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A novel method for isolating Schwann cells using the extracellular domain of Necl1

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Abstract

Myelinating co-cultures of Schwann cells and DRG-neurons are a powerful experimental system to probe 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 co-cultures. An advantage of the myelinating co-culture system is that Schwann cells and the neurons can be genetically manipulated before they are co-cultured. 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 co-cultures should significantly facilitate future studies aimed at the delineation of the molecular mechanisms axon-Schwann cell interactions and myelination.

Keywords

Schwann cells; Myelination; Necl; Axon

Introduction

Myelination is a complex cellular process during which the glial cell (Schwann cells in the peripheral nervous system – PNS - and Oligodendrocytes in the central nervous system - CNS) wraps its membrane multiple times around the underlying axon; the resulting multi-lamellar structure is called myelin. Even though the biochemical and molecular composition of myelinated fibers is rather well characterized (for a review, see Poliak and Peles 2003), the molecular mechanisms governing the onset of myelination remain largely obscure. Recently, several studies report the identification of key-molecules mediating the axon-Schwann cell interactions necessary to induce myelination (Chan et al. 2006; Dzhashvili et al. 2007; Eshed et al. 2005; Spiegel et al. 2007; Taveggia et al. 2005). 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 decades ago (Bunge et al. 1967). The obvious advantage of the culture-experiments is the ease of pharmacological and genetic manipulation of the cells in culture, the temporal control over the manipulation and the relatively short duration of the experiments (as compared to 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,

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and from human biopsies) were published and are used today in many laboratories. The major obstacles upon isolating Schwann cells are (i) to receive a large enough number of cells and (ii) to remove contaminating fibroblasts from the culture. The separation of Schwann cells from fibroblasts could so far be achieved in several ways: by utilizing the differential adhesive properties of Schwann cells and fibroblasts (Kreider et al. 1981), the 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, the direct isolation of Schwann cells by magnetic 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). In the current paper, we described detailed step-by-step protocols for the FACS-isolation of the Schwann cells and their use in myelinating co-cultures.

MATERIALS AND METHODS

A complete list of all media, antibodies, reagents and equipment used in this study can be found in the supplementary 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 (Sambrook et al. 1989). Necl1-Fc was collected from three-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 on an SDS-PAGE and staining the gel with Coomassie-blue (see Figure 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 #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:

- 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, 1 finer pair of forceps for extracting the uterus.
- Fine dissection-tools for extracting the embryos from the uterus and dissecting the DRGs (in a 50ml tube filled with 70% EtOH): 2 pairs of fine forceps and one pair of bowed forceps
- Several 10cm and 6cm tissue culture dishes
- 2 10cm and 2 6cm tissue culture dishes covered with Sylgard

- L15-medium (~100ml per dissection)
- A clean area covered with a paper towel where the rat can be dissected
- A dissection hood containing a dissection-microscope and a light-source; before starting the dissection, clean all of them by wiping with 70% EtOH.
- 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 CO₂-inhalation.
2. Place the anesthetized rat on a paper towel on a clean surface. Decapitate and sterilize the rats 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 10cm tissue culture (make sure to close the lid after placing the uterine horn in the dish). Proceed to the other uterine horn and remove also this one. 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 10cm 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 them in a 6cm tissue-culture dish filled with L15-medium. Once all embryos are stripped of their placentas and amniotic sac, place the 6cm dish on the Styrofoam filled with ice.

Do the next steps one pup at a time under the dissection microscope in a 100mm Petri-dish, covered with Sylgard and filled with L15; use the very fine forceps (FST #5).

