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Purification of Fibroblasts and Schwann Cells from Sensory and Motor Nerves in Vitro

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Corresponding Author:	Fei Ding CHINA
Corresponding Author's Institution:	
Corresponding Author E-Mail:	dingfei@ntu.edu.cn
Order of Authors:	Qianru He
	Fanhui Yu
	Yan Li
	Junjie Sun
	Fei Ding
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Professor F. Ding
Jiangsu Key Laboratory of Neuroregeneration,
Nantong University, 19 Qixiu Road,
Nantong, JS 226001, P. R. China

Dr. Nam Nguyen
Manager of Review, JoVE

Dec 20, 2019

Dear Dr. Nguyen,

We should be very grateful to you for giving us another opportunity to further revise our resubmitted manuscript entitled "Purification of Fibroblasts and Schwann Cells from Sensory and Motor Nerves in Vitro".

We have carefully read the comments and tried the best to deal with them point by point, making a revised version of the manuscript. The three sentences that can be featured in the video in the protocol section are marked in red, lines 86, 92 and 109 respectively. We trust that our revision will meet the expectations of you, and hope the manuscript be accepted for publication in JoVE.

Professor F. Ding is the corresponding author who can be reached at the following address: Jiangsu Key Laboratory of Neuroregeneration, Nantong University, 19 Qixiu Road, Nantong, JS 226001, P. R. China. Phone: ++86-513-85051802; fax: ++86-513-85511585; E-mail: dingfei@ntu.edu.cn.

We appreciate your consideration for publication in JoVE and look forward to hearing from

you as soon as possible.

Best regards,

Yours sincerely

Fei Ding

Professor

TITLE:

Purification of Fibroblasts and Schwann Cells from Sensory and Motor Nerves in Vitro

AUTHORS:

Qianru He, Fanhui Yu, Yan Li, Junjie Sun, Fei Ding

Key Laboratory of Neuroregeneration of Jiangsu and Ministry of Education, Co-Innovation Center of Neuroregeneration, Jiangsu Clinical Medicine Center of Tissue Engineering and Nerve Injury Repair, Nantong University, Nantong, PR China

heqianru@ntu.edu.cn
1352554076@qq.com
hesaidan@126.com
15506660626@163.com

CORRESPONDING AUTHOR:

Fei Ding
dingfei@ntu.edu.cn

KEYWORDS:

sensory Schwann cells, motor Schwann cells, sensory fibroblasts, motor fibroblasts, cell culture, peripheral nervous system

SUMMARY:

Here, we present a method to purify fibroblasts and Schwann cells from sensory and motor nerves in vitro.

ABSTRACT:

The principal cells in the peripheral nervous system are the Schwann cells (SCs) and the fibroblasts. Both these cells distinctly express the sensory and motor phenotypes involved in different patterns of neurotrophic factor gene expression and other biological processes, affecting nerve regeneration. The present study has established a protocol to obtain highly purified rat sensory and motor SCs and fibroblasts more rapidly. The ventral root (motor nerve) and the dorsal root (sensory nerve) of neonatal rats (7-days-old) were dissociated and the cells were cultured for 4-5 days, followed by isolation of sensory and motor fibroblasts and SCs by combining differential digestion and differential adherence methods sequentially. The results of immunocytochemistry and flow cytometry analyses showed that the purity of the sensory and motor SCs and fibroblasts were >90%. This protocol can be used to obtain a large number of sensory and motor fibroblasts/SCs more rapidly, contributing to the exploration of sensory and motor nerve regeneration.

INTRODUCTION

In the peripheral nervous system, the nerve fibers mainly consists of axons, Schwann cells (SCs), and fibroblasts, and also contains a small number of macrophages, microvascular

endothelial cells, and immune cells[1]. SCs wrap the axons in a 1:1 ratio and are enclosed by a connective tissue layer called the endoneurium. The axons are then bundled together to form groups called fascicles, and each fascicle is wrapped in a connective tissue layer known as the perineurium. Finally, the whole nerve fiber is wrapped in a layer of connective tissue, which is termed as the epineurium. In the endoneurium, the whole cell population is comprised of 48% SCs, and a substantial portion of the remaining cells involves fibroblasts[2]. Furthermore, fibroblasts are important components of all nerve compartments, including the epineurium, the perineurium, and the endoneurium[3]. Many studies have indicated that SCs and fibroblasts play a crucial role in the regeneration process after peripheral nerve injuries[4-6]. After transection of the peripheral nerve, the perineurial fibroblasts regulate cell sorting via the ephrin-B/EphB2 signaling pathway between SCs and fibroblasts, further guiding the axonal regrowth through wounds[5]. Peripheral nerve fibroblasts secrete tenascin-C protein and enhance the migration of SCs during nerve regeneration through β1-integrin signaling pathway[7]. However, the SCs and fibroblasts used in the above studies were derived from the sciatic nerve, which includes both sensory and motor nerves.

In the peripheral nervous system, the sensory nerves (afferent nerves) conduct sensory signaling from the receptors to the central nervous system (CNS), while the motor nerves (efferent nerves) conduct signals from the CNS to the muscles. Previous studies have indicated that SCs express distinct motor and sensory phenotypes and secrete neurotrophic factors to support peripheral nerve regeneration[8,9]. According to a recent study, fibroblasts also express different motor and sensory phenotypes and affect the migration of SCs[10]. Thus, the exploration of differences between motor and sensory nerve fibroblasts/SCs allows us to study the complicated underlying molecular mechanisms of peripheral nerve specific regeneration.

At present, there are many ways to purify SCs and fibroblasts, including the application of antimitotic agents, antibody-mediated cytolysis[11,12], sequential immunopanning[13] and laminin substratum[14]. However, all the above methods remove fibroblasts and preserve only the SCs. Highly purified SCs and fibroblasts can be obtained by flow cytometry sorting technology[15], but it is a time-consuming and costly technique. Hence, in this study, a simple differential digestion and differential adherence method for purifying and isolating sensory and motor nerve fibroblasts and SCs was developed in order to obtain a large number of fibroblasts and SCs more rapidly.

