# Cellular therapy after spinal cord injury using neural progenitor cells

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# Chapter 4a

## Purification of Schwann cells by selection of p75 low affinity nerve growth factor receptor expressing cells from adult peripheral nerve

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**ABSTRACT**

The intrinsic capacity of Schwann cells to promote regeneration after limited peripheral nerve lesions has been successfully transferred to extensive peripheral nerve injuries and central nervous system lesions by autologous transplantation strategies. However, both the intrinsic ability of axotomized neurons to regenerate and the permissiveness of the parenchyma surrounding the acute injury site diminish over time. Therefore, the autologous transplantation mode requires a fast and effective method to isolate Schwann cells from peripheral nerve biopsies. Here, we report a method to purify p75 low affinity nerve growth factor receptor (p75LNGFr) expressing Schwann cells from peripheral nerve biopsies in adult rats using magnetic-activated cell separation (MACS). After one week of nerve degeneration in culture, nerve fragments were dissociated resulting in mixed cultures containing Schwann cells and fibroblasts. After incubation with specific anti-p75LNGFr antibodies and secondary magnetic bead conjugated antibodies followed by one cycle of MACS, 95% pure Schwann cell cultures were generated as confirmed by flow-cytometry and immunocytochemistry. In contrast to established methods, MACS separation of p75LNGFr expressing cells allows the reliable purification of Schwann cells within 9 days after biopsy employing direct selection of Schwann cells rather than fibroblast depletion assays. Therefore, this method represents an effective and fast means to generate autologous Schwann cells for clinical transplantation strategies aiming for axon repair and remyelination.

**1. INTRODUCTION**

After limited injury to the peripheral nervous system (PNS), endogenous Schwann cells are recruited to form a scaffold for regenerating axons to grow along, produce growth conducive extracellular matrix components, secrete neurotrophic factors and remyelinate regenerating axons in a phenotypical appropriate manner, which ultimately leads to reinnervation of the target and functional recovery [1].

By transplanting Schwann cells, these favorable properties have been successfully transferred to more severe peripheral nerve injuries and even central nervous system (CNS) lesions. After severe peripheral nerve injuries endogenous recruitment of Schwann cells is not sufficient to promote morphological and functional restoration. The availability of peripheral nerve autograft material, which represents the standard therapy in these cases, is limited. Allografts on the other hand, are subject to graft rejection [2]. Artificially produced guidance channels seeded with autologous Schwann cells could overcome these limitations. Syngenic Schwann cells seeded into guidance channels have been successfully employed to support regeneration in animal models of peripheral nerve injury [3, 4]. In the CNS, transplanted Schwann cells can regenerate and remyelinate axons, which have been completely interrupted

[5-8], or have been demyelinated by toxins or irradiation [9-12]. Furthermore, the regenerative capacity of Schwann cells can be enhanced by *ex vivo* genetic modification to overexpress neurotrophic factors [13-15]. Ideally, Schwann cells are transplanted in an autologous fashion to avoid graft immune rejection. The disadvantage is that autologous Schwann cells will not be available immediately after a nerve lesion. The generation of sufficient quantities of Schwann cells for transplantation from the patient's own peripheral nerve biopsy requires at least three to six weeks according to established protocols [16-18]. However, the regeneration supportive capacity in the PNS and CNS decreases over time due to events such as cellular degeneration, scar formation and down-regulation of growth promoting molecules [19, 20].

The separation of Schwann cells from rapidly dividing fibroblasts, which build the protecting layers (epi-, perineurium) surrounding bundles of nerve fibers, represents the major time consuming factor [21, 22]. Thus far, established methods for Schwann cell purification from peripheral nerve fragments are based on fibroblast depletion as opposed to direct selection of Schwann cells. As an initial step to separate Schwann cells from fibroblasts, nerve fragments are maintained under cell culture conditions on an adhesive substrate such as laminin for several weeks. This procedure allows fibroblasts to migrate out, while Schwann cells remain in the nerve fragment. Each consecutive transfer of these nerve fragments reduces the number of fibroblasts [17]. The number of rapidly dividing fibroblasts can be reduced by adding antimitotic drugs such as cytosine arabinoside (Ara-C) to the cell culture medium or by maintaining serum-free primary cultures [23, 24]. The yield of enriched Schwann cells can be further enhanced by predegeneration of peripheral nerves *in vivo* before biopsy [16, 25]. Besides the time required to enrich Schwann cells, these purification methods are rather unspecific [18]. More specific purification methods, such as fibroblasts depletion using specific antibodies against the cell-surface antigen Thy-1 coupled to either complement activation or immunopanning, have only been reported for neonatal peripheral nerves [26, 27].

