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A Novel Method for Isolating Schwann Cells Using the Extracellular Domain of Necl1

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Myelinating cocultures of Schwann cells and dorsal root ganglion neurons are a powerful experimental system for probing the molecular mechanisms of axon–Schwann cell interaction. The isolation of a pure population of myelination-competent Schwann cells is a prerequisite for this experimental system. We describe here a protocol for a FACS-based isolation of Schwann cells utilizing a specific affinity reagent (Necl1-Fc) and the use of these isolated cells in myelinating cocultures. An advantage of the myelinating coculture system is that Schwann cells and the neurons can be genetically manipulated before they are cocultured. We further show that our method allows the isolation of virally transduced Schwann cells in a single purification step. This protocol for the FACS-based isolation of myelination-competent Schwann cells by Necl1-Fc and the use of these cells in myelinating cocultures should significantly facilitate future studies aimed at delineation of the molecular mechanisms of axon–Schwann cell interactions and myelination.

Key words: Schwann cells; myelination; Necl; axon

Myelination is a complex cellular process during which the glial cell [Schwann cells in the peripheral nervous system (PNS) and oligendrocytes in the central nervous system (CNS)] wraps its membrane multiple times around the underlying axon; the resulting multilamellar structure is called *myelin*. Even though the biochemical and molecular composition of myelinated fibers is rather well characterized (for review see Poliak and Peles, 2003), the molecular mechanisms governing the onset of myelination remain largely obscure. Recently, several studies have reported the identification of key molecules mediating the axon–Schwann cell interactions necessary to induce myelination (Eshed et al., 2005; Taveggia et al., 2005; Chan et al., 2006; Dzhashiashvili et al., 2007; Spiegel et al., 2007). These studies made extensive and elegant use of cell culture systems of in which primary Schwann cells myelinate neurons derived from the dorsal root ganglion (DRG), a method that was described by Bunge and colleagues (1967) decades ago. The obvious advantage of the culture experiments is the ease of pharmacological and genetic manipulation of the cells in culture, temporal control over the manipulation, and relatively short duration of the experiments (compared with generating and maintaining genetically modified rodents).

Several protocols for the isolation of Schwann cells from various mammalian origins (human, mouse, rat) and at different developmental stages (embryonic DRGs, neonatal sciatic nerve, and human biopsies) were published and are used today in many laboratories. The major obstacles to isolating Schwann cells are 1) to receive a large enough number of cells and 2) to remove contaminating fibroblasts from the culture. The separation of Schwann cells from fibroblasts so far has been achieved in several ways: by utilizing the differential adhesive properties of Schwann cells and fibroblasts (Kreider et al., 1981), different growth-rates of the two cells (by antimitotic treatment; Brockes et al., 1979), cell-type-specific immunodepletion of fibroblasts with a Thy1-antibody (Assouline et al., 1983), and more recently, direct isolation of Schwann cells by magnetically activated cell sorting (MACS) based on the expression of the $p75^{NTR}$ receptor (expressed by Schwann cells but not by fibroblasts; Manent et al., 2003; Vroemen and Weidner, 2003).

Here we describe detailed step-by-step protocols for isolation of Schwann cells by fluorescence-activated cells sorting (FACS) and their use in myelinating cocultures. This procedure for purifying Schwann cells is based on the specific binding properties of the extracellular domain (ECD) of Necl1, an axonal cell-adhesion

Additional Supporting Information may be found in the online version of this article.

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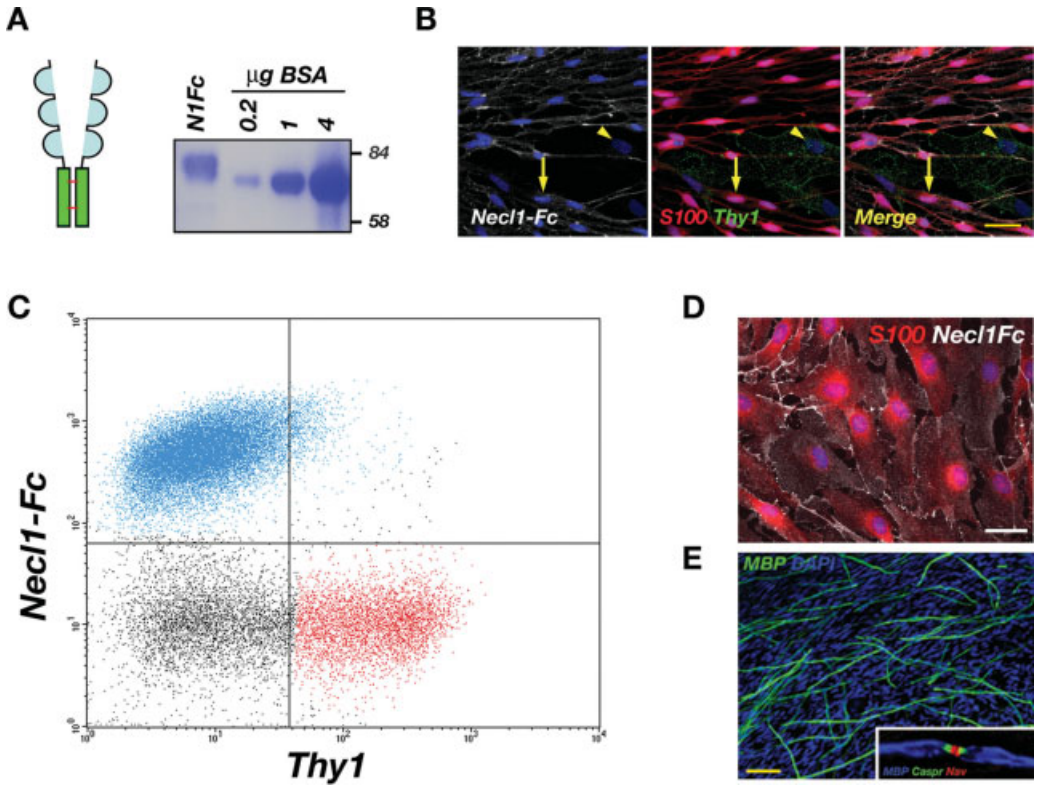

