

Journal of Visualized Experiments

Protocol for isolation, characterization and microRNA-based genetic modification of human dental follicle stem cells

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58089R2
Full Title:	Protocol for isolation, characterization and microRNA-based genetic modification of human dental follicle stem cells
Keywords:	stem cell; dental follicle stem cell; mesenchymal stem cell; non-viral modification; genetic engineering; transient transfection; microRNA
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Additional Information:	
Question	Response
If this article needs to be "in-press" by a certain date, please indicate the date below and explain in your cover letter.	

TITLE:

Isolation, Characterization and microRNA-based Genetic Modification of Human Dental Follicle Stem Cells

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KEYWORDS:

Stem cell, dental follicle stem cell, mesenchymal stem cell, non-viral modification, genetic engineering, transient transfection, microRNA

SUMMARY

This protocol describes the transient genetic engineering of dental stem cells extracted from the human dental follicle. The applied non-viral modification strategy may become a basis for the improvement of therapeutic stem cell products.

ABSTRACT:

To date, several stem cell types at different developmental stages are in the focus for the

treatment of degenerative diseases. Yet, certain aspects, such as initial massive cell death and low therapeutic effects, impaired their broad clinical translation. Genetic engineering of stem cells prior to transplantation emerged as a promising method to optimize therapeutic stem cell effects. However, safe and efficient gene delivery systems are still lacking. Therefore, the development of suitable methods may provide an approach to resolve current challenges in stem cell-based therapies.

The present protocol describes the extraction and characterization of human dental follicle stem cells (hDFSCs) as well as their non-viral genetic modification. The postnatal dental follicle unveiled as a promising and easily accessible source for harvesting adult multipotent stem cells possessing high proliferation potential. The described isolation procedure presents a simple and reliable method to harvest hDFSCs from impacted wisdom teeth. Also this protocol comprises methods to define stem cell characteristics of isolated cells. For genetic engineering of hDFSCs, an optimized cationic lipid-based transfection strategy is presented enabling highly efficient microRNA introduction without causing cytotoxic effects. MicroRNAs are suitable candidates for transient cell manipulation, as these small translational regulators control the fate and behavior of stem cells without the hazard of stable genome integration. Thus, this protocol represents a safe and efficient procedure for engineering of hDFSCs that may become important for optimizing their therapeutic efficacy.

INTRODUCTION:

The human dental follicle is a loose ectomesenchymally-derived connective tissue surrounding the developing tooth^{1,2}. Beside its function to coordinate osteoclastogenesis and osteogenesis for the tooth eruption process, this tissue harbors stem and progenitor cells especially for the development of the periodontium³⁻⁵. Therefore, the dental follicle is considered as an alternative source to harvest human adult stem cells^{6,7}.

Several studies demonstrated that human dental follicle stem cells (hDFSCs) are capable of differentiating into the periodontal lineage including osteoblasts, ligament fibroblasts and cementoblasts⁸⁻¹⁰. Furthermore, these cells were shown to match all characteristics of mesenchymal stromal cells (MSCs) including self-renewing capacity, plastic adherence, expression of specific surface markers (*e.g.*, CD73, CD90, CD105) as well as osteogenic, adipogenic and chondrogenic differentiation potential¹¹⁻¹³. Other studies also revealed a neural differentiation potential of hDFSCs^{2,14-18}.

Due to their promising properties and easy access, hDFSCs became recently relevant for tissue engineering¹⁹⁻²¹. The first studies concentrated on the potential of DFSCs to regenerate bone, periodontal and tooth roots^{19,22-30}. Since the knowledge of the neurogenic capability of hDFSCs, their application as potential treatment for neurodegenerative diseases has been investigated³¹⁻³³. HDFSCs have also gained importance with respect to the the regeneration of other tissues (*e.g.*, corneal epithelium)^{34,35}. The therapeutic potential of hDFSC is not only based on their direct differentiation potential but also on their paracrine activity. Recently, hDFSCs have been shown to secrete a wealth of bioactive factors, such as matrix metalloproteinases (MMPs), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor

(bFGF) and hepatocyte growth factor (HGF), which play a crucial role for angiogenesis, immunomodulation, extra cellular matrix remodeling and reparative processes³⁶.

However, broad clinical translation of stem cell therapy is still impaired by several challenges, such as massive initial cell death and low beneficial stem cell effects^{37,38}. Genetic engineering provides a promising strategy to address these challenges and therefore can greatly enhance the therapeutic efficacy of stem cells^{38–40}. For transient cell manipulation, microRNAs (miRs) are suitable candidates, as these small translational regulators control the fate and behavior of stem cells without the hazard of stable genome integration^{41–43}. To date, several beneficial miRs have been identified promoting stem cell proliferation, survival, homing, paracrine activity as well as their differentiation into several lineages⁴⁴. For instance, miR-133a engineered MSCs showed an increased survival and engraftment in infarcted rat hearts resulting in an improved cardiac function when compared to unmodified MSCs⁴⁵. Likewise, miR-146a overexpressing MSCs were shown to secrete higher amounts of VEGF which in turn led to an enhanced therapeutic efficiency in ischemic tissue⁴⁶.

This manuscript presents a detailed protocol for the selective extraction and genetic engineering of hDFSCs. For this purpose, we described the harvesting and enzymatic digestion of human dental follicles as well as the subsequent isolation of hDFSCs. In order to characterize isolated cells, important instructions for the verification of MSC properties have been included in accordance with the guidelines of the International Society for Cellular Therapy¹³. In addition, we provide a detailed description for the generation of miR-modified hDFSCs by applying a cationic lipid-based transfection strategy and the evaluation of transfection efficiency and cytotoxicity.

PROTOCOL:

HDFSCs are isolated from the dental follicles of extracted wisdom teeth provided by the Department of Oral and Maxillofacial Plastic Surgery of the Rostock University Medical Center. Informed consent and written approval was obtained from all patients. This study was authorized by the local ethics committee of the University of Rostock (Permission No. A 2017-0158).