Magnetic-activated cell separation (MACS) represents a highly effective and fast method to select individual cell populations from a mixed cell population. Cell type specific cell surface antigens are labeled with magnetic bead conjugated antibodies followed by separation on a high gradient magnetic column[28]. A suitable cell surface antigen to select Schwann cells is the p75 low affinity nerve growth factor receptor (p75LNGFr), which is widely expressed on Schwann cells *in vitro*, but not on fibroblasts [29]. A similar approach using magnetic Dynabeads to enrich p75LNGFr expressing olfactory ensheathing cells has been described recently [30]. Vice versa, the cell surface antigen Thy-1 is expressed on fibroblasts, but not on Schwann cells, and thus can be used to enrich Schwann cells by depleting Thy-1 expressing fibroblasts [31].

In the present experiment, we investigated the efficacy of MACS to purify Schwann cells from adult sciatic nerve biopsies by using either specific antibodies (1) against p75LNGFr to select Schwann cells or (2) against Thy-1 to deplete fibroblasts. Results from this study indicate that only MACS selection of p75LNGFr expressing Schwann cells represents a highly effective procedure to establish primary Schwann cell cultures for autologous transplantation strategies in the PNS and CNS.

## 2. MATERIALS AND METHODS

### *2.1 Schwann cell isolation*

Sciatic nerve fragments with a length of approximately 35mm were taken bilaterally from deeply anesthetized (0.5 ml of a combination of ketamine (50 mg/kg), xylazine (2.6 mg/kg) and acepromazine (0.5 mg/kg)) adult Fischer 344 rats (average weight 160-180 g). The sciatic nerves were washed with ice-cold Hank's balanced salt solution (HBSS, PAA Laboratories, Austria) and the epineurium was stripped off with a fine forceps. Each sciatic nerve fragment weighed on average 29.6 mg ± 1.7. Nerves were cut into 1mm pieces and plated on collagen type-I (5 mg/cm$^2$, Pan Biotech, Germany) coated culture dishes in standard medium, which consists of Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal calf serum (FCS; Pan Biotech, Germany) and Penicillin/Streptomycin (100 U of penicillin, 100 mg/ml Streptomycin; Pan Biotech, Germany). The culture dishes with the sciatic nerve pieces were kept in a cell culture incubator at 37°C in a humidified atmosphere containing 5% $CO_2$. After 7 days in culture, the sciatic nerve fragments were transferred into 24 well plates containing 500 µl HBSS with 3% trypsin, 1% collagenase and 1% hyaluronidase (all Sigma, Germany) per well. After two hours of incubation at 37°C, tissue fragments were dissociated by trituration through a glass Pasteur-pipette and a 20-gauge sterile needle. The suspension was centrifuged at 120 x g for 10 min at room temperature. After resuspension in standard medium, trituration through a 22-gauge hypodermic needle followed. The resulting cell suspension was cultured in standard medium for two days on poly-l-ornithin/laminin (P-Orn/Lam; both Sigma, Germany) culture flasks, which were coated as follows: flasks were incubated with 20mg/cm$^2$ poly-l-ornithin in distilled $H_2O$ for 2 hours at 37°C, rinsed with distilled $H_2O$ and incubated with 0.4 mg/cm$^2$ laminin in PBS for 2 hours at 37°C. After 2 days in culture, the number of viable cells total before MACS and flow-cytometry/immunocytochemistry analysis was determined by counting the Trypan blue excluding cells using a hemocytometer.

### *2.2 Schwann cell purification*

Each purification run using MACS was conducted with nerve biopsies pooled from 3-4 rats (6-8 nerve explants). The MACS procedure was performed according to the manufacturer's instructions (Miltenyi Biotec, Germany). The flask containing the unpurified cells was washed with Dulbecco's phosphate buffered saline

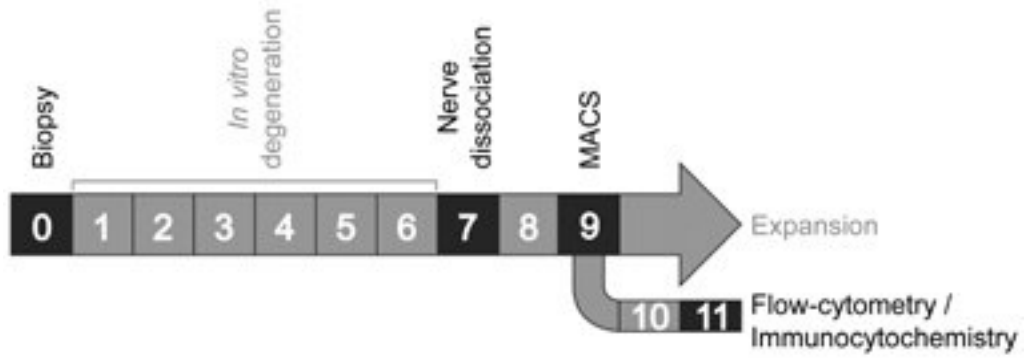


***Figure 1:*** *Time line from peripheral nerve biopsy (day 0) through immunocytochemical/flow-cytometry analysis of MACS selected cells (day 11).*