Fig. 1. FACS purification of myelination-competent Schwann cells by binding of Necl1-Fc. **A:** Production of Necl1-Fc-conditioned medium. A plasmid driving the expression of a fusion protein between the extracellular domain of Necl1 and the constant region of human IgG (e.g., Necl1-Fc) is transiently transfected into HEK293T cells. The amount of Necl1-Fc in the conditioned medium can be assessed by binding the secreted fusion-protein to protein A beads and running the bound protein in SDS-PAGE together with known protein standards (i.e., rising amounts of BSA). A typical preparation contains 1–2 μg Necl1-Fc/ml conditioned medium; this concentration is sufficient for the further uses described in this protocol. **B:** Necl1-Fc specifically binds to Schwann cells (labeled by S100, arrow) but not to fibroblasts (labeled by Thy1, arrowhead), both derived from DRGs; DRG-derived neurons are absent because of a previous trypsinization step. Note the spindle shape of the Schwann cells (DAPI labeling of cell nuclei in blue). **C:** Necl1-Fc labeling of Schwann cells can be used for FACS. Cultures similar to those in B were removed from the culture dishes nonenzymatically and labeled with Necl1-Fc and Thy1; the two markers label two distinct cell populations with Necl1-Fc binding typically to 75–85% of all cells. (For FACS analysis, 10,000 events were analyzed, and quadrants were placed such that untreated control cells stained only with secondary antibodies are within the lower left quadrant.) **D:** Cells sorted based on binding of Necl1-Fc are Schwann cells. FACS based on binding of Necl1-Fc allows the isolation of highly pure Schwann cells (labeled here with S100, Necl1-Fc, and DAPI); even though demonstrated here for DRG-derived Schwann cells, the same purification scheme can potentially be applied also for Schwann cells derived from the sciatic nerve. **E:** Schwann cells purified by Necl1-Fc-based FACS are myelination competent. When the FACS-purified Schwann cells (as described for D) are added back to and cocultured with DRG-derived neurons, they are able to myelinate the underlying axons. Myelin segments are best identified by staining the cultures for marker proteins such as myelin basic protein (MBP), and, importantly, these myelin segments exhibit the hallmarks of nodal specialization (see **inset**) including nodes of Ranvier (indicated by staining for Na_v channels in red) and their flanking paranodes (indicated by Caspr staining in green). Scale bars = 30 μm in B,D; 50 μm in E.

molecule that mediates axon-Schwann cell interactions at the onset of myelination (Spiegel et al., 2007): a fusion-protein of Necl1-ECD and the constant region of human IgG binds specifically to Schwann cells but not neurons or fribroblasts.

MATERIALS AND METHODS

A complete list of all media, antibodies, reagents, and equipment used in this study can be found in the Supporting Information.

Production of Necl1Fc-Conditioned Medium (Duration 6 Days)

Conditioned medium containing the soluble extracellular domain of Necl1 fused to the constant region of human IgG (e.g., "Necl1-Fc") is produced by transient transfection of HEK293T cells with an expression plasmid of the same construct (e.g., pSX-Necl1). The following procedure is based on the one described by Sambrook et al. (1989). Necl1-Fc was collected from 3-day-old cultures grown in DMEM, 1% low-IgG serum, and Pen-Strep mix. The quality of the Necl1Fc-conditioned medium can be checked by binding a small volume of conditioned medium to Sepharose-protein A,

running the bound protein by SDS-PAGE, and staining the gel with Coommassie blue (see Fig. 1A for expected result).

Production of Retroviral Stocks (Duration 3 Days)

A description of the production of ecotropic retroviral virions is beyond the scope of the protocol presented here. An excellent and very detailed protocol for the production of retroviral particles can be found on the web page of Dr. Garry Nolan (Stanford University) and his group (http://www.stanford.edu/group/nolan/protocols/pro_helper_dep.html). Their protocol is based on the ecotropic Phoenix cell line for the helper-free production of retroviral stocks (ATCC No. SD 3444). Viral stocks produced in this packaging cell line are very well suited for infection of rodent Schwann cells.

Dissection of DRGs From E15 Rat Embryos and Establishment of Dissociated DRG Cultures (Duration 5 Hours)

Before starting the dissection, prepare the following: 1) coarse dissection tools (all in a beaker filled with 70% EtOH) for decapitating the pregnant rat, cutting through the skin and muscle, and extracting the uterus: 1 pair of coarse scissors for decapitation and cutting through the skin and muscle, 1 finer pair of scissors for dissecting out the uterus, 1 coarse pair of forceps for holding the skin, and 1 finer pair of forceps for extracting the uterus; 2) fine dissection tools for extracting the embryos from the uterus and dissecting the DRGs (in a 50-ml tube filled with 70% EtOH): 2 pairs of fine forceps and one pair of bowed forceps; 3) several 10-cm and 6-cm tissue culture dishes; 4) two 10-cm and two 6-cm tissue culture dishes covered with Sylgard; 5) L15 medium (~100 ml per dissection); 6) a clean area covered with a paper towel where the rat can be dissected; 7) a dissection hood containing a dissection microscope and a light source (before starting the dissection, clean all of them by wiping with 70% EtOH); and 8) a styrofoam container filled with ice and covered with aluminium foil for cooling the embryos, spinal cords, and DRGs during the dissection.