1. Isolation of hDFSCs

Note: To prevent bacterial contamination, wisdom teeth should not be erupted before extraction

1.1. Preparation of required solutions

1.1.1. Prepare phosphate-buffered saline (PBS)/Penicillin-Streptomycin-Glutamine (P-S-G) solution: mix 495 mL of PBS with 5 mL of P-S-G solution. Store aliquots of 50 mL at 4 °C.

1.1.2. Prepare hDFSC culture medium: mix 445 mL of basal medium with 5 mL of antibiotic agent and 50 mL of fetal bovine serum (FBS). Store at 4 °C.

1.1.3. Prepare Collagenase type I stock solution (30 mg/mL): dilute 300 mg of Collagenase type I in 10 mL of basal medium supplemented with 1% antibiotic agent. Vigorously mix the solution. Filter the solution using a 0.2 μ m filter. Store aliquots of 500 μ L of Collagenase type I stock solution at -20 °C.

1.1.4. Prepare Dispase II stock solution (40 mg/mL): dilute 40 mg of Dispase II in 10 mL of basal medium supplemented with 1% antibiotic agent. Vigorously mix the solution. Filter the solution using a 0.2 μ m filter. Store aliquots of 500 μ L of Dispase II stock solution at -20 °C.

1.2. Surgical procedure

1.2.1. Inject a sufficient amount (maximum: 2 mL) of local anesthetic.

1.2.2. Create and raise a three-sided mucoperiosteal flap. Raising a lingual flap is not required.

1.2.3. Protect the lingual aspect with a periosteal elevator.

1.2.4. Gently mill buccal and distal bone with a round hard metal burr.

1.2.5. Loosen the tissue of the follicle with a periosteal elevator. Carefully remove the complete tooth (germ) and follicle by pulling out the crown (and follicle) with posterior (third-molar) forceps.

1.2.6. Debride and irrigate the socket thoroughly with NaCl solution.

1.2.7. Replace the mucoperiosteal flap with vicryl sutures (see **Table of Materials**).

1.2.8. Remove the tooth follicle from the oral cavity and place them into an aliquot of 50 mL of PBS/P-S-G solution.

Note: The sample can be stored at 4 °C until further processing.

1.3. Enzymatic digestion of the tooth follicle

1.3.1. Pre-warm hDFSC culture medium and PBS/P-S-G solution to room temperature (RT).

1.3.2. Thaw one aliquot of each Collagenase type I and Dispase II stock solutions. Prepare a digestion solution of 3 mg/mL Collagenase type I and 4 mg/mL Dispase II by adding 500 μ L of each Collagenase type I and Dispase II stock solution to 4 mL of basal medium containing 1% antibiotic agent.

1.3.3. Place extracted follicle in a sterile Petri dish and add 10 mL of PBS/P-S-G solution to wash the extracted tissue. Repeat this washing step twice.

176 1.3.4. Mince the extracted follicle to pieces of about 1 mm x 1 mm with a sterile scalpel within
177 the Petri dish containing 10 mL of PBS/P-S-G solution.

178
179 1.3.5. Transfer minced tissue and PBS/P-S-G solution from the Petri dish into a 50 mL conical
180 centrifuge tube. Wash the Petri dish with 10 mL of PBS/P-S-G solution and transfer the solution
181 into the same tube.

182
183 1.3.6. Centrifuge the conical centrifuge tube for 10 min at 353 x g at RT and discard the
184 supernatant.

185
186 1.3.7. Add 5 mL of digestion solution to the pelleted tissue. Gently mix the solution and the
187 tissue. Incubate the mixture at 37 °C and 5% CO₂ for 2 hours in a shaking incubator.

188
189 1.3.8. Centrifuge digested cell/tissue suspension at 353 x g for 10 min at RT. Discard the
190 supernatant and re-suspend the obtained pellet in 6 mL of hDFSC culture medium.

191
192 1.3.9. Seed cell suspension in a 25 cm² cell culture flask and incubate cells at 37 °C, 5% CO₂ and
193 20% O₂.

194
195 Note: If tissue is not completely digested, transfer the remaining tissue to the cell culture flask as
196 well.

197
198 1.3.10. Change medium carefully 24 h after cell seeding. Afterwards change medium every three
199 days until confluency.

200
201 Note: HDFSCs should be plastic-adherent 24 h after cell seeding and can be separated from non-
202 adherent cells and blood components by simply changing the medium.

203 204 1.4. Cell harvesting

205
206 1.4.1. Pre-warm hDFSC culture medium, PBS/P-S-G solution and
207 Trypsin/Ethylenediaminetetraacetic acid (EDTA) solution to RT.

208
209 1.4.2. Discard the supernatant from culture flask and wash confluent cells with 5 mL of PBS/P-S-
210 G solution.

211
212 1.4.3. Add 1 mL of Trypsin/EDTA solution to the culture flask and incubate for 3 min at 37 °C. Stop
213 the digestion process by adding 3 mL of hDFSC culture medium to the culture flask.

214
215 1.4.4. Transfer cell suspension into a 15 mL conical centrifuge tube and centrifuge the suspension
216 for 10 min at 353 x g at RT. Discard the supernatant and re-suspend the pellet in an appropriate
217 amount of hDFSC culture medium.

218

1.4.5. Count cells: gently mix 10 μ L of cell suspension with 10 μ L of Trypan blue solution. Apply 10 μ L into a counting chamber and calculate the hDFSC cell amount.

2. Characterization of hDFSCs

2.1. Immunophenotyping

2.1.1. Preparation of cells for flow cytometric analysis

2.1.1.1. Preparation of required solutions

2.1.1.1.1. Prepare PBS/EDTA (2 mM): mix 996 mL of PBS with 4 mL of EDTA (0.5 M).

2.1.1.1.2. Prepare staining buffer: mix 995 mL of PBS/EDTA (2 mM) with 5 g of BSA. Filter the solution using a 0.22 μ m filter unit and store at 4 °C until usage.