PROTOCOL:

This study was carried out in accordance with the Institutional Animal Care Guidelines of Nantong University. All the procedures including the animal subjects were ethically approved by the Administration Committee of Experimental Animals, Jiangsu Province, China.

1. Isolation and culture of motor and sensory nerve fibroblasts and SCs

1.1. Use seven-day-old Sprague-Dawley (SD) rats (n=4) provided by the Experimental Animal

Center of Nantong University of China. Place the rats in a tank containing 5% isoflurane for 2-3 minutes, allow the animals to breath slowly and have no independent activity, and then sanitize using 75% ethanol prior to decapitation.

1.2. Use scissors to cut the back skin for about 3 cm and remove the spinal column. Open the vertebral canal carefully to expose the spinal cord.

1.3. Maintain the spinal cord in a 60 mm Petri dish with 2-3 mL of ice-cold D-Hanks' balanced salt solution (HBSS).

1.4. Based on the anatomical structure, excise the ventral root (motor nerve) and then the dorsal root (sensory nerve) under a dissecting microscope. Next, place them in an ice-cold D-Hanks' balanced salt solution (HBSS).

1.5. After removing the HBSS, slice the nerves into 3–5 mm pieces with scissors and digest with 1 mL of 0.25% (w/v) trypsin at 37 °C for 18-20 min. Next, supplement with 3-4 mL of DMEM containing 10% fetal bovine serum (FBS) to stop the digestion.

1.6. Pipette the mixture up and down gently about 10 times and centrifuge at 800 x *g* for 5 min. After that, discard the supernatant and suspend the precipitate in 2-3 mL of DMEM supplemented with 10% FBS.

1.7. Filter the cell suspension using a 400 mesh filter, and then inoculate the cells in 60 mm Petri dish and culture at 37 °C in the presence of 5% CO_2. After 4-5 days of culturing, isolate the passage 0 (p0) fibroblasts and SCs after reaching 90% confluence.

1.8. Isolation and culture of SCs (**Figure 1**)

1.8.1. Wash the cells once using 1x PBS. Add 1 mL of 0.25% (w/v) trypsin (37 °C) per 60 mm Petri dish to digest the cells for 8-10 s at room temperature. After that, add 3 mL of DMEM supplemented with 10% FBS to stop the digestion.

1.8.2. Gently blow the mixture to detach the SCs with a pipette. Then collect and centrifuge the SCs at 800 x *g* for 5 min.

1.8.3. Discard the supernatant and suspend the precipitate in 3 mL of DMEM with 10% FBS, 1% penicillin/streptomycin, 2 μM forskolin and 10 ng/mL HRG, and then inoculate the cells in uncoated 60 mm Petri dish. After culturing at 37 °C for 30-45 min, the fibroblasts (a few number of fibroblasts are digested with SCs) attach to the bottom of the dish.

1.8.4. Transfer the supernatant (including the SCs) to another poly-L-lysine (PLL)-coated medium dish and culture at 37 °C for 2 days.

1.9. Isolation and culture of fibroblasts (**Figure 1**)

1.9.1. After removing the SCs (as shown in step 1.8), wash the remaining fibroblasts in the dishes with 1x PBS and then add 1 mL of 0.25% (w/v) trypsin to digest the fibroblasts for 2 min at 37 °C.

1.9.2. Add DMEM supplemented with 10% FBS to end the digestion. Blow the fibroblasts using a pipette, and then collect and centrifuge them at 800 x *g* for 5 min.

1.9.3. Discard the supernatant, suspend the precipitate with 2 mL of DMEM containing 10% FBS, and then inoculate the cells in uncoated 60 mm Petri dish. The fibroblasts after culturing for 30-45 min at 37 °C attach to the bottom of the dish. Discard the supernatant (including a few numbers of SCs). Then add 3 mL of DMEM supplemented with 10% FBS into the fibroblasts dish and culture at 37 °C for 2 days.

1.10. Passage the p1 cells until they reached 90% confluence. Then purify them by differential digestion and differential adherence again as described in sections 1.8 and 1.9.

1.11. Digest the p2 fibroblasts and SCs after culturing for 2 days, and then collect the cells, count and inoculate in 1 x 10^5 numbers/well on PLL-coated slides for immunocytochemistry (ICC).

2. ICC for identification of cell purity

2.1. Culture the cells for 24 h at 37 °C and perform ICC staining after differential digestion and differential adherence of motor and sensory fibroblasts and SCs.

2.2. Wash the motor and sensory fibroblasts and SCs with 1x PBS and fix them in 200 µL/well of 4% paraformaldehyde (pH 7.4) for 18 min at room temperature.

2.3. Block the sample SCs with blocking buffer (0.1% Triton X-100 in 0.01 M PBS containing 5% goat serum) and block the sample fibroblasts with blocking buffer (0.01 M PBS containing 5% goat serum) for 45 min at 37 °C after washing them with PBS thrice.

2.4. Remove the blocking buffer, and incubate with the following primary antibodies: mouse monoclonal anti-CD90 antibody (a specific marker for fibroblasts) (1:1000) for fibroblasts and mouse anti-S100 antibody (a specific marker for SCs) (1:400) for SCs at 4 °C overnight.

2.5. Discard the primary antibodies, wash with PBS thrice, and incubate with the following secondary antibodies: Alexa Fluor 594 goat anti-mouse IgG (1:400) for fibroblasts and 488-conjugated goat anti-mouse IgG (1:400) for SCs at room temperature for 1.5 h.

2.6. Wash the samples thrice with PBS, and stain the nuclei with 5 µg/mL Hoechst 33342 dye for 10 min at room temperature. Wash the sample with 1x PBS thrice and mount them using the mounting medium (20 µL/slide) on the glass slide.

2.7. Take the cell photographs by confocal laser scanning microscope in three random fields for each well. Evaluate the total number of nuclei and CD90-positive cells, S100-positive cells and then calculate the percentage of CD90-positive cells and S100-positive cells, respectively. Perform the staining in triplicate.