1. Anesthetize the E15 pregnant rat by CO_2 inhalation.

2. Place the anesthetized rat on a paper towel on a clean surface. Decapitate and sterilize the rat's belly by spraying it with 70% EtOH.

3. Open the skin over the rat's lower belly; after cutting the skin, clean the scissors and forceps by dipping them into 70% EtOH.

4. Open the now exposed muscle over the lower belly; remove enough muscle until the uterus becomes visible. Be careful not to cut the digestive tract.

5. Using the finer forceps, find the uterus, grasp it, and gently pull out one uterine horn (appears like beads in a sleeve). Detach the uterus at its lower end with the finer pair of scissors and place it in a sterile 10-cm tissue culture (make sure to close the lid after placing the uterine horn in the dish). Proceed to the other uterine horn and remove this one also. Transfer the dish containing the dissected uterus to the dissection hood; add some L15 medium to the dish containing the dissected uterus.

6. Using the finer pair of scissors and the bowed forceps, remove the pups in their placenta from the uterine lining and place them in a second 10-cm tissue culture dish containing L15 medium.

7. Once all pups are removed from the uterine lining, using two fine forceps, peel each embryo out of its placenta and amniotic sac and place it in a 6-cm tissue culture dish filled with L15 medium. Once all embryos are stripped of their placentas and amniotic sac, place the 6-cm dish on ice.

Perform the next steps one pup at a time under the dissection microscope in a 10-cm Petri dish, covered with Sylgard and filled with L15; use the very fine forceps (FST No. 5).

8. With the two pairs of very fine forceps, place the pup in the 10-cm dish and pinch of its head and its tail.

9. Next, turn the pup until it comes to lie on its back. Grip the ribcage and open the stomach; remove all internal organs. Then proceed to open the ribcage and remove the lung. The embryo should now be lying on its back with all its internal organs removed (e.g., like a ''filet'' or open book).

10. With the pup lying on its back, pin it down with one pair of the forceps; with the other forceps, enter the spinal canal from the cervical side and carefully remove the ventral aspect of the vertebral column. Do this by gently cutting/pinching with the forceps first along the right side of the column (where the ribs connect to the vertebrae) and then along its left side. After removing the ventral aspect of the vertebral column, the spinal cord is now exposed along the whole pup when it is lying on its back.

11. While still pinning the pup down on its back with one pair of the forceps, gently enter with the other pair of fine forceps laterally between the ribs/vertebrae and gently spread them. This will loosen the DRG's attachment to the vertebral column.

12. Now remove the spinal cord (with the DRGs attached to it) by gently pulling it with one pair of the forceps out of the carcass, starting at the cervical end; use the other forceps to hold the carcass down while the spinal cord is being removed. Place the removed spinal cord with the attached DRGs in a 6-cm tissue culture dish filled with L15 and keep on ice.

13. Repeat steps 9–14 until all spinal cords are dissected out of the pups.

14. Now proceed to remove the DRGs from the spinal cords. Transfer two spinal cords into a 60-mm dish covered with Sylgard and filled with L15; place the dish under the dissection microscope. Using the two very fine forceps, separate the DRGs from the spinal cord by cutting the dorsal and ventral roots with the fine forceps; once all DRGs are separated from the spinal cord, discard the spinal cord. Proceed until all DRGs, detached from the spinal cords, are in the 60-mm dish under the dissection microscope.

15. Gently swirl the 6-cm dish with circle-like movements until all the DRGs are concentrated in the center of the dish. Using a 200-μl pipette, collect the DRGs and transfer them into a conical 15-ml Falcon tube kept on ice.

16. Spin the DRGs down to the bottom of the tube (1 min at 800 rpm); remove the supernatant and make sure not to aspirate any of the DRGs.

TABLE I. Samples and Controls Used for FACS Purification of Noninfected Schwann Cells

No.	No. of 10-cm dishes	Binding	Fluorophore	Cells for collection	Purpose
1	1	—	Streptavidin-Cy5	—	Control physiological state of cells; control of autofluorescence and background staining of streptavidin-Cy5
2	The rest	Necl1-Fc	Streptavidin-Cy5	Necl1Fc-positive	Purification of SCs

17. Add 2 ml trypsin without EDTA to the DRGs; briefly tap the tube to resuspend the DRGs in the trypsin and place the tube in an incubator at 37°C for 40 min.

18. Stop the trypsinization by adding 2 ml of heat-inactivated FCS; add an additional 4 ml L15 and centrifuge the trypsinized DRGs for 5 min at 800 rpm. Carefully discard the supernatant into a tube labeled ''Waste'' without disrupting the pellet.

19. Resuspend the DRGs in 2 ml NB medium and triturate the DRGs with a Pasteur pipette 20 times (the DRGs start to break apart during this step). Triturate the DRGs an additional 20 times with a fire-polished Pasteur pipette until the cell suspension becomes homogenous and no more cell clumps are visible.