(PBS) and incubated with 40 µl/cm $^2$ Accutase (Innovative Cell Tech, San Diego, USA) for 5 minutes to detach the cells. Accutase, which is a mixture of collagenases, proteases and EDTA, was used instead of trypsin to protect trypsin sensitive cell surface markers. The cells were collected in DMEM containing 10% FCS and were centrifuged at 300 x g for 5 min at 4°C, washed with PBS supplemented with 2mM EDTA (PE) and counted using a hemocytometer (= number of unpurified cells). A sample of unpurified cells (2 x.$10^5$) was seeded on P-Orn/Lam coated culture flask for further analysis.

For MACS Schwann cell selection, the remaining unpurified cells were incubated with 5 µl (equals 0.25 mg IgG1) of the anti-p75LNGFr monoclonal antibodies (Chemicon, Germany) in 95 µl Dulbecco's PBS, 2mM EDTA, 0.5% BSA (PEB) for 10 min at room temperature. At the end of the incubation, 5ml of PE was added and the cells were centrifuged (300 x g for 5 min at 4°C). After one wash with PE, the cells were incubated with 20 µl of the microbead-linked rat anti-mouse IgG1 (at 1:5; Miltenyi Biotec, Germany) in 80 µl PEB for 15 min at 4°C. After 2 rinsing steps with PE, a MS column (Miltenyi Biotec, Germany) was placed in the MiniMACS magnet (Miltenyi Biotec, Germany) and flushed with PEB. A maximum of 5 x $10^6$ cells was resuspended in 500 µl PEB and applied onto the MS+ column. Three rinses with 500 µl PEB followed to wash out unbound cells, which represented the p75LNGFr negative fraction. After removal from the magnet, the column was flushed with 2 ml PE, which allowed the collection of the p75LNGFr positive cell fraction.

For MACS fibroblast depletion, cell suspensions were incubated with a mouse monoclonal anti-Thy-1 IgG1 (at 1:100; Harlan Sera Lab, Germany) and microbead-linked rat anti-mouse IgG1 antibodies as described. The MS column was loaded with a maximum of 5 x $10^6$ labeled cells. Cells not binding to the column attached to the magnet represented the Thy-1 negative cell fraction. Flushing of the column after removal of the magnet allowed the collection of the Thy-1 positive cell fraction.

The number of viable cells in the various fractions was determined by counting the Trypan blue excluding cells using a hemocytometer. For flow-cytometry analysis 2 days later, a sample of purified cells (2 x $10^5$) was plated on P-Orn/Lam coated culture flasks and standard medium was added. The remaining Schwann cells were expanded in standard medium supplemented with 2 µM forskolin (Sigma, Germany) and 0.2% bovine pituitary extract (Clonetics, Germany).

### *2.3 Flow-cytometry analysis of MACS selected cells*

A sample of unpurified cells or cells (2 x $10^5$ cells each) purified by either p75LNGFr selection or Thy-1 depletion was harvested within 48 hours after MACS using Accutase, washed with PE and immunolabeled with the anti-Thy-1 mouse antibody (at 1:100 in PEB) used for the fibroblast depletion assay. The cells were washed with PE, stained with a secondary fluorescein-conjugated donkey anti mouse IgG antibody (at 1:1000 in PEB; Jackson Immunoresearch, Germany) and washed with PE. The total number of 10,000 events was analyzed on a FACSCalibur flow-cytometer (Becton-Dickinson, Germany) directly after the staining procedure. Data were processed with the WinMDI 2.8 software (J. Trotter, USA). The percentage of fibroblasts in the various samples was determined by measuring the fraction of Thy-1 positive cells in the fluorescence intensity dotplot compared to the total amount of intact cells.