8. Using the two pairs of very fine forceps, place the pup in the 100mm dish and pinch of its head and its tail.
9. Then 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. While lying on its back, pin the pup 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 60mm 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 60mm dish covered with Sylgard and filled with L15; place the dish under the dissection-microscop. Using the two very fine forceps (FST #5), 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 60mm dish under the dissection microscope.
15. Gently swirl the 60mm dish with circle-like movements until all the DRGs are concentrated in the center of the dish. Using a 200 μ l-pipette, collect the DRGs and transfer them into conical 15ml Falcon-tube kept on ice.
16. Spin the DRGs down to the bottom of the tube (1 minutes at 800 rpm); remove the supernatant and make sure not to aspirate any of the DRGs.
17. Add 2ml of Trypsin w/o EDTA to the DRGs; shortly tap the tube to resuspend the DRGs in the Trypsin and place the tube in an incubator at 37°C for 40 minutes.
18. Stop the trypsinization by adding 2ml of heat-inactivated FCS; add additional 4ml of L15 and centrifuge the trypsinized DRGs for 5 minutes 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 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 μ l of the cell-suspension into an Eppendorf-tube, mix with 25 μ l of Trypan-blue for live-dead staining and count the cells using a Hemacytometer. The dissociated DRGs of one E15 rat pup yield typically $\sim 4 \times 10^5$ cells.

Culturing the dissociated DRG-cells until the 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 co-cultured: since the DRG-neurons have a strong proliferative effect on the Schwann cells and due to 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 Nec11-Fc.

1. Plate the cells resulting from the dissociated DRGs in 6-well plates previously coated with Matrigel and PDL (see above); plate 2×10^5 cells per well in 2ml NB-medium.
2. The next day, remove the medium and replace with 1.5 ml fresh NB-medium per well.
3. 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 confluent covered (mainly by Schwann cells growing along the neurites of the DRG-neurons

and rapidly filling up the gaps between the neurites). It usually takes 5–8 days until the 6-wells are confluent.

Option: at this stage of proliferation, Schwann cells can efficiently be infected with retroviral constructs due to 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 three 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 two hours, 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 2ml PBS[−] and then add 0.5 ml Trypsin diluted 1:2 in PBS[−]. Let the diluted Trypsin act for at least 2–3 minutes and then start detaching the cells by vigorously tapping the 6-well 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 6-well plate into two 100 mm Primaria-dishes already containing 10 ml of SCPM. This step typically yields ~8 10cm Primaria plates.
6. The next day, remove the medium and replace with fresh SCPM. Continue to replace the medium with fresh SCPM every day until the 100 mm dishes are nearly confluent (usually 2–4 days). When the 100 mm 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 to threaten to overgrow the culture. This is usually due to low-quality NRG1β1-conditioned medium, which does not stimulate fast-enough 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 Nec11-Fc for FACS (Duration: 3 hours)

In a typical FACS-purification of non-infected Schwann cells, labeling is done with Nec11-Fc (pre-clustered with a biotinylated anti-human Fc secondary antibody and followed by binding of Cy5-coupled Streptavidin). See Table 1 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 Nec11-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 Cy5TM are an excellent choices due to the non-overlapping emission-spectra). See Table 2 for the samples and controls needed for the isolation of Schwann cells infected with a GFP-construct.

Non-enzymatic removal of Schwann cells with 0.1 mM EDTA

1. Wash the dishes twice with 10ml PBS[−]
2. Add 2ml of EDTA 0.1 mM in PBS[−] (sterile-filtered) to each plate and incubate for 5×10 minutes 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 15ml Falcon-tubes according to the experimental set-up (0.5×2 plates per tube, depending on the FACS-sample – see Table 1 + Table 2) and triturate several times with a 5ml pipette.