20. For counting the cells, transfer 25 μl of the cell suspension into an Eppendorf tube, mix with 25 μl trypan blue for live-dead staining, and count the cells with a hematocytometer. The dissociated DRGs of one E15 rat pup yield typically, $\sim 4 \times 10^5$ cells.

Culturing the Dissociated DRG Cells Until Isolation of the Schwann Cells by FACS (Duration 10–12 Days)

The cell suspension resulting from the dissociated DRGs contains three major cell populations: DRG neurons, Schwann cells, and fibroblasts. In the beginning, all cells are cocultured: since the DRG neurons have a strong proliferative effect on the Schwann cells and because of the serum-free defined NB medium, the culture will soon become strongly enriched for Schwann cells. The neurons are then removed by trypsinizing the culture and replating the cells on Primaria plates (on which neurons cannot grow). The Schwann cells are finally separated from the fibroblasts by FACS based on their specific ability to bind Necl1-Fc.

1. Plate the cells resulting from the dissociated DRGs in six-well plates previously coated with Matrigel and PDL (Supporting Information); plate 2×10^5 cells per well in 2 ml NB medium.

2. On the next day, remove the medium and replace with 1.5 ml fresh NB medium per well.

3. On the next day, remove the medium and replace with 1.5 ml BN medium per well.

4. Continue to replace the BN medium every second day until the wells are confluently covered (mainly by Schwann cells growing along the neurites of the DRG neurons and rapidly filling the gaps between the neurites). It usually takes 5–8 days until the six wells are confluent.

Option: at this stage of proliferation, Schwann cells can efficiently be infected with retroviral constructs because of the strong proliferative effect of the DRG neurons on the associated Schwann cells. The infections are performed in three cycles, one cycle on each of the first 3 days in culture. For infections, the culture medium is removed from the well and undiluted viral sup (supplemented with 8 μg/ml Polybrene) is added to the cells; after 2 hr, the viral sup is removed and replaced with the culture medium.

5. When the wells are 100% confluent with cells, trypsinize the culture: remove the BN medium from each well, wash it once with 2 ml PBS^{--}, and then add 0.5 ml trypsin diluted 1:2 in PBS^{--}. Let the diluted trypsin act for at least 2–3 min and then start detaching the cells by vigorously tapping the six-well plate on its side. Once all cells are detached, stop the trypsinization by adding 1.5 ml SCPM to each well. Triturate the cells several times with a 1-ml pipette until the cell suspension is homogenous. Transfer the cell solution from three wells of the six-well plate into two 10-cm Primaria dishes already containing 10 ml SCPM. This step typically yields about eight 10-cm Primaria plates.

6. On the next day, remove the medium and replace with fresh SCPM. Continue to replace the medium with fresh SCPM every day until the 10-cm dishes are nearly confluent (usually 2–4 days). When the 10-cm Primaria dishes are nearly confluent, proceed to the purification of Schwann cells by FACS.

Troubleshooting: During this phase of the cultures, usually only Schwann cells and fibroblasts are left. Even though the Schwann cells are initially in large numerical excess, the fibroblasts sometimes tend to proliferate very fast (SCPM contains FBS) and threaten to overgrow the culture. This usually is due to low-quality NRG1β1-conditioned medium, which does not stimulate sufficiently fast proliferation of the Schwann cells. If the fibroblasts should start to overgrow the culture, immediately proceed to the FACS purification of Schwann cells.

Labelling of Schwann Cells With Necl1-Fc for FACS (Duration 3 Hours)

In a typical FACS purification of noninfected Schwann cells, labeling is done with Necl1-Fc (preclustered with a biotinylated anti-human Fc secondary antibody and followed by binding of Cy5-coupled streptavidin). See Table I for the samples and controls used in such an experiment. If previously infected Schwann cells are to be FACS purified, the separation is based on two positive selections: the infection and the binding of Necl1-Fc to Schwann cells. It is advisable to choose two fluorophores with distinct emission spectra to avoid ''bleed through'' from one channel to the other (i.e., GFP and Cy5 are an excellent choices because of the nonoverlapping emission spectra). See Table II for the samples and con-

TABLE II. Samples and Controls Needed for the Isolation of Schwann Cells Infected With a GFP Construct

No.	Infection	No. of 10-cm dishes	Binding	Fluorophoere	Cells for collection	Purpose
1	—	0.5		GFP, Cy5	—	Control physiology Control autofluorescence
2	GFP	0.5	—	GFP, Cy5	—	Control leakage from GFP channel to Cy5 channel
3	—	0.5	Necl1-Fc	GFP, Cy5	—	Control leakage from Cy5 channel to GFP channel
4	GFP	The rest	Necl1-Fc	GFP, Cy5	GFP-/ Necl1Fc-positive	Experiment

trols needed for the isolation of Schwann cells infected with a GFP construct.

Nonenzymatic Removal of Schwann Cells With 0.1 mM EDTA

1. Wash the dishes twice with 10 ml PBS^{--}.

2. Add 2 ml of EDTA 0.1 mM in PBS^{--} (sterile-filtered) to each plate and incubate for 5–10 min at room temperature, then start removing the Schwann cells by tapping onto the dish and observing their detachment under the microscope.

3. Collect the detached Schwann cells into 15-ml Falcon tubes according to the experimental setup (0.5–2 plates per tube, depending on the FACS sample; see Tables I, II) and triturate several times with a 5-ml pipette.

4. Add SCPM to a total volume of 14 ml, and spin down the detached cells for 7 min at 1,000 rpm and 4°C; discard the supernatant.