### *2.4 Immunocytochemistry*

Cells from each sampling fraction were plated in standard medium on P-Orn/Lam coated Permanox Chamber Slides (Nunc, Germany) for 2 days. The cells were fixed with ice-cold 4% paraformaldehyde in PBS for 30 minutes, rinsed 3 times with Tris-buffered saline (TBS), blocked with TBS containing 3% donkey blocking serum (Pan Biotech, Germany) and incubated overnight with the primary antibody in TBS containing 3% blocking serum at 4°C. The following primary antibodies were used: anti-p75 low affinity NGF receptor (rabbit polyclonal; Chemicon, Germany; at 1:2000), anti-27C7 (mouse monoclonal; generous gift from K. Wewetzer, University of Hannover, Germany; at 1:200) and mouse anti-Thy-1 (mouse monoclonal; Harlan Sera Lab, Germany; at 1:2000). Cells were rinsed twice with TBS and incubated for 2 hours with the corresponding secondary fluorescein or rhodamine-X linked donkey anti-mouse/rabbit IgG antibodies (Jackson Immunoresearch, Germany; at 1:1000) in TBS containing 3% donkey blocking serum. After 5 rinses with TBS, incubation with Hoechst 33342 (2 mg/ml; Sigma) as a nuclear counterstain followed. The chambers were removed from the slides and coverslipped using Prolong Antifade (Molecular Probes, Netherlands) as mounting medium. Phase contrast images of unfixed cell cultures were taken on an inverted Olympus IX70 phase contrast microscope (Olympus, Germany), equipped with a Color View 12 digital camera (Soft Imaging

***Table 1:*** *Flow-cytometry analysis of Thy-1 expressing fibroblasts after MACS p75LNGFr Schwann cell selection.*

| Exp. # | *unpurified fraction* | *MACS p75LNGFr positive fraction* | *MACS p75LNGFr negative fraction* |
|---|---|---|---|
| | | *(Thy-1 expressing cells in percent (%))* | |
| *1* | *18.56* | *4.92* | *91.37* |
| *2* | *63.36* | *5.20* | *98.40* |
| *3* | *35.58* | *1.98* | *59.25* |
| *4* | *60.33* | *10.26* | *95.04* |
| *5* | *64.58* | *5.76* | *94.61* |
| *6* | *77.72* | *1.79* | *98.06* |
| *Mean* | *53.4* | *5.0* | *89.5* |
| *SD* | *21.9* | *3.1* | *15.0* |

***Table 2:*** *Flow-cytometry analysis of Thy-1 expressing fibroblasts after MACS Thy-1 fibroblast depletion*

| Exp. # | *unpurified fraction* | *MACS Thy-1 positive fraction* | *MACS Thy-1 negative fraction* |
|---|---|---|---|
| | | *(Thy-1 expressing cells in percent (%))* | |
| *1* | *23.10* | *88.98* | *14.63* |
| *2* | *18.56* | *85.09* | *3.08* |
| *3* | *63.36* | *93.41* | *63.86* |
| *4* | *39.63* | *91.72* | *26.80* |
| *5* | *35.58* | *77.60* | *2.65* |
| *6* | *60.33* | *95.16* | *59.48* |
| *7* | *64.58* | *80.62* | *42.27* |
| *8* | *77.72* | *88.42* | *76.76* |
| *Mean* | *47.9* | *87.6* | *36.2* |
| *SD* | *21.6* | *6.2* | *28.7* |

System, Germany). Immunocytochemistry samples were analyzed using a Leica DMR fluorescence microscope (Leica, Germany). At every individual filter setting, 8 bit monochrome pictures were taken using a Spot CCD camera model 2.2.1 (Diagnostic Instruments, Michigan USA).

## 3. RESULTS

### *3.1 Schwann cell isolation*

After obtaining sciatic nerve biopsies from adult rats, nerve fragments were kept in culture for 7 days before dissociation (Fig. 1). After 7 days of *in vitro* degeneration, dissociation of peripheral nerve fragments and 2 days in culture, 0.86 x $10^6$ cells ± 0.09 x $10^6$ (n=6) total viable cells per animal were counted, which corresponds to 1.5 x $10^4$ cells per mg nerve tissue. Out of all dissociated cells less than 5% were non-viable cells. Dissociation before day 7 post biopsy resulted in a low yield of Schwann cells (data not shown). Peripheral nerve homogenates plated on