4. Add SCPM up to a total volume of 14ml and spin down the detached cells for 7 minutes at 1000 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 15ml-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 minutes at 1000 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. Preclustering of Necl1-Fc (prepare 1ml of preclustered Necl1-Fc for the control FACS-samples and 2.5ml of pre-clustered Necl1-Fc for test-sample to be FACS-purified): dilute biotinylated 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 0.45µm low protein binding filter. Gently rock the tube at room-temperature for 30 minutes.
7. Do now the binding of the pre-clustered Necl1-Fc to the Schwann cells: add the Necl1-Fc conditioned medium pre-clustered with the biotinylated anti human Fc secondary antibody to the cell-pellet left after the washing step (see 5. above) and re-suspend the cells in it. Place on ice and let the binding continue for 25 minutes; 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 0.5ml per control-sample and 1ml per sample for sorting)
9. Wash the cells by adding 12 ml PBS⁻ + 1% low IgG serum; then centrifuge the cells for 7 minutes at 1000 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 1ml-pipettor. Place the tubes containing the cells on ice, protect from light and let the Cy5-coupled Streptavidin bind to the biotinylated secondary antibody for 20 minutes; mix the cells every few minutes by gently flicking the tube.
11. Wash the cells by adding 12 ml PBS⁻ + 1% low IgG serum; then centrifuge the cells for 7 minutes at 1000 rpm and 4°C. Carefully discard the supernatant without disturbing the cell-pellet.
12. Resuspend the cells in HBSS + 1% low IgG serum: re-suspend the control-samples in 0.5ml and the sample for sorting in 1–2ml (the cell-concentration should not exceed 1×10^6 cells/ml)
13. Triturate the cells several times with a 1ml Gilson-pipette until a single-cell suspension is reached and transfer them to a sterile FACS-tube (e.g. a sterile 5ml 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-sorting of Necl1Fc-labelled of Schwann cells

Prepare already 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 stuck to the walls of the tube without medium). The FACS-

purification itself is done on a FACS equipped with a cell-sorting device (Beckton-Dickinson FACSVantage or similar). Sorting speed is kept rather low (200–300 cells per second) and only cells positive for Nec11-Fc are collected. After the FACS-purification, plate 3×10^5 cells on a 10cm Primaria dish in 10ml SCPM. Check the cells the next day and replace their medium with fresh SCPM. Continue to expand the purified Schwann cells for another 1–2 passages and freeze then stocks for storage in liquid nitrogen until further use.

Myelinating co-culture of Schwann cells and DRG-neurons

Neuronal co-cultures grown in 4-well dishes should be handled with care in order to avoid the detachment and/or drying-out of the cultures. The 4-well dishes are placed inside a larger cell-culture tray (with lid, up to 8 4-well dishes per tray) containing a small water-reservoir (some milliliters of DDW inside the lid of a 50ml Falcon tube, placed in the middle of the tray). Be particularly gently when changing the medium of the cultures in the 4-well dishes: use a manual 1ml pipette to aspirate the medium and an electronic pipette (set to its lowest speed) to add the fresh medium. Change the medium in each 4-well dish separately. Unless otherwise indicated, when changing the medium, slightly tilt the 4-well dish towards you, aspirate the old medium such that $\sim 80 \mu\text{l}$ of it are left in the well and then add $250 \mu\text{l}$ of the fresh medium.

1. Dissect and dissociate the DRGs as described above; per 13mm coverslip (in 4-well dishes, coated with Matrigel and PDL) plate 3×10^4 DRG-derived cells in $90 \mu\text{l}$ NBN in a drop in the middle of the coverslip.
2. The next morning, add 0.25 ml NB + FudR x1 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 0.25ml NB (without FudR) to each well.
4. Repeat this FudR-cycle for at least one more time; perform at least 2 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 confluency, aspirate the SCPM and replace with 10ml of 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 RSC's 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 15ml tube. Centrifuge the cells for 5 minutes at 1000 rpm, discard the supernatant and re-suspended the cells in 1 ml BN-medium.
7. Count the Schwann cells in a hemacytometer and dilute them in BN-medium to a concentration of 2×10^5 cells/ml. Aspirate the NB-medium from the 4-well dishes containing the DRG-neurons and add 0.5ml of the Schwann cell-suspension to each well (e.g. 1×10^5 starved Schwann cells to each well of purified DRG-neurons).
8. The next day, exchange the medium in the 4-well dishes and add fresh BN-medium
9. The co-cultures are grown for additional 5–10 days in BN whereby the medium is changed every second day. As a rule of thumb, the denser the Schwann cells in the co-culture are before the induction of myelination, the more Schwann cells will form myelin-segments.
10. After growing the co-cultures 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 co-cultures 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. PFA-fixation for immunostaining: aspirate all medium from the wells, wash once with DMEM and then add 0.25 ml of 4% PFA to each well. Fix the cultures for 10–15 minutes in 4% PFA, then wash them three times in PBS; keep the fixed cultures at 4°C until used for staining.