From now on, all following steps are carried out on ice and in a cooled centrifuge.

5. Unite the pellets of each FACS sample in one 15-ml tube. Then, wash the cells by adding 12 ml PBS^{--} + 1% low IgG to each tube and resuspending the cells, followed by centrifuging the cells for 7 min at 1,000 rpm and 4°C. Carefully discard the supernatant without disturbing the cell pellet.

Do now the preclustering of Necl1-Fc needed for the binding step:

6. For preclustering of Necl1-Fc (prepare 1 ml of preclustered Necl1-Fc for the control FACS samples and 2.5 ml of preclustered Necl1-Fc for test sample to be FACS purified), dilute biotinilated anti-human Fc secondary antibody 1:500 in Necl1-Fc conditioned medium (see above for preparation of this conditioned medium) and sterilize by filtering through a syringe-mounted 0.45-μm low-protein-binding filter. Gently rock the tube at room temperature for 30 min.

7. Now perform the binding of the preclustered Necl1-Fc to the Schwann cells: add the Necl1-Fc conditioned medium preclustered with the biotinylated anti-human Fc secondary antibody to the cell pellet left after the washing step (see step 5 above) and resuspend the cells in it. Place on ice and let the binding continue for 25 min; mix the cells every few minutes by gently flicking the tube.

8. During the binding step, prepare the diluted Cy5-coupled streptavidin: Cy5-coupled streptavidin is diluted 1:500 in PBS^{--} + 1% low-IgG serum (prepare 0.5 ml per control sample and 1 ml per sample for sorting).

9. Wash the cells by adding 12 ml PBS^{--} + 1% low-IgG serum, and then centrifuge the cells for 7 min at 1,000 rpm and 4°C. Carefully discard the supernatant without disturbing the cell pellet.

10. Add the diluted Cy5-coupled streptavidin to the cells and dissolve the cell pellet with a 1-ml pipettor. Place the tubes containing the cells on ice, protect them from light, and let the Cy5-coupled streptavidin bind to the biotinylated secondary antibody for 20 min; mix the cells every few minutes by gently flicking the tube.

11. Wash the cells by adding 12 ml PBS^{--} + 1% low-IgG serum, and then centrifuge the cells for 7 min at 1,000 rpm and 4°C. Carefully discard the supernatant without disturbing the cell pellet.

12. Resupend the cells in HBSS + 1% low-IgG serum: resuspend the control samples in 0.5 ml and the sample for sorting in 1–2 ml (the cell concentration should not exceed 1×10^6 cells/ml).

13. Triturate the cells several times with a 1-ml pipette until a single-cell suspension is reached, and transfer them to a sterile FACS tube (e.g., a sterile 5-ml starter tube with a lid); place the tubes containing the cells on ice and protect from light. The cells are now ready for the FACS.

FACS of Necl1Fc-Labelled of Schwann Cells

Prepare in advance autoclaved glass tubes covered with aluminum foil for the collection of the sorted cells. Immediately before use, add in a tissue-culture hood 0.5 ml of SCPM to each collection tube and cover its walls by ''rolling'' the SCPM along them (this will help to avoid having collected cells stick to the walls of the tube without medium). The FACS purification itself is done on an FACS equipped with a cell-sorting device (Beckton-Dickinson FACSVantage or similar). Sorting speed is kept rather low (200–300 cells/sec), and only cells positive for Necl1-Fc are collected. After the FACS purification, plate 3×10^5 cells on a 10-cm Primaria dish in 10 ml SCPM. Check the cells on the next day, and replace their medium with fresh SCPM. Continue to expand the purified Schwann cells for one or two more passages, then freeze the stocks for storage in liquid nitrogen until further use.

Myelinating Coculture of Schwann Cells and DRG Neurons

Neuronal cocultures grown in four-well dishes should be handled with care to avoid the detachment and/or drying of the cultures. The four-well dishes are placed inside a larger cell-culture tray (with lid, up to eight four-well dishes per tray) containing a small water reservoir (some milliliters of DDW inside the lid of a 50-ml Falcon tube, placed in the middle of the tray). Be particularly gently when changing the

medium of the cultures in the four-well dishes: use a manual 1-ml pipette to aspirate the medium and an electronic pipette (set to its lowest speed) to add the fresh medium. Change the medium in each four-well dish separately. Unless otherwise indicated, when changing the medium, slightly tilt the four-well dish toward you, aspirate the old medium such that ~80 μl of it is left in the well and then add 250 μl of the fresh medium.

1. Dissect and dissociate the DRGs as described above; per 13-mm coverslip (in four-well dishes, coated with Matrigel and PDL), plate 3×10^4 DRG-derived cells in 90 μl NB in a drop in the middle of the coverslip.

2. On the next morning, add 0.25 ml NB + FudR to each well; make sure that the coverslips are completely submerged in the medium.

3. Two days later, completely remove the NB + FudR from the wells and add 0.25 ml NB (without FudR) to each well.

4. Repeat this FudR cycle for at least one more time; perform at least two complete medium changes after the last FudR treatment before adding the starved Schwann cells in BN.

5. Neuregulin-starvation of Schwann cells, when the Schwann cells are close to confluence, aspirate the SCPM and replace with 10 ml DMEM + 10% FCS + Pen/Strep. Keep the Schwann cells for 3 days in this medium; after this neuregulin starvation, Schwann cells usually take a spindle-like shape (unlike their flat shape when grown in SCPM).