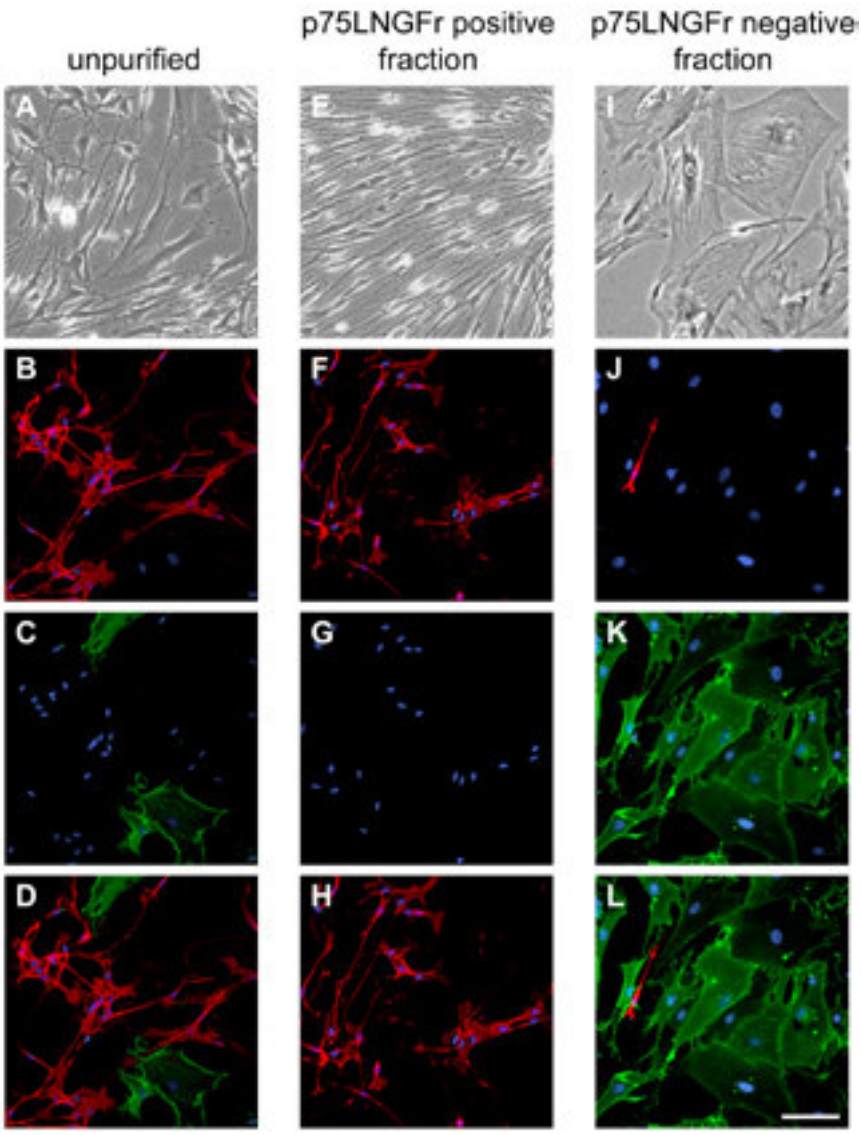


***Figure 2:*** *Immunocytochemical analysis before and after MACS p75LNGFr Schwann cell selection from peripheral nerve biopsies. A-D show unpurified cultures right after peripheral nerve dissociation, E-H illustrate the p75LNGFr positive and I-L the p75LNGFr negative fraction (A, E, I phase-contrast micrographs; all others immunofluorescent micrographs). (A) Peripheral nerve homogenates in culture consist of bipolar Schwann cells with small cigar-shaped nuclei and flattened fibroblasts with prominent oval nuclei, which is mirrored by (B) p75LNGFr expressing Schwann cells and (C) Thy-1 expressing fibroblasts. (D) Overlay of C and D. Of note, the Hoechst 33342 nuclear counterstain is always co-localized with either Thy-1 or p75LNGFr. (E) Following MACS p75LNGFr selection, cells are found almost exclusively with the typical bipolar Schwann cell morphology in the p75LNGFr positive fraction. (F) These cells express p75LNGFr, (G) but not Thy-1. (H) Overlay of F and G. (I) In contrast, the p75LNGFr negative fraction contains cells with fibroblast morphology. This is paralleled by (J) the lack of p75LNGFr and (K) abundance of Thy-1 immunoreactivity. (L) Overlay of J and K. Thy-1 immunoreactivity (fluorescein; green), p75LNGFr immunoreactivity (rhodamine; red), Hoechst 33342 nuclear counterstain (blue). Scale bar A, E, I 75µm; B-D, F-H, J-L, 100mm.*

