment) as soon as most of the individual myelinating cells get released from their association to extracellular matrix components. A determination of cell viability by means of staining with fluorescent dyes can prove useful at this and other steps of the process. This can be achieved by means of co-staining with propidium iodide, which selectively labels the nuclei of dead cells, and Syto24 (green), which labels the nuclei of all cells (reference control) and visualization by fluorescence microscopy (*see* Fig. 2d). Typically, assessment of propidium iodide incorporation immediately after overnight dissociation renders less than 10% propidium iodide-positive cells [16].
10. Mechanical dissociation is of relevance to reduce cell clumping and produce a single cell suspension for plating. Immediately isolated SCs have a tendency to adhere to each other and form aggregates of various sizes that may not adhere to the substrate. Caution should be taken at this step given the fact that excessive mechanical dissociation can compromise the viability of the cells.
11. Floating myelin debris and cell clumps can be observed by phase contrast and fluorescence microscopy using combined staining with a general nuclear marker and a selective myelin stain, such as FluoroMyelin™ (*see* Fig. 3b). If the digested cell suspension does not look homogeneous and tissue fragments or large cell clumps are observed, the steps of mechanical dissociation and centrifugation can be repeated. An optional filtering step using 70 μm nylon filters might be performed prior to centrifugation to remove myelin debris. However, avoid excessive manipulation and mechanical dissociation so as not to compromise the viability of the cells. Cell counting prior to myelin removal is challenging due to interference with myelin autofluorescence. We have found that fluorescence staining with the nuclear stains Syto24 and propidium iodide (red) is a useful combination to estimate the yields and viability during and after dissociation [16].
12. The drop-plating method allows the recovery of high densities of adherent cells per unit area. It contributes to the survival of the adherent cells by limiting fluid shearing motion while preventing the attachment of myelin debris. Plating the cells as drops in 100 mm dishes is recommended for in vitro expansion,

but plating in other formats is preferred for other uses such as determination of purity and viability (*see* Fig. 2c) [16].

13. At this point, cells begin to attach and extend processes on the laminin substrate, while most myelin debris remains floating. The separation of myelin from the viable adherent cells represents a major advantage of the drop-plating method. Gentle washes with DMEM can be performed to remove loosely attached myelin and other cellular impurities in this and all subsequent steps. Avoid the use of vacuum to aspirate the culture medium. Instead, use a glass Pasteur pipette with a thin tip to manually remove/replace the medium. It is advisable to leave a small volume of medium in each plate so as to prevent cell detachment by the flow created with the addition of new medium.
14. At 3 days post-plating, most SCs remain viable and display a typical elongated, spindle-shaped morphology. At this stage, most of the cells are actively proliferating and express the SC markers S100, $p75^{NGFR}$, GFAP, and O4 (cell surface sulfatide). The percentage of Thy-1-expressing cells should be less than 5% (*see* Fig. 3d–e) [16]. See Chapter 6 for a standard immunostaining protocol using live or fixed SC cell cultures.
15. SCs typically acquire a bipolar morphology and align along each other by forming a pattern reminiscent of a fingerprint. Adult rat SC cultures can be passaged up to 5–8 times without losing their morphology, growth properties, and ability for in vitro differentiation [16]. Extended culture can lead to an increase in the percentage of fibroblasts after 2–3 rounds of expansion.
16. Mechanical tapping of the dish usually helps to accelerate cell detachment. Check the progression of the dissociation by phase contrast microscopy, and stop the trypsinization as soon as detachment is observed. Confluent SC cultures typically detach as bundles. This is a time-sensitive step that is determined empirically in each case as assessed by microscopic observation. Avoid over-trypsinization of the cells, as this reduces cell survival.
17. Once the cells are in suspension, cell counting is useful to determine an appropriate dilution for use or replating. Viability assays can be performed using trypan blue exclusion assays [16]. This method should render a percentage of live cells higher than 90–95% if the time and magnitude of trypsinization are strictly monitored.
18. It has been estimated that confluent cultures can render ~100–150 $\times 10^3$ cells/cm^2. Subculture the cells by replating the cell suspensions in high proliferation medium at a ratio of 1:3–1:10 according to experimental needs.

19. SCs are particularly sensitive to the presence of DMSO in the freezing medium. Therefore, it is essential to remove the freezing medium as soon as possible after thawing of the stocks. A dilution of 1:10–1:20 with low proliferation medium is recommended in order to reduce the cryoprotector concentration and minimize cytotoxicity prior to centrifugation and replating.
20. Monitor the condition of the cells by phase contrast microscopy 2–3 h after plating. At this time, it is possible to observe some cells attached to the substrate extending branched processes, particularly if PLL-/laminin-coated dishes are used. Lack of attachment may be indicative of poor viability or a problem with the substrate. It is recommended to change the medium the following day after plating in case dead cells or excessive debris are observed.

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