6. After starvation, remove the RSCs from the culture dishes by trypsinizing them with diluted trypsin (diluted 1:1 in PBS^{-}); collect the cells in DMEM + 10% FCS + Pen/Strep and transfer them to a 15-ml tube. Centrifuge the cells for 5 min at 1,000 rpm, discard the supernatant, and resuspended the cells in 1 ml BN medium.

7. Count the Schwann cells in a hemacytomer and dilute them in BN medium to a concentration of 2×10^5 cells/ml. Aspirate the NB medium from the four-well dishes containing the DRG neurons and add 0.5 ml of the Schwann cell suspension to each well (e.g., 1×10^5 starved Schwann cells to each well of purified DRG neurons).

8. On the next day, exchange the medium in the four-well dishes and add fresh BN medium.

9. The cocultures are grown for additional 5–10 days in BN with the medium being changed every second day. As a rule of thumb, the denser the Schwann cells in the coculture before the induction of myelination, the more Schwann cells will form myelin segments.

10. After growing the cocultures for 5–10 days in BN, induce myelination by switching the medium to BNC: completely remove the BN medium from the wells and add 0.25 ml BNC (containing the freshly added ascorbic acid) to each well. Continue to grow the cocultures for additional 10–14 in BNC during which fresh medium is added every second day.

11. After 10–14 days, process the cultures for further use. Paraformaldehyde (PFA) fixation for immunostaining: aspirate all medium from the wells, wash once with DMEM, and then add 0.25 ml 4% PFA to each well. Fix the cultures for 10–15 min in 4% PFA, then wash them three times in PBS; keep the fixed cultures at 4°C until they are used for staining.

RESULTS

We previously described the expression of the nectin-like (Necl/SynCAM/CADM) cell adhesion molecules in the PNS (Spiegel et al., 2007). We further showed that the extracellular domain of Necl1 specifically binds to Necl4 on the surface of Schwann cells. In the presented protocol, we use this binding specificity of Necl1 for labeling Schwann cells with a chimeric protein containing the extracellular domain of Necl1 fused to the Fc region of human IgG (e.g., Necl1-Fc; Fig. 1A). Necl1-Fc specifically binds to Schwann cells but binds neither DRG neurons (Spiegel et al., 2007) nor fibroblasts (Fig. 1B). We utilized this binding specificity of Necl1-Fc to develop a protocol for the isolation of Schwann cells by FACS (Fig. 1C). We prefer this novel affinity reagent over commercially available antibodies because 1) it can be easily produced in large amounts and at low costs, 2) it is highly specific for Schwann cells compared with known markers such as Thy1 for fibroblasts (Fig. 1B,C) and $p75^{NTR}$ for Schwann cells (95% of the Necl1-Fc-positive cells are $p75^{NTR}$ positive and vice versa; data not shown), 3) it can be detected by FACS, and 4) the FACS-purified cells isolated based on the binding of Necl1-Fc are Schwann cells able to myelinate isolated DRG neurons in coculture very well (Fig. 1E).

Cocultures of DRG neurons together with Schwann cells that were infected with retroviral vectors expressing dominant-negative (Howe and McCarthy, 2000; Spiegel et al., 2007) or knock-down (Chan et al., 2006) constructs proved to be a particularly useful tool for the molecular dissection of axon–Schwann cell interactions. FACS has the great advantage that cells with complex marker combinations can be isolated in one step, provided that each marker is labeled with a different fluorophore. The protocol presented here is well suited to isolating Schwann cells that were infected with various retroviral constructs, including green fluorescent protein (GFP) fusion proteins and knock-down vectors with a fluorescent protein as reporter. For the purpose of demonstration, we describe here the isolation of Schwann cells that were infected with a retroviral construct driving the expression of enhanced GFP (eGFP). Retroviral infection was performed as previously described (Howe and McCarthy, 2000; Spiegel et al., 2007) using three consecutive cycles during the first 3 days in culture. This infection scheme yields very high rates infection (>90% of the Schwann cells in culture) because of the strong proliferative effect of DRG neurons on associated Schwann cells (cell division is a prerequisite for the successful infection of target cells with retroviral MMLV-based vectors). Even though retroviral infection is not selective—e.g., fibroblasts also are infected (Fig. 2A)—the subsequent Necl1Fc-based FACS purification (Fig. 2B) allows the highly specific isolation of GFP-positive Schwann cells (Fig. 2C). Importantly, these GFP-positive Schwann cells are still myelination competent (Fig. 2D).