RESULTS

We previously described the expression of the Nectin-like (Nec1/SynCAM/CADM) cell adhesion molecules in the PNS (Spiegel et al. 2007). We further showed that the extracellular domains of Nec11 specifically bind to Nec14 on the surface of Schwann cells. In the presented protocol, we use this binding-specificity of Nec11 for labeling of Schwann cells with a chimeric protein containing the extracellular domain of Nec11 fused to the Fc-region of human IgG (e.g. Nec11-Fc; Figure 1a). Nec11-Fc specifically binds to Schwann cells but bind neither DRG-neurons (Spiegel et al. 2007) nor fibroblasts (Figure 1b). We utilized this binding-specificity of Nec11-Fc to develop a protocol for the isolation of Schwann cells by fluorescence activated cell sorting (FACS, Figure 1c). We prefer this novel affinity-reagent over commercially available antibodies since (i) it can be easily produced in large amounts and at low costs, (ii) it is highly specific for Schwann cells when compared to known markers such as Thyl for fibroblasts (Figure 1b+c) and p75NTR for Schwann cells (95% of the Nec11-Fc positive cells are p75NTR-positive and vice-versa, data not shown), (iii) it can be detected by FACS, and (iv) the FACS-purified cells isolated based on the binding of Nec11-Fc are Schwann cells able to myelinate isolated DRG-neurons in co-culture very well (Figure 1e).

Co-cultures 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-sorting 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 isolate Schwann cells that were infected with various retroviral constructs, including GFP-fusion proteins and knockdown 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 three days in culture. This infection-scheme yields very high rates infection (>90% of the Schwann cells in culture) due to 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. also fibroblasts are infected (Figure 2a) – the subsequent Nec11Fc-based FACS-purification (Figure 2b) allows the highly specific isolation of GFP-positive Schwann cells (Figure 2c). Importantly, these GFP-positive Schwann cells are still myelination-competent (Figure 2d).

The general scheme of our protocol is as follows (see Figure 3): DRGs are dissected from embryos, dissociated with trypsin and the resulting cells – neurons, Schwann cells and fibroblasts – are co-cultured in a defined medium. Due to the strong mitogenic effect of DRG-neurons on Schwann cells (Dong et al. 1995), the later 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

confluency, the cells are trypsinized and re-plated 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 desired so) and used in myelinating co-cultures together with DRG-neurons.

DISCUSSION

Isolation of DRG-derived Schwann cells by FACS-sorting 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} are the low costs of Necl1-Fc: Necl1-Fc conditioned medium is produced by standard CaPO₄-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/second) but sorting can be continued for several hours (Schwann cells are still viable after 3–4 hours of sorting). Given that typically 75–85% of the cells were found positive for Necl1Fc-binding (with the rest being Thy1-positive, data not shown), 1–3×10⁶ purified Schwann cells can be collected in one preparation; this results in 3–10 10cm-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 additional two passages, it should be possible to freeze down an extensive stock of primary Schwann cells, all at passage 4.