2.1.1.1.3. Prepare paraformaldehyde (PFA) stock solution (4%): dilute 4 g PFA in 100 mL of PBS and heat the solution to 80 °C. Mix the solution and adjust the pH value to 7.3. Aliquot obtained PFA solution in respective amounts (1.5 mL) and store at -20 °C until usage.

CAUTION: Because PFA fumes are toxic, prepare the PFA solution in a ventilated fume hood.

2.1.1.2. After cell harvesting (1.4), transfer 14 samples of 5×10^4 cells into 1.5 mL microcentrifuge tubes. Centrifuge the suspensions at 300 x g for 10 min at 4 °C. Discard the supernatant.

Note: Perform the following work in a rather shaded room. Keep cells and reagents on ice unless stated otherwise.

2.1.1.3. Re-suspend cells in certain amounts of staining buffer (4 °C) and add FcR blocking reagent (4 °C) as indicated in **Table 1**.

2.1.1.4. Add the following antibodies onto the inner side of the respective sample as indicated in **Table 1**: Allophycocyanin (APC) mouse anti-human CD29; APC mouse IgG1 κ isotype control; Peridinin-chlorophyll protein (PerCP)-Cyanine5.5 mouse anti-human CD44; PerCP-Cyanine5.5 mouse IgG2b κ isotype control; V500 mouse anti-human CD45; V500 mouse IgG1 κ isotype control; Phycoerythrin (PE) mouse anti-human CD73; PE mouse IgG1 κ isotype control; PerCP-Cyanine5.5 mouse anti-human CD90; PerCP-Cyanine5.5 mouse IgG1 κ isotype control; mouse anti-human CD105: Alexa Fluor (AF)488; mouse IgG1 negative control: AF488; PE-Cyanine7 mouse anti-human CD117; PE-Cyanine7 mouse IgG1, κ isotype control. After antibodies have been added to each sample, spin down antibodies and mix gently. Incubate the solutions for 10 min at 4 °C.

2.1.1.5. Add 1 mL of PBS (4 °C) and centrifuge the samples at 300 x g for 10 min at 4 °C. Discard the supernatant.

2.1.1.6. Re-suspend cells in 100 μ L of PBS (4 °C) and add 33 μ L of PFA (4%). Mix the solutions and store on ice or at 4 °C until flow cytometric analysis.

2.1.2. Flow cytometric measurement of cells

Note: Perform the following work in a rather shaded room. Keep cells on ice until measurement.

2.1.2.1. To examine the expression of surface antigens, transfer samples into tubes suitable for flow cytometric measurements.

2.1.2.2. Measure at least 2×10^4 events using a flow cytometer. Analyze as depicted in **Figure 2**.

2.2. Multipotent differentiation potential of hDFSCs

2.2.1. Use a commercially available Human Mesenchymal Stem Cell Functional Identification Kit to confirm adipogenic, osteogenic and chondrogenic differentiation potential of hDFSCs. Apply donkey anti-goat AF488 secondary antibody for staining of fatty acid binding protein 4 (FABP4) and aggrecan as well as donkey anti-mouse AF488 secondary antibody for staining of osteocalcin. For staining of nuclei, use mounting medium with 4',6-Diamidino-2-phenylindole (DAPI).

Note: Prepare samples without primary antibody as negative controls for microscopic analyses.

2.2.2. Analyse expression of proteins by laser scanning confocal microscopy. Microscopy settings: 40x objective with oil immersion; excitation laser 488 nm (for AF488) and 405 nm (for DAPI).

3. Transfection of hDFSCs

3.1. After cell harvesting (1.4), seed hDFSCs on a 24-well cell culture plate 24 h prior to transfection.

Note: Starting cell density is approximately 4×10^4 cells per well. Cells should reach ~80% confluence on day of transfection.

Note: Seed one additional cell sample as control for flow cytometric analysis.

3.2. Preparation of transfection complexes

Note: To prevent contamination from RNases, clean the workspace directly before preparation of transfection complexes using RNase decontamination solution. Use only RNase-free material and solutions.

Note: Perform the following work in a rather shaded room.

3.2.1. Prepare miR stock solution (50 μ M): re-suspend Cy3-labelled precursor miR (5 nmol) in 100 μ L nuclease-free water. Aliquot obtained miR stock solution in respective amounts (5 μ L) and store in the dark at -20 $^{\circ}$ C until usage.

3.2.2. Dilute 40 pmol of miR (0.8 μ L of the 50 μ M miR stock solution) in 66.7 μ L of reduced serum medium. Mix the solution gently

3.2.3. Dilute 0.67 μ L of cationic lipid-based transfection reagent in 66.7 μ L of reduced serum medium. Mix the solution gently and incubate for 5 min at RT.

3.2.4. After incubation, add the pre-diluted transfection reagent to the pre-diluted miR. Mix the solution gently and incubate for 15 min at RT.

3.3. Add the prepared transfection complexes dropwise directly to the culture medium on cells. Mix gently by rocking the 24-well cell culture plate back and forth.

3.4. Incubate the cells at 37 $^{\circ}$ C, 5% CO₂, and 20% O₂ for 24 h.

4. Analysis of Transfection

Note: Perform the following work in a rather shaded room.

4.1. Cell harvesting after transfection

Note: Collect all cell solutions of one sample in the same 15 mL conical centrifuge tube.

4.1.1. 24 h after transfection, collect the supernatant of samples in respective centrifuge tubes.

4.1.2. Wash cells with 1 mL of PBS, transfer PBS into the respective centrifuge tube and add 500 μ L Trypsin/EDTA (RT) to cells. Incubate the solutions for 3 min at 37 $^{\circ}$ C.

4.1.3. Stop trypsinization by adding 1 mL of culture medium (RT) to the cells and transfer the solution into the respective centrifuge tube. Centrifuge cells at 300 x g for 10 min at 4 $^{\circ}$ C.