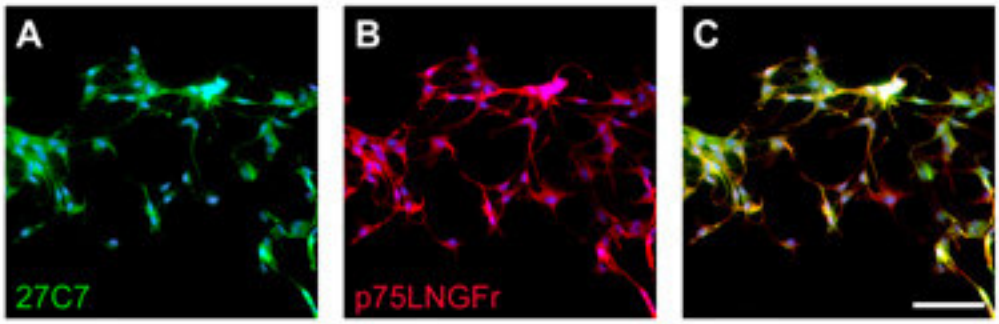


***Figure 3:*** *p75LNGFr expressing cells are Schwann cells. (A) Immunocytochemical analysis with the Schwann cell specific antibody 27C7, which recognizes non-myelinating Schwann cells, confirms the Schwann cell phenotype of (B) p75LNGFr expressing cells. (C) Overlay of A and B. 27C7 immunoreactivity (fluorescein; green), p75LNGFr immunoreactivity (rhodamine; red), Hoechst 33342 nuclear counterstain (blue). Scale bar A-C 100mm.*

P-Orn/Lam coated culture flasks after dissociation, contained both, typical fusiforme bipolar Schwann cells with small cigar-shaped nuclei and flattened fibroblasts with prominent oval nuclei (Fig. 2A). The immunocytochemical analysis confirmed the presence of Schwann cells by p75LNGFr expression and of fibroblasts by Thy-1 expression (Fig. 2B-D). The typical phase-contrast appearance was less prominent in immunocytochemically stained cells due to paraformaldehyde fixation induced alterations of Schwann cell and fibroblast morphology. Flow-cytometry analysis of the primary cultures within 48 hours after enzymatic digestion revealed that 53.4% ± 21.9 (n=6) were Thy-1 expressing fibroblasts (Table 1; Fig. 4A).

### *3.2 Schwann cell purification by positive selection*

Nine days after the sciatic nerve biopsy (Fig. 1), dissociated sciatic nerve fragments were purified by MACS using p75LNGFr antibodies to select Schwann cells. This procedure was repeated for 6 different nerve biopsies (Table 1). Two days later, p75LNGFr selected cells revealed the typical Schwann cell morphology on coated culture flasks (Fig 2E). Immunoreactivity for p75LNGFr and the absence of Thy-1 expression confirmed the Schwann cell identity (Fig. 2F-H). In contrast, cells obtained from the p75LNGFr negative fraction appeared as large, flattened cells with prominent nuclei resembling the typical fibroblast morphology in phase-contrast (Fig. 2I). These cells expressed Thy-1, which identifies fibroblasts, but not p75LNGFr (Fig. 2J-L). Finally, co-localization of p75LNGFr and 27C7 expression, a marker for non-myelinating Schwann cells [32], reconfirms the Schwann cell identity of p75LNGFr expressing cells (Fig. 3). Cells, neither expressing p75LNGFr or Thy-1, were not found (Fig. 2D).

Qualitative morphological data from MACS p75LNGFr selected cells were paralleled by quantitative analysis of Thy-1