In the presented protocol we isolate Schwann cells from dissociated rat DRGs. The advantage of this source is that the original pool of Schwann cells rapidly expands due to 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 (further discussed 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). Since the procedure depends in its core on the binding-specificity of Necl1-Fc, and since 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-sorting of Necl1Fc-labelled Schwann cells works also 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 co-culture: the longer the co-culture 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 base-line) 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 base-line rather low, and vice-versa if the expected effect is inhibition of myelination. In general, myelination in the described co-cultures is robust and the number of myelinated segments is comparable between 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 due to the strong proliferative effect that the DRG-neurons exert on the associated Schwann cells. After three circles of infection (consecutively during the first three 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 additional nine passages after the FACS-sorting, 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 co-culture 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 this 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 did 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 co-cultures can circumvent these limitations. Even though myelinating co-cultures were described already 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 lenti-viral vectors, shRNA) make myelinating co-cultures attractive both for mechanistic studies as well as for the functional evaluation of genes suspected to play a role in myelination. The latter is of particular importance in a time when more and more screens produce more and more candidates potentially involved in myelination (D'Antonio et al. 2006; Nagarajan et al. 2002; Spiegel et al. 2006). Myelinating co-cultures 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 mechanistical question should be answered in a more refined way. The insights gained in myelinating cultures allow 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 non-infected) 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 use myelinating cultures for their research, which should ultimately lead to a better understanding of the molecular mechanisms of myelination.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. FACS-Purification of myelination-competent Schwann cells by binding of Nec11-Fc
A. Production of Nec11-Fc conditioned medium: a plasmid driving the expression of a Fusion-protein between the extracellular domain of Nec11 and the constant region of human IgG (e.g. Nec11-Fc) is transiently transfected into HEK293T-cells. The amount of Nec11-Fc in the conditioned medium can be assessed by binding the secreted fusion-protein to ProteinA-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 of Nec11-Fc per ml of conditioned medium; this concentration is sufficient for the further uses described in this protocol. **B.** Nec11-Fc binds specifically to Schwann cells. Nec11-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 due to a previous trypsinisation-step. Note the spindle-shape of the Schwann cells. (Dapi-labeling of cell-nuclei in blue) **C.** Nec11-Fc labeling of Schwann cells can be used for FACS. Cultures similar to those in B. were removed from the culture-dishes non-enzymatically and labeled with Nec11-Fc and Thy1; the two markers label two distinct cell-populations with Nec11-Fc binding typically to 75–85% of all cells. (For FACS-analysis, 10000 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 Nec11-Fc are Schwann cells. FACS-sorting based on binding of Nec11-Fc allows the isolation of highly pure Schwann cells (labeled here with S100, Nec11-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 Nec11-Fc based FACS are myelination-competent. When the FACS-purified Schwann cells (as described in D.) are added back to and co-cultured 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: B, D, 30 μm; E, 50 μm)

Figure 2. Necl1Fc-based FACS-sorting 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-positive Schwann cells (arrow, labeled in red and white respectively) and S100-/Necl1Fc-negative cells (arrow-head, 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, 10000 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-positive cells together with DRG-neurons, stained for myelin-markers. GFP-expressing Schwann cells myelinate (nucleus marked by arrow) and make green myelin-sheaths (arrow-heads in inlet) as indicated by staining with antibodies against compact-myelin MBP (labeled in blue), paranodal Caspr (in white) and nodal Na_v-channels respectively (in red). (Scale-bars A, C 30 μ m; D. 50 μ m)

Figure 3. Flowchart for the FACS-purification of DRG-derived Schwann cells and their use in myelination co-cultures

Table 1

#	# of 10cm 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	Nec11-Fc	Streptavidin-Cy5	Nec11Fc-positive	Purification of SC's

Table 2

#	Infection	# of 10cm dishes	Binding	Fluorophore	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	Nec11-Fc	GFP, Cy5	-	- Control leakage from Cy5-channel to GFP-channel
4	GFP	The rest	Nec11-Fc	GFP, Cy5	GFP-/Nec11Fc-positive	Experiment