Note: From this time, keep cells and reagents on ice unless stated otherwise.

4.2. Preparation of cells for flow cytometric analysis

4.2.1. Discard the supernatant. Re-suspend cells in 100 μ L of staining buffer (4 $^{\circ}$ C) and transfer cell solution to a 1.5 mL microcentrifuge tube.

4.2.2. Add 0.5 μ L of amine reactive dye to the samples in order to distinguish between live and dead cells. Gently mix the solution and incubate for 10 min at 4 $^{\circ}$ C.

4.2.3. Add 1 mL of PBS (4 °C) to cells and centrifuge at 300 x g for 10 min at 4 °C. Discard the supernatant.

4.2.4. Re-suspend cells in 100 µL of PBS (4 °C) and add 33 µL of PFA (4%). Mix the solutions and store on ice or at 4 °C until flow cytometric analysis.

4.3. Flow cytometric measurement of cells

Note: Perform the following work in a rather shaded room. Keep cells on ice until measurement.

4.3.1. Transfer samples into tubes suitable for flow cytometric measurements.

4.3.2. Examine cell viability and miR uptake efficiency using a flow cytometer. Measure at least 2 x 10⁴ events. Use the gating strategy depicted in **Figure 4**.

Note: Use untransfected cells as negative control to arrange the gating for Cy3 positive cells and to calculate cell death caused by transfection.

REPRESENTATIVE RESULTS:

Here, we present a detailed isolation instruction to harvest hDFSCs from human dental follicle tissue. Due to the easy access of the dental follicle during routine surgery, it is a promising source for the extraction of adult stem cells.

The isolated hDFSCs showed all characteristics described for the definition of MSCs¹³. In fact, cells were plastic-adherent under described culture conditions and displayed a fibroblast-like morphology (**Figure 1**). Flow cytometric analyses revealed that hDFSCs expressed a panel of certain surface antigens, including CD29, CD44, CD73, CD90 and CD105, while CD45 and CD117 were absent (**Figure 2**). Moreover, the adipogenic, osteogenic and chondrogenic differentiation potential of cells under specific *in vitro* culture conditions was confirmed by immunostaining of fatty acid binding protein 4 (FABP4), osteocalcin and aggrecan (**Figure 3**).

The described cationic lipid-based transfection strategy enabled efficient transient genetic modification of hDFSCs with a miR-uptake in ~100% of viable cells 18 h post-transfection (**Figure 4B**). Moreover, transfected (**Figure 4A**) and untransfected (**Figure 4C**) samples showed comparable amounts of dead cells proving gentle cell processing conditions.

FIGURE AND TABLE LEGENDS:

Figure 1: Representative light microscope picture of hDFSCs. Cells show a fibroblast-like morphology under standard culture conditions.

Figure 2: Representative flow cytometric immunophenotyping of hDFSCs. Flow cytometric analysis of cells after staining for specific cell surface markers (blue). Corresponding isotype controls were used as negative controls (grey).

Figure 3: Representative verification of the adipogenic, osteogenic and chondrogenic differentiation potential of hDFSCs. After differentiation, adipocytes, osteocytes and chondrocytes were identified by immunostaining of (A) fatty acid binding protein 4 (FABP4) (green), (B) osteocalcin (green) and (C) aggrecan (green). Nuclei were stained with DAPI (blue).

Figure 4: Representative gating strategy for analysis of transfection. Schematic representation of gating strategy used for the quantification of (A) cytotoxicity (blue: dead cells) and (B) Cy3-labeled miR uptake efficiency (red: Cy3⁺ cells) 18 h post-transfection. Untransfected cells (C, D) were used as control.

Table 1: Pipetting layout for immunophenotyping of hDFSCs.

DISCUSSION:

Adult stem cells are currently in focus for the treatment of several degenerative diseases. In particular, bone marrow (BM)-derived stem cells, including hematopoietic stem cells (HSCs) and MSCs, are under intensive clinical investigation⁴⁷. However, BM harvesting is an invasive procedure causing pain at the site of donation and may lead to adverse events⁴⁸. Recently, the postnatal dental tissue has emerged as a novel and easily accessible source for stem cells. These dental stem cells were shown to meet all MSC characteristics and showed higher proliferation capacity as BM-derived stem cells⁴⁹. Here, we presented a detailed protocol for the extraction, characterization and engineering of hDFSCs.

The described isolation procedure has been developed on human dental follicles of impacted wisdom teeth, as this tissue is commonly extracted and disposed of as medical waste¹⁹. Nevertheless, other dental tissues, including dental pulp^{50,51}, periodontal ligament⁵², exfoliated deciduous teeth⁵³, and root apical papilla⁵⁴, can be utilized for the extraction of dental stem cells.

Genetic engineering of stem cells by inserting miRs is a novel strategy to overcome certain difficulties in stem cell-based therapies, such as low stem cell survival^{43,55–57}. This protocol presented crucial instructions for the efficient introduction of synthetic miR into hDFSCs using a commercially available cationic lipid-based transfection reagent. The application of cationic liposomal formulations for the delivery of therapeutic reagents, such as drugs and nucleic acids, has been investigated in numerous clinical trials^{58,59}. However, cationic liposomes are potentially cytotoxic in a dose-dependent manner by causing *e.g.*, damage to the integrity of the cell membrane or alterations in gene expression^{59–61}. Therefore, particular attention must be paid to toxic effects on cells induced by transfection.

Notably, indicated transfection conditions have been optimized for miR-mediated genetic modification of hDFSCs in respect of efficiency and cytotoxicity. Nevertheless, other studies demonstrated the successful application of this transfection reagent for the delivery of additional nucleic acids, including plasmid DNA, mRNA and siRNA, into different cell types^{62–68}. Results of these studies revealed that ideal delivery conditions varied significantly and have to be defined

for each cell type.

ACKNOWLEDGMENTS:

This work was supported by the FORUN Program of the Rostock University Medical Centre (889018) and the DAMP Foundation (2016-11). In addition, P.M. and R.D. are supported by the BMBF (VIP+ 00240).