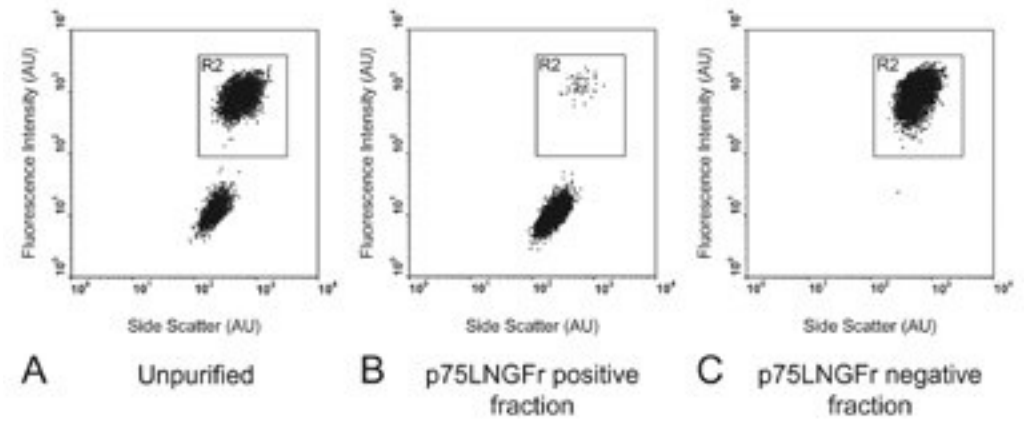


***Figure 4:*** *Flow-cytometry analysis of MACS p75LNGFr separated cells. Dot plots were obtained by flow-cytometry analysis of Thy-1 expressing cells, showing the side scatter signal versus the fluorescence signal intensity in arbitrary units (AU). The dot plots were gated to select the viable cells from the cell population. The boxed areas labeled R2 contain the Thy-1 positive fibroblasts. (A) The unpurified peripheral nerve homogenates almost equally consist of Thy-1 positive (R2 labeled box) and Thy-1 negative cells. (B) The majority of cells of the MACS p75LNGFr positive fraction is located outside of the boxed area indicating that they do not express Thy-1 . (C) Almost all cells from the MACS p75LNGFr negative fraction express Thy-1 (inside boxed area), which identifies them as fibroblasts*

positive cells as determined by flow-cytometry two days after MACS (Table 1; Fig. 4). Cultures derived from one cycle of MACS p75LNGFr selection contained 5.0% ± 3.1 (n=6) Thy-1 positive fibroblasts, compared to 53.4% before MACS, indicating a high specificity (95.0%) for Schwann cells. As a parameter for the sensitivity of this method, cultures from the MACS p75LNGFr negative fraction contained only 10.5% Thy-1 negative cells. This fraction most likely represents Schwann cells not captured by the column. The absolute number of cells retrieved in the p75LNGFr positive fraction further confirms the high sensitivity. From 1.37 x $10^6$ unpurified cells (sample from pooled cells, counted before MACS), 0.58 x $10^6$ cells in the MACS p75LNGFr positive fraction were identified as Thy-1 negative by flow-cytometry, thus representing the yield of Schwann cells after one cycle of p75LNGFr MACS selection. Considering that our unpurified biopsy homogenates contain on average 46.6% Schwann cells (= 0.64 x$10^6$ cells; flow-cytometry Thy-1 negative unpurified cells), 91.0 % of all Schwann cells present in the peripheral nerve biopsies following dissociation can be retrieved. This means, transferred to the total numbers of cells obtainable from one single rat, that out of 0.86 x $10^6$ cells 0.40 x $10^6$ represent Schwann cells, from which 0.37 x $10^6$ cells can be retrieved.

After adding the mitogens forskolin and bovine pituitary extract to the medium, the Schwann cells could be expanded with a cell doubling time of 5 days over at least 4 passages, without observing fibroblasts overgrowing them (data not shown).

### *3.3 Schwann cell purification by fibroblast depletion*

Identical to the MACS p75LNGFr selection protocol, sciatic nerve fragments were dissociated 7 days after the nerve biopsy was taken and were purified by MACS Thy-1 depletion two days later. The separation procedure was repeated in 8 different experiments (Table 2). In contrast to the described MACS p75LNGFr selection assay, Schwann cell purification by MACS depletion of Thy-1 expressing fibroblasts did not prove to be effective in this experiment. The MACS column was able to bind Thy-1 expressing fibroblasts, since the Thy-1 positive fraction had a higher percentage of Thy-1 positive fibroblasts compared to unpurified cells after one cycle of MACS Thy-1 depletion as assessed by flow-cytometry (Table 2). However, the Thy-1 negative fraction still contained 36.2% ± 28.7 (n=8) Thy-1 positive cells indicating a low sensitivity to detect Thy-1 expressing fibroblasts. The specificity of MACS for Thy-1 expressing fibroblasts was only 87.6% ± 6.2 (n=8), suggesting that unlabeled cells were captured by MACS Thy-1 selection. The quantitative flow-cytometry results were confirmed by phase-contrast and immunocytochemical analysis of the MACS Thy-1 depletion assay (data not shown).

## 4. DISCUSSION

The results of the present study demonstrate that Schwann cells can be purified by MACS from adult peripheral nerve biopsies using the expression of the cell surface antigen p75LNGFr for selection. This method allows the fast and reliable purification of adult Schwann cells directly through positive selection rather than indirectly through depletion of fibroblasts. MACS depletion of Thy-1 expressing fibroblasts lacks the sensitivity and specificity to establish Schwann cell cultures from adult peripheral nerve biopsies.

