

Label-Free and High-Throughput Removal of Residual Undifferentiated Cells From iPSC-Derived Spinal Cord Progenitor Cells

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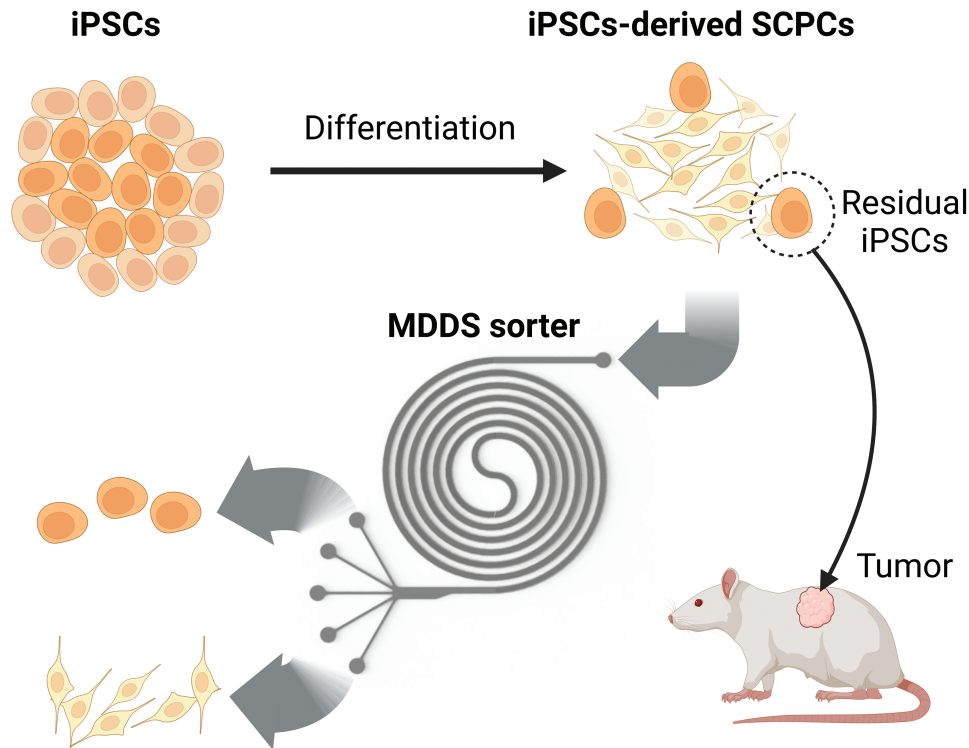
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Abstract

The transplantation of spinal cord progenitor cells (SCPCs) derived from human-induced pluripotent stem cells (iPSCs) has beneficial effects in treating spinal cord injury (SCI). However, the presence of residual undifferentiated iPSCs among their differentiated progeny poses a high risk as these cells can develop teratomas or other types of tumors post-transplantation. Despite the need to remove these residual undifferentiated iPSCs, no specific surface markers can identify them for subsequent removal. By profiling the size of SCPCs after a 10-day differentiation process, we found that the large-sized group contains significantly more cells expressing pluripotent markers. In this study, we used a sized-based, label-free separation using an inertial microfluidic-based device to remove tumor-risk cells. The device can reduce the number of undifferentiated cells from an SCPC population with high throughput (ie, >3 million cells/minute) without affecting cell viability and functions. The sorted cells were verified with immunofluorescence staining, flow cytometry analysis, and colony culture assay. We demonstrated the capabilities of our technology to reduce the percentage of OCT4-positive cells. Our technology has great potential for the “downstream processing” of cell manufacturing workflow, ensuring better quality and safety of transplanted cells.

Key words: cellular therapy; induced pluripotent stem cells; spinal cord injury; neural stem/progenitor cell; sorting technologies.

Graphical Abstract



Significance Statement

Our paper describes a new method to safely manufacture cell-based therapies using a size-based, label-free microfluidic separation technology. This technique removes residual undifferentiated iPSCs from their differentiated progenitor cells, reducing the risk of tumor formation post-transplantation. Our technology has a high throughput without compromising cell viability and function. This technique has broad clinical applications where cell purity and quality are critical. Our technology has great potential for the “downstream processing” of cell manufacturing workflow, ensuring better quality and safety of transplanted cells by reducing the number of potentially tumor-forming cells.