3. Flow cytometry analysis (FCA) for identification of cell purity

3.1. Evaluate the purity of motor and sensory fibroblasts and SCs as described previously by FCA[16]. Briefly, digest the p2 motor and sensory fibroblasts and SCs with 0.25% (w/v) trypsin, resuspend the cell pellets and incubate them in fixation medium at room temperature for 15 min.

3.2. Incubate the cells with permeabilization medium and probe using mouse monoclonal anti-CD90 antibody (0.1 μg/10^6 cells, 200 μL) for fibroblasts and mouse anti-S100 antibody (1:400, 200 μL) for SCs at room temperature for 30 min, respectively.

3.3. Incubate the cells with 488-conjugated goat anti-mouse IgG for 30 min. Use FACS caliber to perform flow cytometry, and analyze the data using Cell Quest software.

3.4. Incubate the cells only with 488-conjugated goat anti-mouse IgG (fibroblasts Group) and mouse IgG1 kappa [MOPC-21] (FITC) - Isotype control (SCs group, which serves as negative control.

4. Statistical analysis

4.1. Present all data as means ± SEM. Assess statistical differences in the data by unpaired *t*-test using GraphPad Prism 6.0. Set statistical significance at $p<0.05$. Perform all assays in triplicate.

REPRESENTATIVE RESULTS

Light microscopic observation

The SCs and fibroblasts are the two main cell populations obtained in the primary cell culture from nerve tissues. After inoculation for 1 h, most of the cells adhered to the bottom of the dish, and the cell morphology changed from round to oval. After culturing for 24 h, the SCs exhibited a bipolar or tripolar morphology and the length of these ranged from 100 to 200 μm. After 48 h, aggregation and proliferation of cells occurred, in which many cells were aggregated in an end-to-end, shoulder-to-shoulder, whirlpool or fence-like arrangement. The other fibroblasts that are larger than SCs exhibited flat and irregular shape. With prolonged culture time, the number of SCs and fibroblasts was gradually increased. The SCs were clustered together between spaces of fibroblasts or located on the surface of fibroblasts (**Figure 2A, 2B**). The cells are subjected to differential digestion and differential adherence to isolate fibroblasts and SCs. After digestion for 8-10 s, SCs appeared as round and are blown off easily, while the fibroblasts are flat and attached to the bottom of the dish

(**Figure 2C, 2D**). After differential digestion and differential adherence twice, the primary cultured SCs and fibroblasts were isolated and the typical cell morphology of motor and sensory SCs and fibroblasts was observed (**Figure 2E-2H**).

Evaluation of purity of motor and sensory fibroblasts and SCs by ICC

The sensory and motor fibroblasts were labeled using CD90 and visualized using a confocal laser scanning microscope (**Figure 3A, 3D**). Hoechst 33342 dye was used to label the cell nucleus (**Figure 3B, 3E**). The merged images of fibroblast immunostaining and nuclear staining (**Figure 3C, 3F**) indicated that 92.51% and 92.64% of CD90 and Hoechst co-labeled cells were present in the motor and sensory fibroblasts (**Figure 3G**), respectively.

The sensory and motor SCs were labeled using S100 and visualized using a confocal laser scanning microscope (**Figure 4A, 4D**), respectively. Hoechst 33342 dye was used to label the cell nucleus (**Figure 4B, 4E**). The merged images of SC immunostaining and nuclear staining (**Figure 4C, 4F**) indicated the presence of 91.61% and 93.56% of S100 and Hoechst co-labeled cells in motor and sensory SCs (**Figure 4G**), respectively.

Evaluation of purity of sensory and motor fibroblasts and SCs by FCA

After differential digestion and differential adherence twice, the primary cultured fibroblasts and SCs were isolated. As shown in **Figure 5A-5C**, almost >90% of cells in the motor and sensory Fb culture were fibroblasts, which was indicated by M2, while the remaining <10% (indicated by M1) were SCs. **Figure 4C-4F** showed that >92% of all cells in the motor and sensory SC culture were SCs, which was indicated by M2, while the remaining <8% (indicated by M1) were fibroblasts.

FIGURE LEGENDS

Figure 1: The schematic diagram showing the separation and purification steps of sensory and motor Fibroblasts and SCs.

Figure 2: Phase-contrast micrograph showing the cell morphology of (A) primary cultured motor and (B) sensory fibroblasts and SCs. After digestion for 10 s, the cell morphology of the SCs was round (as indicated by the arrows), while the fibroblasts remained flat and were attached at the bottom of the dish (**C**: Motor Fibroblasts and SCs; **D**: Sensory Fibroblasts and SCs). After differential digestion and differential adherence, the SCs and Fibroblasts were isolated and the typical cell morphology of motor and sensory Fibroblasts (**E**: Motor Fibroblasts; **F**: Sensory Fibroblasts) and SCs (**G**: Motor SCs; **H**: Sensory SCs) were shown. (Scale bar: 50 μm)

Figure 3: Identification of purity of motor and sensory fibroblasts by ICC. Fluorescence microscopic photographs of cultured motor (**A-C**) and sensory Fibroblasts (**D-F**) showing immunostaining with antibodies against CD90 (**A** and **D**, red), Hoechst 33342 nuclear staining (**B** and **E**, blue), and merging of both staining (**C** and **F**). (**G**) Statistical analysis of purity of cultured Fibroblasts. (Scale bar: 100 μm)

Figure 4: Identification of purity of motor and sensory SCs by ICC. Fluorescence microscopic photographs of cultured motor (**A-C**) and sensory SCs (**D-F**) showing immunostaining with antibodies against S100 (**A** and **D**, red), Hoechst 33342 nuclear staining (**B** and **E**, blue), and merging of both staining (**C** and **F**). (**G**) Statistical analysis of purity of cultured SCs. (Scale bar: 100 μm)

Figure 5: Identification of purity of sensory and motor fibroblasts and SCs by FCA. The FCA graph showing the percentage of CD90-positive cells/S100-positive cells (M2) of cultured motor and sensory Fibroblasts/SCs. Statistical analysis of purity of cultured Fibroblasts (**A**) and SCs (**B**).