The main advantage of MACS p75LNGFr Schwann cell selection is the fast establishment of primary Schwann cell cultures from adult peripheral nerve biopsies within 9 days after biopsy. This time factor becomes particularly relevant for autologous transplantation strategies, since the intrinsic ability of axotomized neurons to regenerate and the permissiveness of the parenchyma surrounding the acute injury site diminish over time[19, 20]. Routinely used methods to enrich Schwann cells from peripheral nerve biopsies such as the repetitive transfer of peripheral nerve fragments in culture, the treatment of cultures with cytotoxic agents to eliminate proliferating fibroblasts, and the predegeneration of peripheral nerve before biopsy, require at least three to six weeks to achieve sufficient purification of Schwann cells [16, 17]. These time requirements may already be beyond the time point, where optimal axonal regeneration can be achieved. Enriching Schwann cells by dissociation of peripheral nerve biopsies earlier than 7 days after biopsy was not possible.

As confirmed by others, immediate dissociation of peripheral nerve fragments causes substantial damage to Schwann cells resulting in poor cell yields[17]. Periph-

eral nerve fragments require a minimum of time to degenerate, which will allow Schwann cells to retract their processes from the surrounding cells and to loosen junctions with extracellular matrix components within the peripheral nerve [22].

The MACS p75LNGFr selection protocol as described allows a high degree of purification without losing significant quantities of Schwann cells. Purification by MACS selection requires a balance between the specificity of Schwann cell selection and an optimal cell yield. Increasing the number of rinses before eluting the column in the positive selection assay would enhance the specificity but at the same time the overall cell yield would decrease. A more efficient means to increase the specificity without decreasing the cell yield is to purify cell homogenates repeatedly on fresh columns. In the present experiment, the degree of Schwann cell enrichment after one cycle of MACS p75LNGFr selection was sufficient for several passages of Schwann cell cultures without observing fibroblast expansion.

The total cell yield of 0.86 x $10^6$ cells per animal or 1.5 x $10^4$ cells per mg nerve tissue obtainable with the described MACS purification protocol lies within the range of previously published data [17, 18, 33].

A concern about MACS cell selection is that microbeads still bound to the cell surface would influence viability or adherence of the purified cells. However, none of these effects were observed in the present study. The small size of the microbeads (ca. 50 nm) prevents the steric interference or mechanical stress on the cells, which occurs when cells are bound to larger magnetic particles like Dynabeads [34]. Because of the small size and the biodegradable constitution, the bound microbeads are removed by the normal membrane turnover.

In contrast, established purification methods using cytotoxic agents such as Ara-C to eliminate fibroblasts from peripheral nerve biopsies have to accept considerable side effects. Cytotoxic agents unspecifically eradicate proliferating cells and therefore proliferating Schwann cells will be eliminated as well [35]. The overall cell yield of Schwann cells will decrease and the time required to generate sufficient quantities of Schwann cells will increase. In addition, Ara-C can induce DNA damage [36] and interfere with the overall protein synthesis [37]. Thus, long-term effects on surviving Schwann cells are likely, which raises safety concerns regarding the application of purified Schwann cells for transplantation in humans.

In analogy to previous studies, which used the expression of Thy-1 as a marker to select fibroblasts either through immunopanning [27] or complement activation induced lysis [26], peripheral nerve homogenates were processed using MACS depletion of Thy-1 expressing fibroblasts. This approach did not prove to be effective, since it showed a very low sensitivity to detect fibroblasts. These data are in line with previous experiments, in which we were not able to purify Schwann cells sufficiently from adult peripheral nerve tissue using the complement activation induced lysis with Thy-1 as the selection antigen

(N.W., unpublished observation). Thus far, Thy-1 antibody labeling and subsequent complement activated lysis to deplete fibroblasts have only been applied successfully to neonatal peripheral nerve tissue [26, 27]. The difference between neonatal and adult peripheral nerve tissue can be attributed to the fact that peri- and epineurium, which represent the main fibroblast containing layers within the peripheral nerve, are just starting to develop in neonatal as compared to adult peripheral nerves. This makes fibroblast depletion methods much more efficient for neonatal nerve tissue [21].

MACS selection of p75LNGFr expressing Schwann cells from adult peripheral nerve biopsies represents a fast, effective and safe means to enrich Schwann cells in culture for clinical transplantation strategies. MACS is already an approved method for the purification of CD34 positive cells from the autologous peripheral blood for clinical applications [38]. The first clinical phase-I trail using autologous Schwann cells to remyelinate inflammatory lesions within the central nervous system caused by multiple sclerosis (personal communication, Jeffery Kocsis) emphasizes the clinical relevance of Schwann cell transplantation paradigms. Of course, MACS selection of p75LNGFr expressing Schwann cells has to be validated in the human system, before this method can be applied to clinically relevant regenerative strategies aiming for axon repair and remyelination.

## 5. ACKNOWLEDGMENTS

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