Introduction

As a potential source to generate various cell types for cell therapy, the conversion of patient-derived somatic cells into induced pluripotent stem cells (iPSCs) bypasses many ethical issues that are associated with the use of human embryonic stem cells (hESCs).¹ Although these iPSCs hold great potential in regenerative medicine, the tumorigenicity of iPSCs remains a significant hurdle for the safe therapeutic application of iPSC-derived differentiated cells.²⁻⁵ Due to their unlimited self-renewal and pluripotency, the residual undifferentiated iPSCs can form teratomas in a dose-dependent manner.^{2,6,7} The potential tumorigenicity risk of the residual undifferentiated iPSCs depends on the number of undifferentiated cells, the features of the cell lines,³ and culture adaptation.⁸ Therefore, it is crucial to evaluate the tumorigenicity of iPSC-derived differentiated cells for each cell line and batch to ensure their safe therapeutic use.

Several removal methods of residual iPSCs or ESCs have been reported. These include cytotoxic antibodies,⁹⁻¹¹ fluorescence-activated cell sorting (FACS),¹²⁻¹⁴ magnetic-activated cell sorting (MACS),^{15,16} and small molecules.^{17,18} In general, these methods rely heavily on cell-surface markers, which lack sufficient specificity for pluripotent cells. On the other hand, alternative removal methods that use gene

manipulation or chemical inhibitors^{13,19-21} may not be suitable for high-throughput cell therapy manufacturing.

Microfluidic spiral sorting is a cutting-edge technique used for the high-throughput separation of cells based on physical properties like size, shape, and density, making it ideal for label-free sorting.^{22,23} This technology has proven effective in selecting specific subpopulations for stem cell therapy.²⁴⁻²⁷ Given that the size of iPSCs decreases during differentiation,²⁸ we suggest that microfluidic spiral sorting could be a valuable tool for removing residual iPSCs from the iPSC-derived population based on size differences. By using this technique, we can further improve the purity and quality of stem cell populations, paving the way for more efficient and effective stem cell therapies.

This study used a high-throughput microfluidic multidimensional double spiral (MDDS) sorter,²⁹⁻³¹ to eliminate residual undifferentiated cells from iPSC-derived spinal cord progenitor cell (SCPC) populations. These SCPCs hold significant promise for treating spinal cord injuries.³²⁻³⁴ The sorter takes advantage of the differences in cell size by applying stronger drag forces on the larger undifferentiated cells. This enables the efficient separation of undifferentiated cells from the heterogeneous population of cells in microfluidic channels. The sorter uses label-free separation and is simple, cost-effective,

and high throughput, making it an ideal solution for the rapid and large-scale production of safer SCPCs. In accordance with the safety quality control (QC) strategy designed to detect residual contaminating iPSCs in iPSC-derived neural stem/progenitor cells intended for clinical trials,³⁵ the expression of OCT4, the primary marker for iPSCs, was analyzed. Here, SOX1 was used as a marker to validate the presence of neural progenitor cells, while the expression of OCT4 was analyzed using immunostaining, flow cytometry, and colony culture assays³⁶ to quantify the purity of our microfluidics-based sorted cells.