DISCUSSION:

The two major cell populations of peripheral nerves included SCs and fibroblasts. The primarily cultured fibroblasts and SCs can accurately assist in modeling the physiology of fibroblasts and SCs during peripheral nerve development and regeneration. The study showed that P7 rat sciatic nerve cells contained about 85% of S100-positive SCs, 13% of OX7-positive fibroblasts and only 1.5% of OX42-positive macrophages[13]. Although the number of fibroblasts is less than SCs, the initial proliferation rate of fibroblasts is faster than that of SCs. Therefore, Ara-C is the most commonly used antimitotic agent for removing fibroblasts in many studies. However, Ara-C is not specific for cell mitosis, and it can also inhibit the proliferation of SCs during vigorous stage of division. With the antibody-mediated cytolysis or immunopanning method, the fibroblasts either were lost or died. Although flow cytometry sorting technology can be used to obtain high-purity fibroblasts and SCs at the same time, it needs a large number of cells, and the cell acquisition rate remained low. To the best of our knowledge, this was the first study to show the purification method of sensory and motor SCs and fibroblasts.

In this study, a large number of sensory and motor fibroblasts and SCs was obtained at the same time by combining differential digestion as well as differential adherence sequentially, causing no harm to the cells. The results of ICC staining and FCA showed that all the sensory and motor SCs/fibroblasts were of high purity (>90%). The critical step of this method is to control the time of differential digestion. If the time is too short, it makes SCs hard to detach, and if the time is too long, it causes some fibroblasts to digest with SCs. In Weiss's study, ice cold Accutase is used to detach SCs from fibroblasts, but this is more expensive than trypsin[17]. Differential digestion with trypsin can effectively assist in separating SCs and fibroblasts. The limitation of this protocol is that it is not easy to excise sensory and motor nerves from neonatal rats, which requires more practice with the dissecting microscope. The protocol for culturing is very useful for studying the biological characteristics of sensory and motor nerve fibroblasts and SCs, and for the mechanism of sensory and motor nerve regeneration or fibroblasts and SCs transplantation to promote nerve regeneration.

ACKNOWLEDGMENTS:

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DISCLOSURES

The authors have nothing to disclose.

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Figure 1

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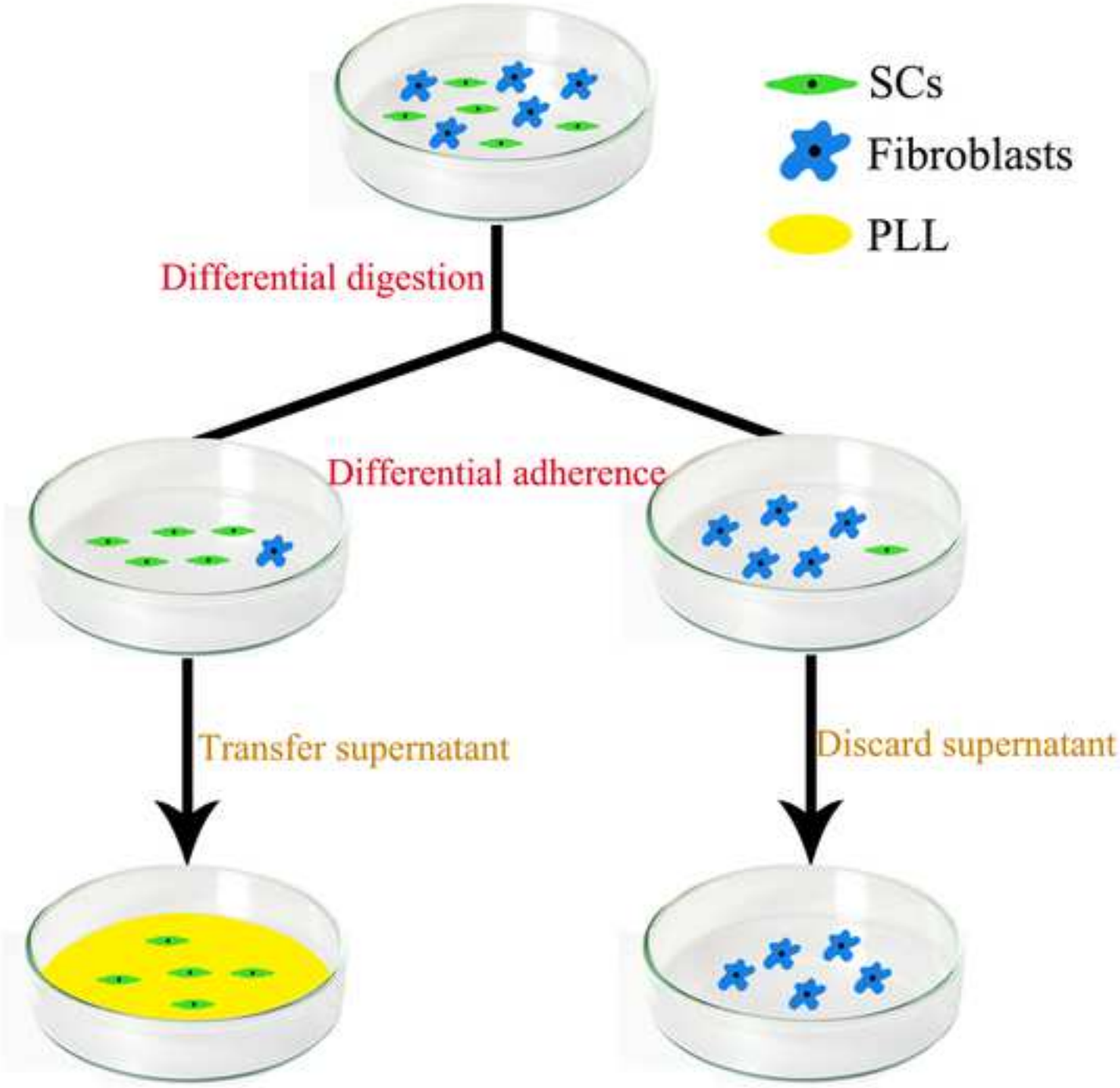