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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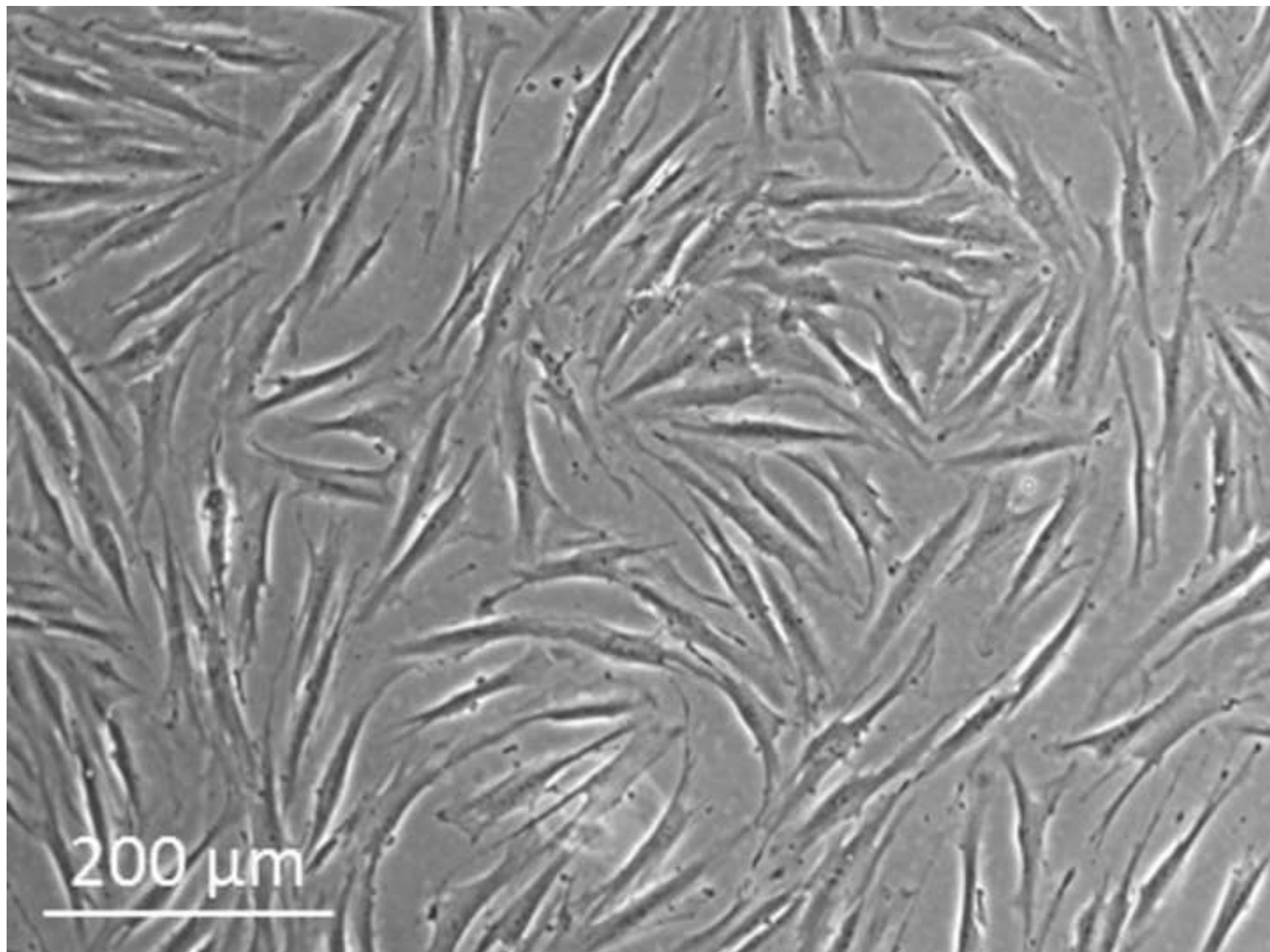