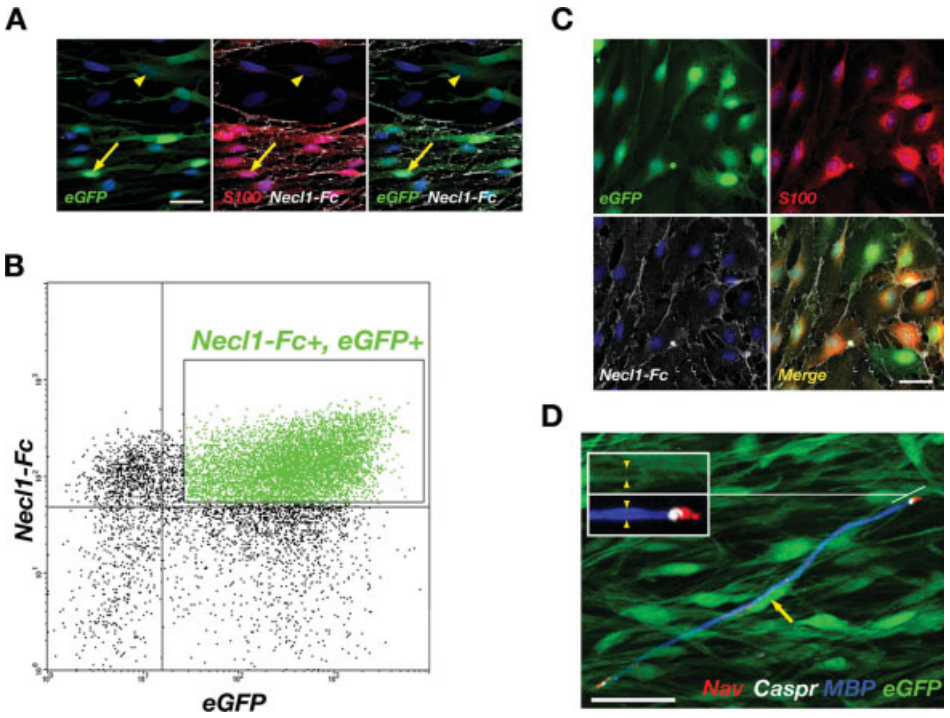

Fig. 2. Necl1Fc-based FACS can be used for the direct purification of retrovirally infected myelination-competent Schwann cells. **A:** eGFP expression and Necl1Fc binding in cultures derived from retrovirally infected dissociated DRGs. Both S100-/Necl1Fc-positve Schwann cells (arrow, labeled in red and white, respectively) and S100-/Necl1Fc-negative cells (arrowhead, presumably fibroblasts) are infected by a retroviral construct driving the expression of eGFP. **B:** FACS analysis of cultures; typically, ~90% of the Schwann cells (e.g. Necl1Fc$^+$ cells) are infected and express the gene of interest (here eGFP, labeled in green). (For FACS analysis, 10,000 events were analyzed and quadrants were placed such that untreated control cells stained only with secondary antibodies are within the lower left quadrant.) **C:** S100 staining of the purified and expanded eGFP-/Necl1Fc-positive cells. All cells are S100-positive Schwann cells and exhibit varying levels of eGFP expression. **D:** Myelinating cocultures of eGFP-/Necl1Fc-positve cells together with DRG neurons, stained for myelin markers. GFP-expressing Schwann cells myelinate (nucleus marked by arrow) and make green myelin sheaths (arrowheads in **inset**) as indicated by staining with antibodies against compact-myelin MBP (labeled in blue), paranodal Caspr (in white), and nodal Na_v channels (in red). Scale bars = 30 μm in A,C; 50 μm in D.

The general scheme of our protocol is as follows (see Fig. 3): DRGs are dissected from embryos and dissociated with trypsin, and the resulting cells (neurons, Schwann cells, and fibroblasts) are cocultured in a defined medium. Because of the strong mitogenic effect of DRG neurons on Schwann cells (Dong et al., 1995), the latter will readily proliferate and constitute the main cell population in culture; if desired, Schwann cells can be efficiently infected with retroviral vectors during this phase of strong proliferation. Once this culture has reached confluence, the cells are trypsinized and replated on a substrate permissive only for Schwann cells and fibroblasts. Subsequently, the Schwann cells are labeled with Necl1-Fc and sorted by FACS; the resulting pool of pure Schwann cells is then expanded, stored (if so desired), and used in myelinating cocultures together with DRG neurons.

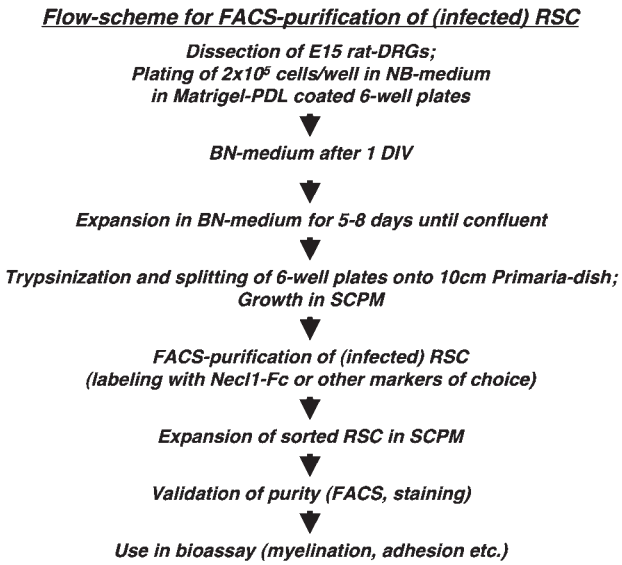

Fig. 3. Flow chart for the FACS purification of DRG-derived Schwann cells and their use in myelination cocultures.