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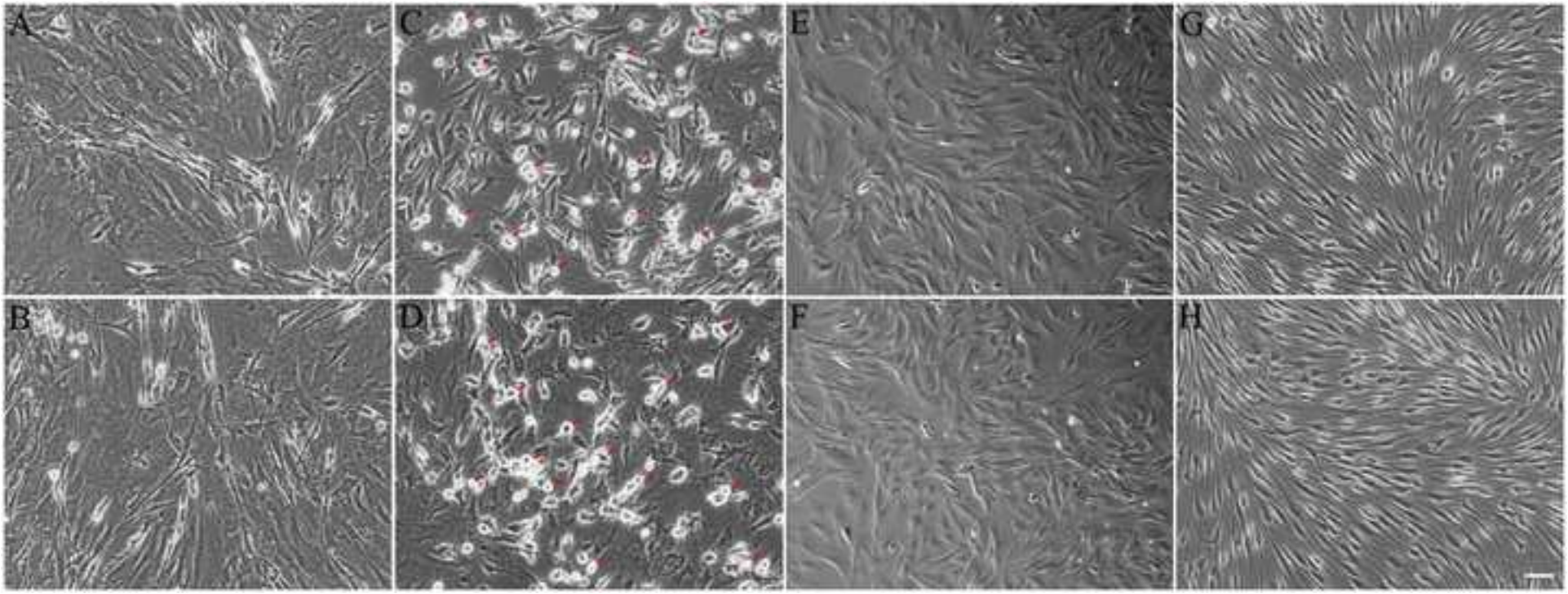

Figure 3

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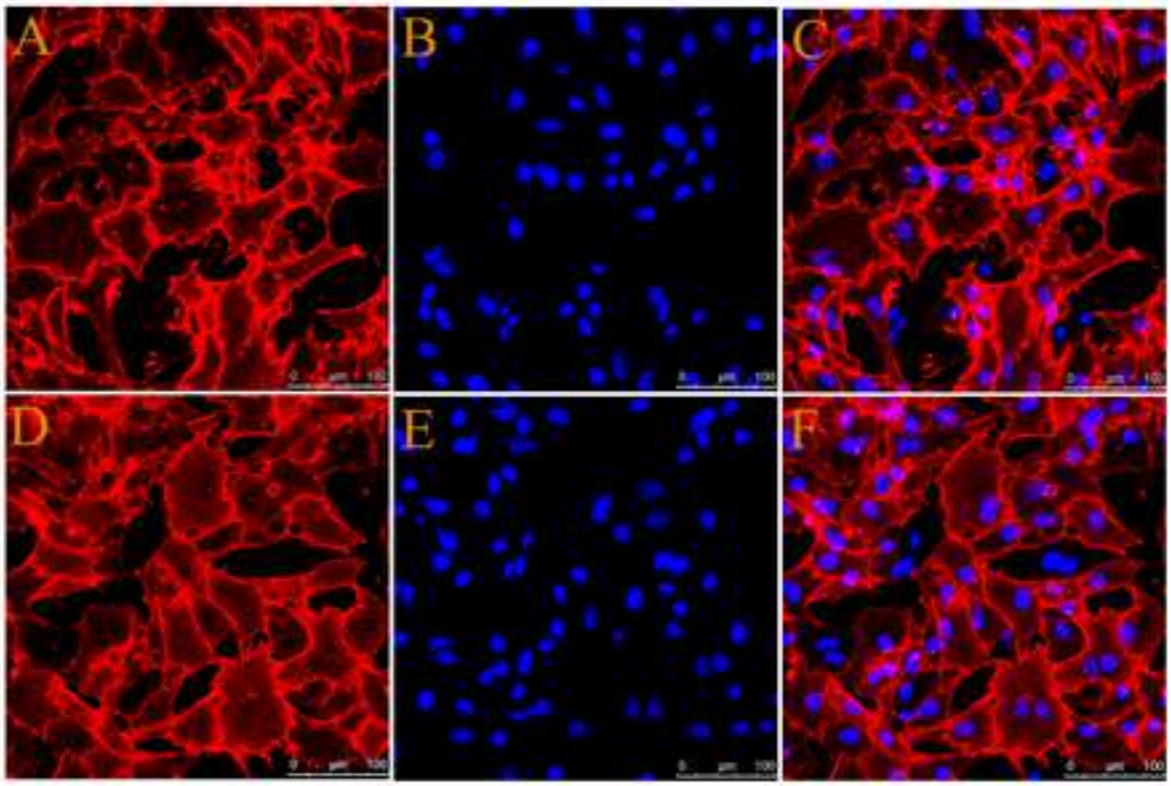