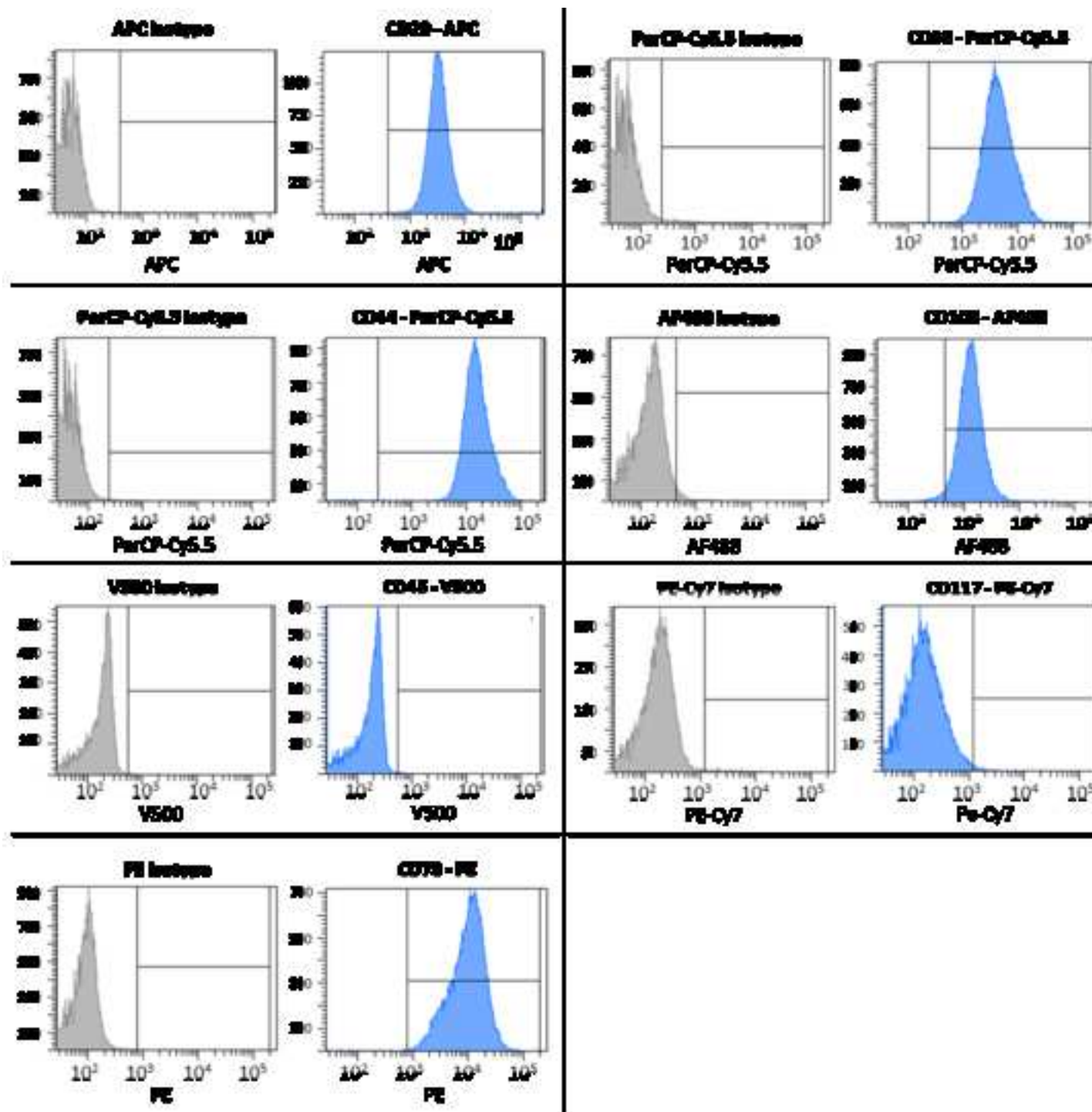
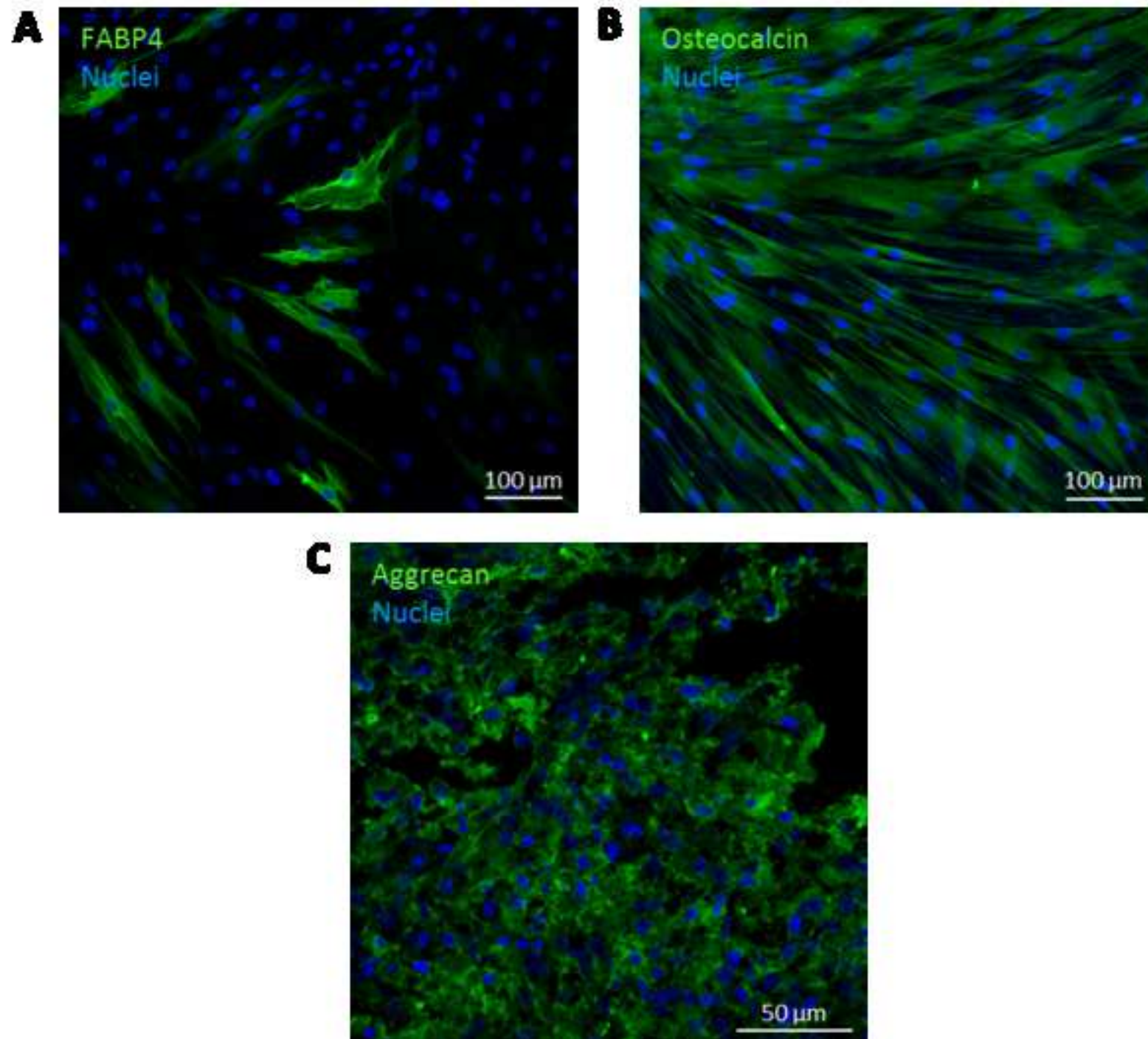
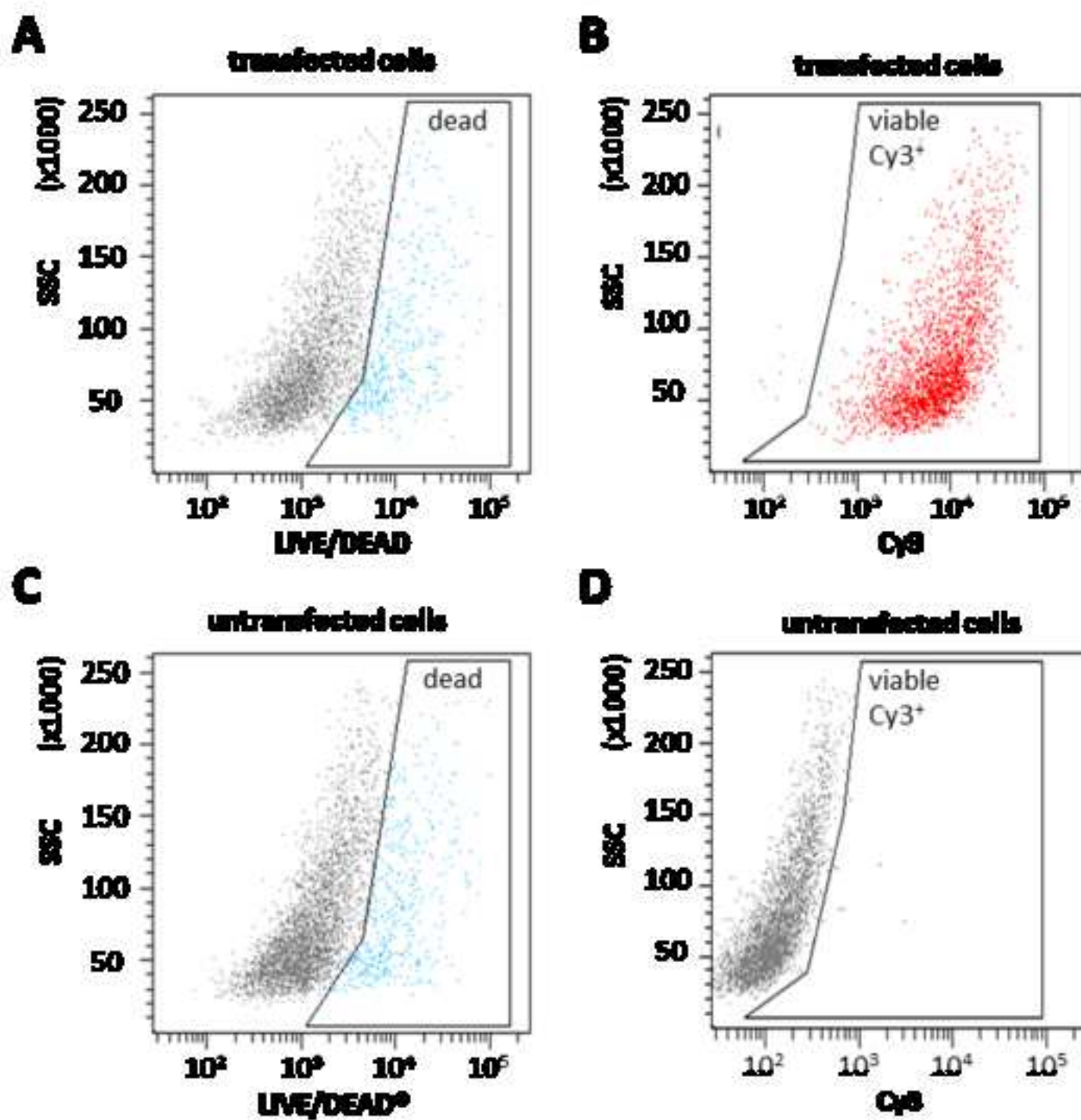