Materials and Methods

SCPC Differentiation From Human iPSCs

The iPSC lines were maintained in StemMACS iPS-Brew XF cell culture medium on Matrigel Matrix coating (Corning, USA). The cord lining epithelial cells (CLEC)-derived iPSCs were developed by CellResearch Corporation and are hypothesized to have the immune-privileged properties of the CLECs.^{37,38} BJ-iPSCs were derived from BJ-fibroblasts using modified mRNA, which represent the conventional fibroblast-derived iPSCs and have been used to generate spinal motor neurons.^{39,40} The iPSCs were routinely passaged every 5–7 days using ReLeSR Passaging Reagent (STEMCELL Technologies, Canada). The iPSCs were then differentiated into SCPCs based on a modified protocol to generate spinal motor neurons.^{41,42} Briefly, when the iPSCs were 70%–80% confluent, the iPSCs were dissociated into single-cell suspension using Accutase (Nacalai Tesque Inc., Japan). The dissociated iPSCs were then counted and reseeded at a density of 800 000 cells/well in a 6-well plate in a neural induction medium supplemented with ROCK inhibitor Y-27632 (5 μ M). The neural induction medium consisted of DMEM/F12 (50%, Biological Industries, Israel), neural medium (50%), NeuroBrew-21 (1 $\times$), N2 (1 $\times$), nonessential amino acid (1 $\times$, Thermo Fisher Scientific, USA), glutamax (0.5 $\times$, Thermo Fisher Scientific), LDN-193189 (0.5 μ M), and CHIR-99021 (4.25 μ M). On day 3, retinoic acid (10 μ M, Sigma-Aldrich, USA) was added to the medium. On day 4, the cells were reseeded at a lower density (2 million cells per 100 mm dish) in the same medium and maintained with daily medium change until day 10. On day 10, the cells were characterized and termed as SCPCs, which were then frozen or used for experiments. Unless specified, all other culture components were purchased from Miltenyi Biotech (Germany).

Microfluidic MDDS Sorter Design and Fabrication

The polydimethylsiloxane (PDMS) MDDS sorter device was fabricated via the standard soft-lithographic technique. 3D CAD software (SolidWorks 2020, USA) was used to design an aluminum mold for making the PDMS MDDS sorter replica, and the aluminum mold was fabricated via a micro-milling process (Whits Technologies, Singapore). The optimal channel dimensions and channel configuration were determined based on the observation of trajectories of particles with various sizes (7–30 μ m) in the devices with different channel dimensions. The MDDS device is composed of 2 sequentially interconnected spiral channels with distinct dimensions. The design of the first spiral channel incorporates a comparatively smaller dimension to generate an increased inertial lift force, subsequently directing all target particles or cells toward the inner wall side of the channel. In contrast, the second spiral

channel features an enlarged dimension, allowing particles to attain disparate equilibrium positions as dictated by the balance between inertial lift and Dean drag forces, ultimately facilitating particle separation based on size.³¹ Through empirical selection, an optimal device was identified, in which the first spiral channel exhibits a rectangular cross-section with an 800 μ m width, 100 μ m height, and 3 loops, while the second channel demonstrates a trapezoidal cross-section, encompassing an 800- μ m width, heights of 120 and 180 μ m for the inner- and outer-wall sides, respectively, and 3 loops.

A 10:1 mixture of PDMS base and curing agent (Sylgard 184, Dow Corning, Inc., USA) was used to make the PDMS replica. After curing on the hot plate for 10 minutes at 80 $^{\circ}$ C, the PDMS replica was bonded to a glass substrate using a plasma machine (Femto Science, South Korea).

SCPCs Sorting Using MDDS Sorter

Prior to sorting, the MDDS sorter was incubated with 70% ethanol for at least 30 minutes for sterilization. It was then rinsed with 1 $\times$ phosphate-buffered saline (PBS) and medium. The cells, at a concentration of 0.5–1 million cells/mL, were loaded into a 20-mL syringe and injected into the device at flow rates of 2 mL/minute for the low-speed mode and 3 mL/minute for the high-speed mode using a syringe pump (PHD ULTRA Syringe Pumps, Harvard Apparatus, USA). To implement the recirculation strategy, a dual check valve (Quosina, USA) was used to retract the sorted cells into the input syringe by withdrawing them at a flow rate of 10 mL/minute from the outlet reservoir using the syringe pump.