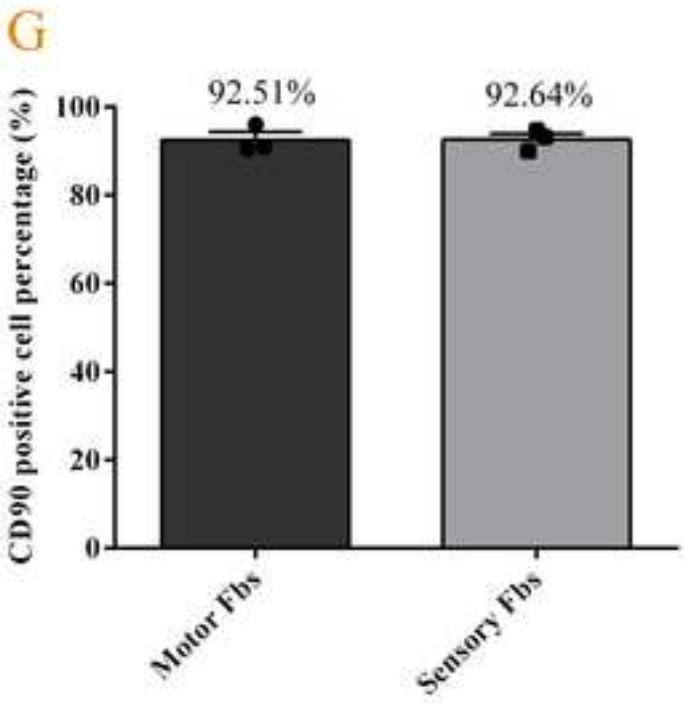

Figure 4

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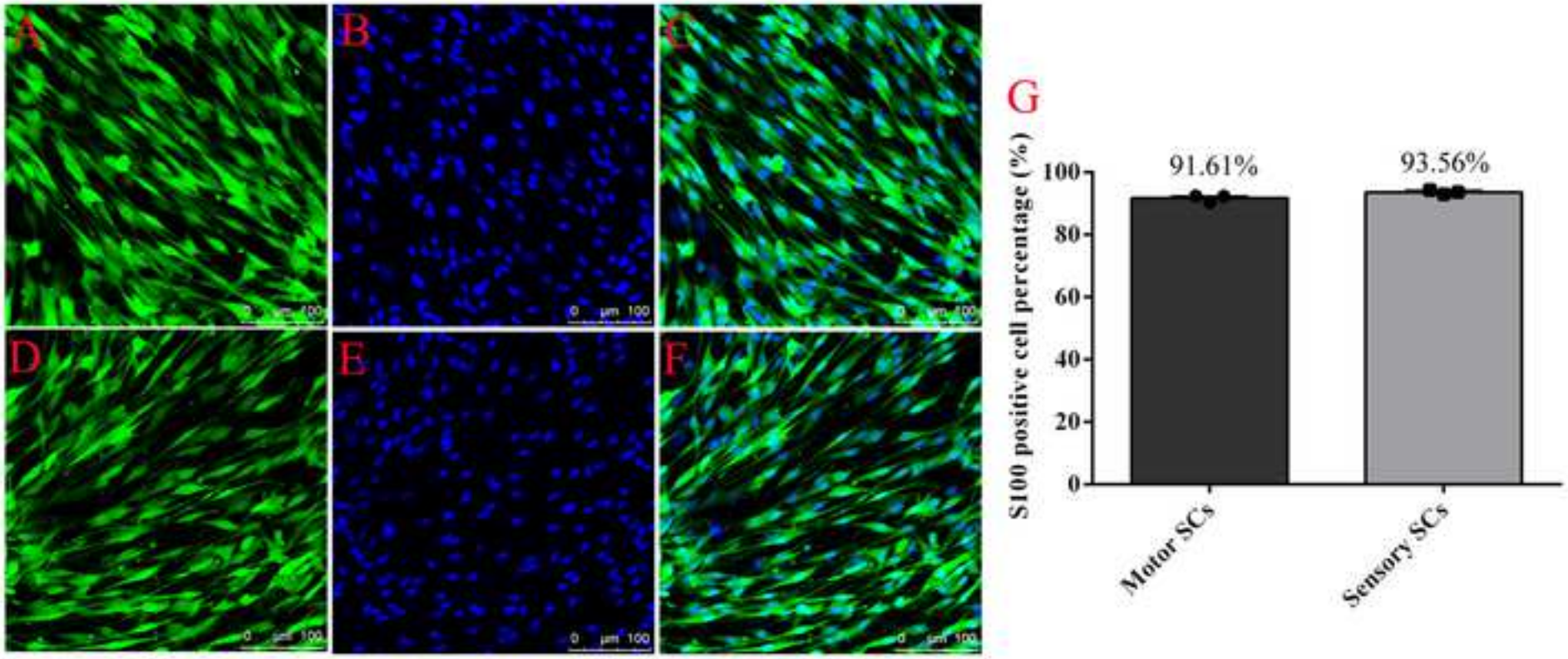
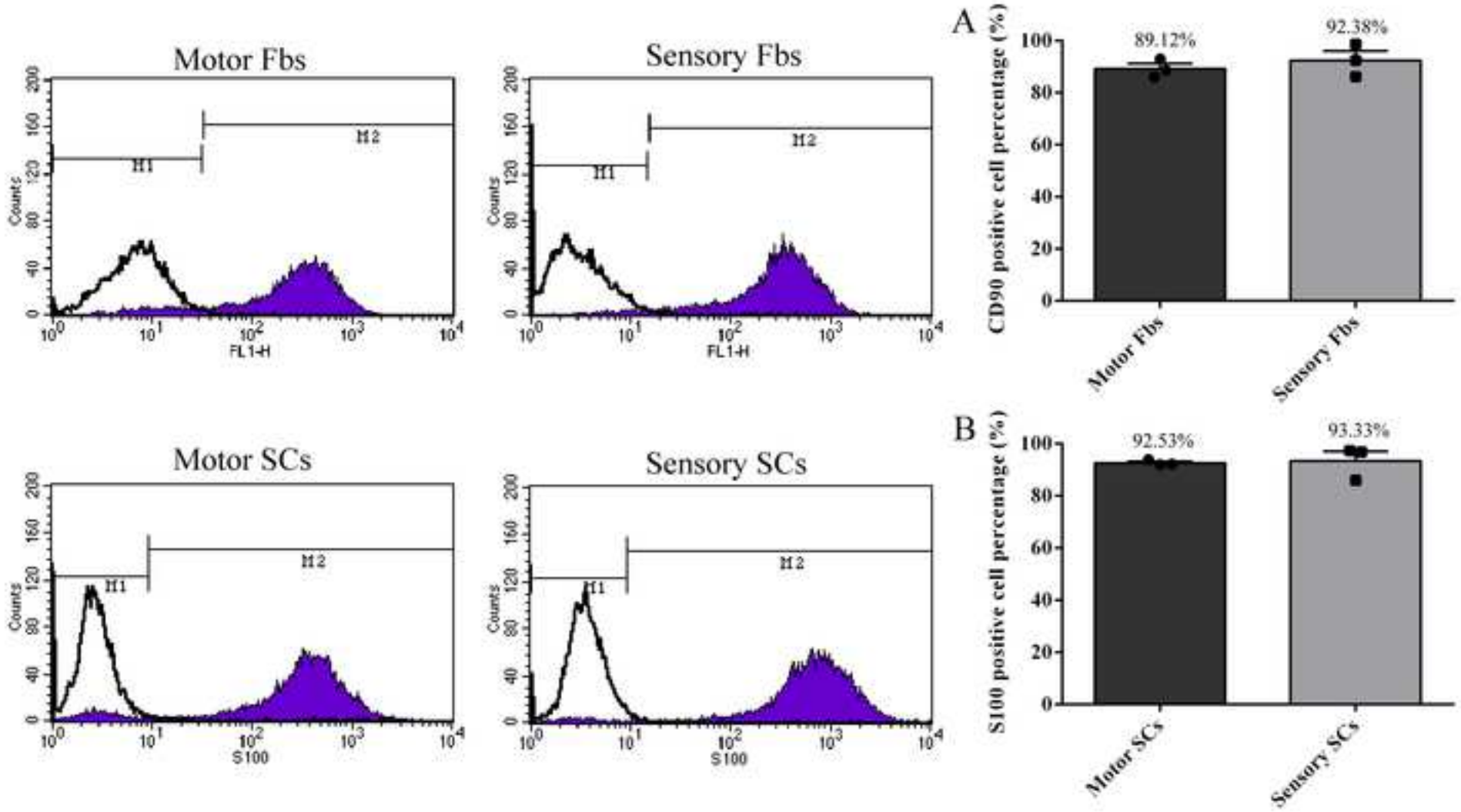