Figure 2







Sample	MACS buffer [μl]	FcR blocking reagent [μl]	Antibodies [μl]				
			CD29 APC	CD44 PerCP-Cy5.5	CD45 V500	CD73 PE	CD90 PerCP-Cy5.5
1	30	10	10				
2	37.5	10		2.5			
3	37.5	10			2.5		
4	30	10				10	
5	35	10					5
6	35	10					
7	37.5	10					
8	30	10					
9	30	10					
10	37.5	10					
11	30	10					
12	40	10					
13	30	10					
14	37.5	10					

		Isotype controls [μl]					
CD105 AF488	CD117 PE-Cy7	APC IgG1	PerCP-Cy5.5 IgG2b	V500 IgG1	PE IgG1	PerCP-Cy5.5 IgG1	AF488 IgG1
5							
	2.5						
		10					
			10				
				2.5			
					10		
						10	
							5

[illegible]

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Mouse anti Human CD105 Antibody: Alexa Fluor 488	Bio-Rad	MCA1557A488	Clone SN6, monoclonal
Mouse IgG1 Negative Control Antibody: Alexa Fluor 488	Bio-Rad	MCA928A488	monoclonal
APC Mouse Anti-Human CD29 Antibody	BD Biosciences	559883	Clone MAR4, monoclonal
APC Mouse IgG1, κ Isotype Control Antibody	BD Biosciences	555751	Clone MOPC-21, monoclonal
PE Mouse Anti-Human CD73 Antibody	BD Biosciences	550257	Clone AD2, monoclonal
PE Mouse IgG1, κ Isotype Control Antibody	BD Biosciences	555749	Clone MOPC-21, monoclonal
PE-Cy7 Mouse Anti-Human CD117 Antibody	BD Biosciences	339217	Clone 104D2, monoclonal
PE-Cy7 Mouse IgG1, κ Isotype Control Antibody	BD Biosciences	557872	Clone MOPC-21, monoclonal
PerCP-Cy5.5 Mouse Anti-Human CD44 Antibody	BD Biosciences	560531	Clone G44-26, monoclonal
PerCP-Cy5.5 Mouse IgG2b, κ Isotype Control Antibody	BD Biosciences	558304	Clone 27-35, monoclonal
PerCP-Cy5.5 Mouse Anti-Human CD90 Antibody	BD Biosciences	561557	Clone 5E10, monoclonal
PerCP-Cy5.5 Mouse IgG1, κ Isotype Control Antibody	BD Biosciences	55095	Clone MOPC-21, monoclonal
V500 Mouse Anti-Human CD45 Antibody	BD Biosciences	560777	Clone HI30, monoclonal
V500 Mouse IgG1, κ Isotype Control Antibody	BD Biosciences	560787	Clone X40, monoclonal
FcR Blocking Reagent, human	Miltenyi Biotec	130-059-901	