DISCUSSION

Isolation of DRG-derived Schwann cells by FACS based on binding of Necl1-Fc yields a highly pure population of Schwann cells, comparable to the purity of

Schwann cells that were FACS isolated after labeling with an antibody directed against $p75^{NTR}$. A major advantage of using Necl1-Fc instead of an antibody directed against $p75^{NTR}$ is the low cost of Necl1-Fc: Necl1-Fc-conditioned medium is produced by standard $CaPO_4$ transfection of HEK293T cells with an expression plasmid of Necl1-Fc. Such conditioned medium can readily be produced in large amounts at low costs, and its quality is easily determined by SDS-PAGE and following Commassie staining. The quantity of Schwann cells purified by FACS depends on the speed of sorting and on the patience of the FACS operator: adherent cells (such as Schwann cells) are sorted at rather low speed (200–300 cells/sec), but sorting can be continued for several hours (Schwann cells are still viable after 3–4 hr of sorting). Given that typically 75–85% of the cells were positive for Necl1Fc binding (with the rest being Thy1 positive; data not shown), $1–3 \times 10^6$ purified Schwann cells can be collected in one preparation; this results in three to ten 10-cm Primaria plates of highly pure Schwann cells at passage 2. It should be noted that, even in preparations with relatively more fibroblasts at passage 1, the purity of the sorted Schwann cells was not impaired. The isolated Schwann cells readily proliferate provided that their culture medium (e.g., SCPM) contains sufficient levels of NRG. After the FACS-purified Schwann cells are expanded during an additional two passages, it should be possible to freeze down an extensive stock of primary Schwann cells, all at passage 4.

With the protocol presented, we isolate Schwann cells from dissociated rat DRGs. The advantage of this source is that the original pool of Schwann cells rapidly expands because of the strong proliferative effect that the DRG neurons in the culture exert on the associated Schwann cells. Besides increasing the number of Schwann cells, this strong proliferative effect is particularly advantageous for high-efficiency infection of the Schwann cells with retroviral constructs (discussed further below). Even though we focus in the procedure described above on DRG-derived rat Schwann cells, it can potentially be applied also to Schwann cells from other species (mouse, potentially also human) and other sources (sciatic nerve, biopsies). Because the procedure depends at its core on the binding specificity of Necl1-Fc, and because we detect strong and specific binding of Necl1-Fc also to sciatic-nerve-derived rat and DRG-derived mouse Schwann cells (Spiegel et al., 2007; and unpublished results), it is likely that FACS of Necl1Fc-labelled Schwann cells also works on these sources. Importantly, the Schwann cells isolated by FACS based on their labeling with Necl1-Fc are able to myelinate in culture the axons of FudR-purified DRG neurons. The extent of myelination (e.g., the amount of myelin segments) depends very much on the time course of the coculture: the longer the coculture continues before and after the induction of myelination by the addition of ascorbic acid to the culture medium, the more myelin segments will form. The desired extent of myelination (e.g., the baseline) depends on the nature of the experiment and on the manipulation (genetic or pharmacologic) of the cells in culture: if the expected effect of the manipulation is an increase in the number of myelinated segments, it might be desirable to keep the baseline rather low, and vice versa if the expected effect is inhibition of myelination. In general, myelination in the described cocultures is robust, and the numbers of myelinated segments are comparable among different samples of the same treatment. If no myelination should be observed even in untreated samples (e.g., neither Schwann cells nor DRG neurons were manipulated), the most likely cause is contamination of the cultures with *Mycoplasma*; such a contamination can be readily detected with a PCR-based *Mycoplasma*-detection kit.

Retroviral infection (e.g., with MMuLV-based vectors) of Schwann cells in cultures of dissociated DRGs is highly effective because of the strong proliferative effect that the DRG neurons exert on the associated Schwann cells. After three cycles of infection (consecutively during the first 3 days after the dissection and plating of the DRGs), typically more than 90% of the Schwann cells in cultures are infected. Furthermore, this infection is stable: when tested after an additional nine passages after the FACS, still >90% of the Schwann cells exhibited expression of the retroviral construct (data not shown). This makes infections with retroviral vectors particularly suitable for the heterologous expression of constructs in Schwann cells that are destined for coculture experiments (these experiments continue for a minimum of at least 14 days: 4 days before and 10 days after the addition of ascorbic acid). The choice of the viral system most suitable for a particular experiment again depends on the detailed goals of the experiment.

Research in the field of myelination and myelin biology has traditionally been conducted in the intact animal. Particularly, biochemical, electron microscopic, and immunohistochemical studies have yielded a large body of data on the composition and structure of myelinated nerves. The extensive use and study of genetically modified animals has further contributed to the characterization of key molecules necessary for the development and maintenance of the myelin sheath. Nevertheless, the animal models often do not allow for a refined understanding of the exact molecular mechanisms underlying the onset and progression of myelination. In the intact animal, the temporal overlap of different processes (migration, ensheathment, and myelination) is often too high, the spatial resolution is often too low (e.g., the anatomical complexity is too high), and molecular redundancy seems to be very high in the intact animal; myelinating cocultures can circumvent these limitations. Even though myelinating cocultures were described decades ago (Bunge et al., 1967), only recent years have seen the widespread use of this technique in the field. The emergence and availability of powerful molecular tools (i.e., retro- and lentiviral vectors, shRNA) make myelinating cocultures attractive both for mechanistic studies and for the functional evaluation of genes suspected to play a role in myelination. The latter

is of particular importance since more and more screens produce more and more candidates potentially involved in myelination (Nagarajan et al., 2002; D'Antonio et al., 2006; Spiegel et al., 2006). Myelinating cocultures infected with various viruses are a good and cost-effective alternative to the generation of genetically modified mice, especially when several candidate genes should be tested or a mechanistic question should be answered in a more refined way. The insights gained in myelinating cultures allow us then to go back to analyze myelination in the intact animal in a more educated way.

In the present protocol, we introduce a new method for purifying Schwann cells (both infected and noninfected) and summarize several well-established techniques for their use in myelinating cultures. It is our hope that this protocol will encourage more researchers in the field to use myelinating cultures for their research, which should ultimately lead to a better understanding of the molecular mechanisms of myelination.

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