Figure 5



Name of Material/Equipment	Company	Catalog Number	Comments/Description
Alexa Fluor 594 Goat Anti-Mouse IgG(H+L)	Life Technologies	A11005	Dilution: 1:400
CoraLite488-conjugated Affinipure Goat Anti-Mouse IgG(H+L)	Proteintech	SA00013-1	Dilution: 1:400
Confocal laser scanning microscope	Leica Microsystems	TCS SP5	
Cell Quest software	Becton Dickinson Biosciences		
D-Hank's balanced salt solution	Gibco	14170112	
DMEM	Corning	10-013-CV	
Dissecting microscope	Olympus	SZ2-ILST	
Fetal bovine serum (FBS)	Gibco	10099-141C	
Forskolin	Sigma	F6886-10MG	
Fluoroshield Mounting Medium	Abcam	ab104135	
Fixation medium/Permeabilization medium	Multi Sciences (LIANKE) Biotech, Co., LTD	GAS005	
Flow cytometry	Becton Dickinson Biosciences	FACS Calibur	
Mouse IgG1 kappa [MOPC-21] (FITC) - Isotype Control	Abcam	ab106163	Dilution: 1:400
Mouse monoclonal anti-CD90 antibody	Abcam	ab225	Dilution: 1:1000 for ICC, 0.1µg for 10^6 cells for
Mouse anti-S100 antibody	Abcam	ab212816	Dilution: 1:400
Polylysine (PLL)	Sigma	P4832	
Recombinant Human NRG1-beta 1/HRG1-beta 1 EGF Domain Protein	R&D Systems	396-HB-050	
0.25% (w/v) trypsin	Gibco	25200-072	

Flow Cyt

Editorial comments:

The manuscript has been modified and the updated manuscript, 60952_R0.docx, is attached and located in your Editorial Manager account. Please use the updated version to make your revisions.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: We should be very grateful to you for giving us opportunity to further revise our submitted manuscript. Following your suggestion, we submit a highlighted version.

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Response: In this manuscript, we didn’t reuse any figures from a previous publication.

3. Please use 12 pt font and single-spaced text throughout the manuscript.

Response: The font and line spacing have been modified according to your requirements.

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). Any text that cannot be written in the imperative tense may be added as a “Note.”

5. Please add a one-line space between each of your protocol steps.

6. Please avoid long steps (more than 4 lines).

Response: The protocol section has been modified according to your requirements.

7. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

Response: Following your suggestion, “Discussion” sections are rephrased and marked in red.

8. Figure 2: Please add a title for the whole figure in Figure Legend.

9. Figure 3: Please add a title for the whole figure in Figure Legend.

10. Figure 4: Please add a title for the whole figure in Figure Legend.

Response: The titles have been added in Figure legends of new Figure 3-5.

11. Please do not abbreviate journal titles for references.

Response: Journal titles have been modified in references section.