Immunofluorescent Staining

The sorted cells were seeded at a density of 80 000 cells/well in a 96-well plate. After the cells were attached to the wells overnight, the cells were fixed in 4% paraformaldehyde (PFA, Santa Cruz Biotechnology, USA) for 15 minutes at room temperature. Thereafter, the cells were permeabilized in 0.1% Triton X-100 (Sigma-Aldrich) for 15 minutes, followed by 1 hour incubation in the blocking buffer (5% fetal bovine serum [FBS, Gibco, USA] and 1% bovine serum albumin [BSA, Sigma-Aldrich] in PBS [Gibco]) at room temperature. The cells were then incubated in primary antibodies diluted in the blocking buffer at 4 $^{\circ}$ C overnight. The primary antibodies used were OCT4 (1:500, sc-5279, Santa Cruz Biotechnology), SOX1 (1:500, 4194S, Cell Signaling Technology, USA), and HOXB4 (1:200, ab133521, Abcam, UK). On the next day, the cells were washed with PBS and incubated in respective secondary antibodies (donkey anti-mouse AF 488/donkey anti-rabbit AF 568, 1:1000, Thermo Fisher Scientific) and DAPI diluted in blocking buffer (1:1000, Thermo Fisher Scientific) for 1 hour at room temperature. The cells were then washed with PBS before imaging. Imaging was performed on a Leica DMi8 microscope or Opera Phenix High-Content Screening System using 10 $\times$ or 20 $\times$ objectives.

Flow Cytometry Analysis

Cells were collected in 15 mL centrifuge tubes and adjusted to 2×10^6 cells/tube. They were then fixed in 4% PFA and incubated at room temperature for 15 minutes. Thereafter, the fixed cells were pelleted by centrifugation at 1962 $\times$ g for 5 minutes and washed once with PBS. Next, cells were stained by incubation at room temperature for at least 2 hours with a primary antibody in perm/block buffer (0.5% saponin, Sigma-Aldrich, USA; 1% BSA). The primary

antibodies used were: OCT4 (1:500, sc-5279, Santa Cruz Biotechnology) and SOX1 (1:500, 4194S, Cell Signaling Technology). Cells were centrifuged and washed twice with PBS. They were then stained by incubation in the dark at room temperature for at least 45 minutes with a secondary antibody in perm/block buffer. The secondary antibodies used were donkey Alexa Fluor 488-conjugated anti-rabbit IgGs (A-21206, Thermo Fisher Scientific) and donkey Alexa Fluor 555-conjugated anti-Mouse IgGs (A-31570, Thermo Fisher Scientific). After that, cells were washed with PBS and resuspended in PBS. Before running with the Cytoflex flow cytometer (Beckman Coulter, USA), cells were filtered through a 45- μ m cell strainer to remove cell clumps and kept on ice. Compensation was performed and applied to the analysis. Due to the relatively weak expression level of the OCT4 marker, the percentage of OCT4⁺ cells was obtained using fluorescence minus one (FMO) control (ie, FMO-DAPI-SOX1 control).

The removal rate of OCT4⁺ and OCT4⁺SOX1⁻ cells is calculated based on the number of input cells and the number of cells in the Large group, multiplied by their respective percentages of OCT4⁺ and OCT4⁺SOX1⁻:

Removal rate =

$$\frac{N_{\text{Large group}} \times \% \text{ OCT4}^+ / \text{OCT4}^+ \text{SOX1}^-_{\text{Large group}}}{N_{\text{Input cell}} \times \% \text{ OCT4}^+ / \text{OCT4}^+ \text{SOX1}^-_{\text{Input cells}}} \times 100\%$$

where $N_{\text{Large group}}$ and N_{Input} are the number of cells in the Large group post-sorting and the number of input cells prior to sorting, respectively.

EdU-Based Proliferation Assay

The proliferation of SCPC and hiPSC was measured with the Click-iT EdU flow cytometry assay kit (Invitrogen, USA). EdU (5-ethynyl-2'-deoxyuridine) is a nucleoside analog to thymidine and is incorporated into DNA during active DNA synthesis. The EdU was added (10 μ M) to the culture medium before seeding for incubation. After ~24 hours of incubation, cells were harvested and rinsed with PBS containing 1% BSA. Thereafter, the cells were fixed with Click-iT fixative for 15 minutes in the dark at room temperature and rinsed in PBS with 1% BSA. The cells were then permeabilized with 1 $\times$ Click-iT saponin-based permeabilization for 15 minutes at room temperature in the dark. After that, they were incubated for 30 minutes at room temperature in the dark with a Click-iT reaction cocktail. Cells were washed and resuspended with 1 $\times$ Click-iT saponin-based permeabilization and wash reagent before being analyzed by Cytoflex flow cytometer (Beckman Coulter).