UltraPure EDTA	Thermo Fisher Scientific	15575-020	0.5M, pH 8.0
Steritop	Merck Millipore	SCGPT05RE	0.22 µm, radio-sterilized, polyethersulfone
BSA	Sigma-Aldrich	A7906	
PFA	Merck Millipore	1040051000	
Human Mesenchymal Stem Cell Functional Identification Kit	R&D Systems	SC006	
RNase decontamination solution; RNaseZap RNase Decontamination Solution	Thermo Fisher Scientific	AM9780	
Cy3-labelled precursor miR; Cy3 Dye-Labeled Pre-miR Negative Control #1	Thermo Fisher Scientific	AM17120	5 nmol
Pre-miR miRNA Precursor Negative Control #1	Thermo Fisher Scientific	AM17110	5nmol
Cationic lipid-based transfection reagent; Lipofectamine 2000 Transfection Reagent	Thermo Fisher Scientific	11668019	
Reduced serum medium; Opti-MEM I Reduced Serum Medium	Thermo Fisher Scientific	31985070	
Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific	A-11055	polyclonal
Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific	A-21202	polyclonal

Mounting medium; Fluoroshield with DAPI	Sigma-Aldrich	F6057-20ML	histology mounting medium
ELYRA PS.1 LSM 780 confocal microscope	Zeiss		
BD FACS LSRII flow cytometer	BD Biosciences		
BD FACSDiva Software 6.1.2	BD Biosciences		
ZEN2011 software	Zeiss		
Trypsin/EDTA solution (0.05%/0.02%)	Biochrom	L2143	in PBS, w/o: Ca ²⁺ , Mg ²⁺
Amine reactive dye; LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit	Thermo Fisher Scientific	L10119	
PBS (1x)	Thermo Fisher Scientific	10010023	pH: 7.4; w/o: Ca and Mg
P-S-G (100x)	Thermo Fisher Scientific	10378016	
Basal medium; Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12	Thermo Fisher Scientific	11039021	
Antibiotic, ZellShield FBS	Biochrom	W 13-0050	
	Thermo Fisher Scientific	10500064	
Collagenase type I	Thermo Fisher Scientific	17100017	
Dispase II	Thermo Fisher Scientific	17105041	
Filter, Sterifix syringe filter 0.2 µm	Braun	4099206	
50 mL conical centrifuge tube	Sarstedt	62,547,254	
15 mL conical centrifuge tube	Sarstedt	62,554,502	

Cell culture flask 75 cm ²	Sarstedt	833,910,002	
Cell culture flask, 25 cm ²	Sarstedt	833,911,002	
Freezing medium, Biofreeze	Biochrom	F 2270	
	Thermo Fisher		
Cryotubes	Scientific	377267	1.8 mL
Trypan blue solution	Sigma-Aldrich	T8154	0.4 %
Counting chamber			
	Paul Marienfeld		
Local anesthetic, Xylocitin	Mibe		
(lidocaine hydrochloride) 2% with			
epinephrine (adrenaline) 0.001%			
NaCl solution	Braun		0.9 %
Vicryl satures, Vicryl rapide	Ethicon		3 - 0



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Protocol for isolation, characterization and micro-RNA based genetic modification of human dental follicle stem cells

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To

The Review Editor

Journal of Visualized Experiments



15.05.2018

Dear Dr. Steindel,

thank you very much for the opportunity to revise our manuscript JoVE-58089 “*Protocol for isolation, characterization and microRNA-based genetic modification of human dental follicle stem cells*” and resubmit it for publication in your journal. As you will see, we have entirely addressed the editorial comments.

Please find our responses to the editorials` comments, below:

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Editorial comments:

We thank the editor for general instructions regarding our manuscript. We have revised the initial draft in full accordance to each of these specifications.

1. 1.4.2: Is there some particular reason you have not marked this for filming? 3.1, which is filmed, immediately follows from it and there is still room.

We did not include this step for filming because it shows only a simple cell culture procedure (cell harvesting and seeding). However, of you wish to include this step in the video we will be happy to do so.

2. 1.4.2.3: What is an ‘appropriate amount’ of medium?

For cell counting, a dilution with a suitable concentration should be prepared. For optimal results, the concentration should be around 1.000.000 cells per mL.

3. 2.1.1.2: Do you mean one tube per sample or one tube for all samples?

To characterize hDFSCs from one patient 14 samples of 5×10^4 cells should be taken. Transfer each sample into a separate 1.5 mL microcentrifuge tube.

Changed to: “After cell harvesting (1.4.2), transfer 14 samples of 5×10^4 cells into 1.5 mL microcentrifuge tubes.”

4. 2.1.1.4: What do you mean by ‘shake down antibodies’?

Changed to: “spin down antibodies”

5. Results: 1.4.3.1 is no longer in the manuscript (2nd paragraph).

We deleted this step number from the results part.

6. Figure 2: This is varyingly referred to as an ‘analysis’ (Results) and ‘gating’ (legend, step 2.1.2.2); it looks more like an ‘analysis’ than a ‘gating strategy’.

We changed “gating strategy” to “flow cytometric analysis”.

7. Figure 3: A, B, C are no longer in the Figure.

We included A, B; C in the Figure.

Sincerely,



Prof. Robert David, PhD