12. Please sort the items in alphabetical order according to the name of material/equipment.

Response: The material/equipment have been sorted in alphabetical order.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript describes a simple protocol of differential adhesion/selection by substrate to separate Schwann cells and fibroblasts from postnatal rat nerves. The methodology described here is standard for primary cultures of Schwann cells, in particular those derived from rat tissues. The paper is well presented and fairly clear to follow.

Major Concerns:

The use of differential attachment/detachment to separate Schwann cells and fibroblasts

has been used for a long time and extensive literature exists on the matter of isolation of these cells based on differential substrate affinity. The protocol described here has been recently described in detail in two papers by Weiss et al published in GLIA (2016) and Methods in Mol Biol (2018). The argument used by the authors to explain the advantage of their method (and the purification method itself) are nearly identical to the one used by Weiss et al. This is a concern in the realm of peer-review publications. Though the issue of novelty per se seems not be be a decisive factor for publication in JoVE, this Reviewer understands that simple reproduction of prior published methods by others should not merit independent publication. It is concerning that this paper does not add a novel aspect in the methods or protocols.

Response: Thank you for the comment. Although our method is similar to that in Weiss's paper, there are still some differences, such as: 1. Previous studies have focused on the culture of Schwann cells or fibroblasts from the sciatic nerve (mixed nerve). This study is the first time to show the culture methods of Schwann cells and fibroblasts from the sensory and motor nerve. 2. The digestion method of nerve fascicles is different from Weiss's paper. In addition, ice cold StemPro™ Accutase™ Cell Dissociation Reagent was used to detach the SCs from Fbs in Weiss's paper. In our study, 0.25% (w/v) preheat trypsin was used to detach the SCs from Fbs.

Minor Concerns:

The authors should consider to tackle the points described below.

The culture medium of rat Schwann cells is not supplemented with growth factors. It is doubtful that media formulation would be able to sustain the growth of purified Schwann cells over time.

Response: Thank you for the comment. When SCs and Fbs grow together, they can provide nutrition support for each other, and DMEM supplemented with 10% FBS is used for cell culture, but growth factors (2 μM forskolin and 10 ng/ml HRG) need to be added in medium if SCs are purified. We have modified the protocol 1.8 and marked in red.

In 1.5, add the volume of trypsin solution per mass of tissue.

Response: The volume of trypsin has been added in protocol 1.5.

In 1.9, how many times (or at which ratio) are the cells passaged?

Response: The cells are passaged 2 times from primary culture to purification. We have added passage 0 (p0), p1, p2 in protocol section.

Reviewer #2:

Minor Concerns:

Line 18: 7-days-old rats are not newborn.

Line 52: Transection

Response: We would like to thank you for your pertinent comment. We have revised these mistakes.

Reviewer #3:

This paper describes simple methods to isolate and purify peripheral nerve sensory and motor Schwann cells and fibroblasts in vitro by combining differential digestion and differential adherence sequentially. The manuscript is well written and easy to read with proper figures and citations. This reviewer recommends acceptance with no revisions.

Response: Thank you for overall positive assessments of our manuscript.

Reviewer #4:

Manuscript Summary:

In this study, the authors reported a simple method to purify fibroblasts and Schwann cells from sensory and motor nerves of 7-day-old SD rats.

Major Concerns:

1. The English should be improved. There are so many grammatical errors and incomplete sentences in the text. The authors need to ask a native English speaker to review the sentences.

2. The protocol is a bit difficult to understand. Schematic presentation of experimental

procedure would be helpful for the readers.

Response: We appreciate your important suggestions. The manuscript has been revised and marked in red. New Figure 1 is schematic diagram.

3. The photomicrographs are of poor quality. They are grainy at enlarged views, and scale bars are unclear. Higher resolution images are required. In addition, double immunofluorescence micrographs (CD90 and S100) would be preferable in Figs. 2 and 3.

Response: According to your valuable suggestion, we have revised some figures quality and added clear scale bars. Both CD90 and S100 antibodies used in this experiment are generated from mouse, so double immunostaining is not allowed. Hoechst 33342 dye was used to label the total cells, Fbs and SCs were labeled using CD90 and S100 antibodies, respectively. We quantified the number of CD90/s100 and Hoechst dye co-labeled cells and calculated the percentage of co-labeled cells in Fbs and SCs, respectively. The data obtained from the experiment is accurate and reliable. We can provide all the raw data. If necessary, we would like to order new antibody to perform the double immunofluorescence.

4. In addition to fibroblasts and Schwann cells, peripheral nerves contain perineurial cells and microvascular endothelial cells.

Response: Thank you for the comment. The introduction section was rephrased are

marked in red.

5. In addition to the use of Ara-C, serum-free culture can inhibit the proliferation of fibroblasts.

Response: Thank you for the comment.

Minor Concerns:

1. In the abstract, the author mentioned newborn rats (7-day-old), but 7-day-old rats are neither newborns nor adults. Methods for purifying Schwann cells from neonatal animals have been established.

Response: Thank you for the suggestion. The abstract has been modified.

2. The authors failed to describe how rats were anesthetized for euthanasia prior to the dissection.

Response: We have added “SD rats are anesthetized with isoflurane” in protocol 1.1.

3. The specificity of the primary antibodies used in this study should be described.

Response: The protocol 2.4 has been modified. The detailed description of the antibodies

is listed in the table of materials. Mouse monoclonal anti-CD90 antibody recognizes the Thy-1.1 antigenic determinant which is a monomorphic determinant within rat strains. It is used to label fibroblasts in many articles, such as: Differential Gene Expression in Primary Cultured Sensory and Motor Nerve Fibroblasts. Front Neurosci, 12: p. 1016 (2018).

S100 is a specific marker for SCs. It is used to mark SCs in many articles, such as: 1. Shen M, et al. Isolation of rat Schwann cells based on cell sorting. Mol Med Rep 16:1747-1752 (2017). 2. Szmydynger-Chodobska J, et al. The Involvement of Pial Microvessels in Leukocyte Invasion after Mild Traumatic Brain Injury. PLoS One 11: e0167677 (2016).