Colony Culture Assay

Cells were seeded in a 12-well plate at an initial density of 17 000 cells/cm². They were cultured in hiPSC medium for 6 days with daily change of medium. Cells were fixed and stained with DAPI and OCT4 antibodies before imaging under a Leica DMi8 microscope.

Statistical Analysis

The data were subjected to normality and homogeneity of variances testing using the Shapiro-Wilk and Levene's tests, respectively. In cases where the data met the assumptions of normality and homogeneity, a one-way ANOVA followed by the Tukey post hoc test was conducted to compare three

or more groups. Otherwise, a nonparametric Kruskal-Wallis test followed by the Dunn post hoc test was used. The results were presented as mean $\pm$ SD, with statistical significance indicated by ns (not significant), * $P \leq .05$, ** $P \leq .01$, or *** $P \leq .001$. Origin software (OriginLab Corporation, USA) was performed for the statistical analysis.

Results

SCPCs Size Profiling and Its Correlation With Pluripotent Biomarker

The differentiation efficiency of SCPCs was assessed on day 10 based on the expression of pluripotent marker OCT4 and neural progenitor marker SOX1, in comparison to iPSCs. High OCT4 expression and no SOX1 expression were observed in iPSCs. In contrast, while most SCPCs expressed SOX1, indicating successful differentiation into neural progenitors, some cells still expressed OCT4 (as highlighted by circles in [Supplementary Fig. S1](#)), suggesting incomplete differentiation or the presence of residual iPSCs.

To gain insight into the biophysical changes that occur during differentiation, we investigated changes in cell size by analyzing microscopy images and size histograms of suspended CLEC-iPSCs ([Fig. 1A](#)) and day 10 CLEC-SCPCs ([Fig. 1B](#)). Our results showed that SCPCs were more homogeneous, with approximately 74% of SCPCs ranging from 10 to 14 μ m in diameter. On the other hand, the size distribution of iPSCs was slightly wider (ie, approximately 76% of iPSCs ranging from 15-21 μ m in diameter). Furthermore, iPSCs were significantly larger than day 4 SCPCs ($P < .001$), with a mean difference of 4.5 μ m. Interestingly, from day 4 to day 10, the nucleus size slightly decreased ($P = .016$). We confirmed a similar observation in BJ-iPSCs and BJ-SCPCs ([Supplementary Fig. S2A](#)), indicating that the size decrease is not cell line dependent.

To verify our findings, we used flow cytometry, where the forward scatter intensity is approximately correlated to cell size and commonly used for gating cell types of different sizes. By gating out the FSC/SSC plot, we found that most of these cells fall into the OCT4⁺ position of the OCT4/SOX1 plot ([Fig. 1C](#)). Moreover, the intensity of OCT4 expression shows a good correlation to the cell size, as shown in [Fig. 1D](#), with Spearman's rank correlation coefficient of 0.68. This result suggests that cell size is positively correlated with OCT4 expression, and the residual iPSCs retained their size morphology even after 10 days of differentiation.

Other than cell size, the nucleus area of the cells was significantly decreased during the differentiation process. [Figure 1E](#) shows the fluorescent images of DAPI staining in the iPSCs, and SCPCs that have been differentiated for 4 and 10 days. By analyzing the nucleus area, we found that the iPSCs appeared to have a larger nucleus area than SCPCs on day 4 and day 10 ([Fig. 1F](#)).

Sized-Based Separation of SCPCs and Spiked iPSCs With the MDDS Sorter

The size-based separation was achieved using a MDDS sorter, as illustrated in [Fig. 2A](#). The sorter divided cells into 4 outlets: S2 (the smallest cells) to S5 (the largest cells), while cell fragments and debris were directed solely to outlet S1. This method was first demonstrated by performing a spiking experiment where a cell population containing 87% day 10 SCPCs spiked with 13% iPSCs was separated.^{9,13,15,43} The

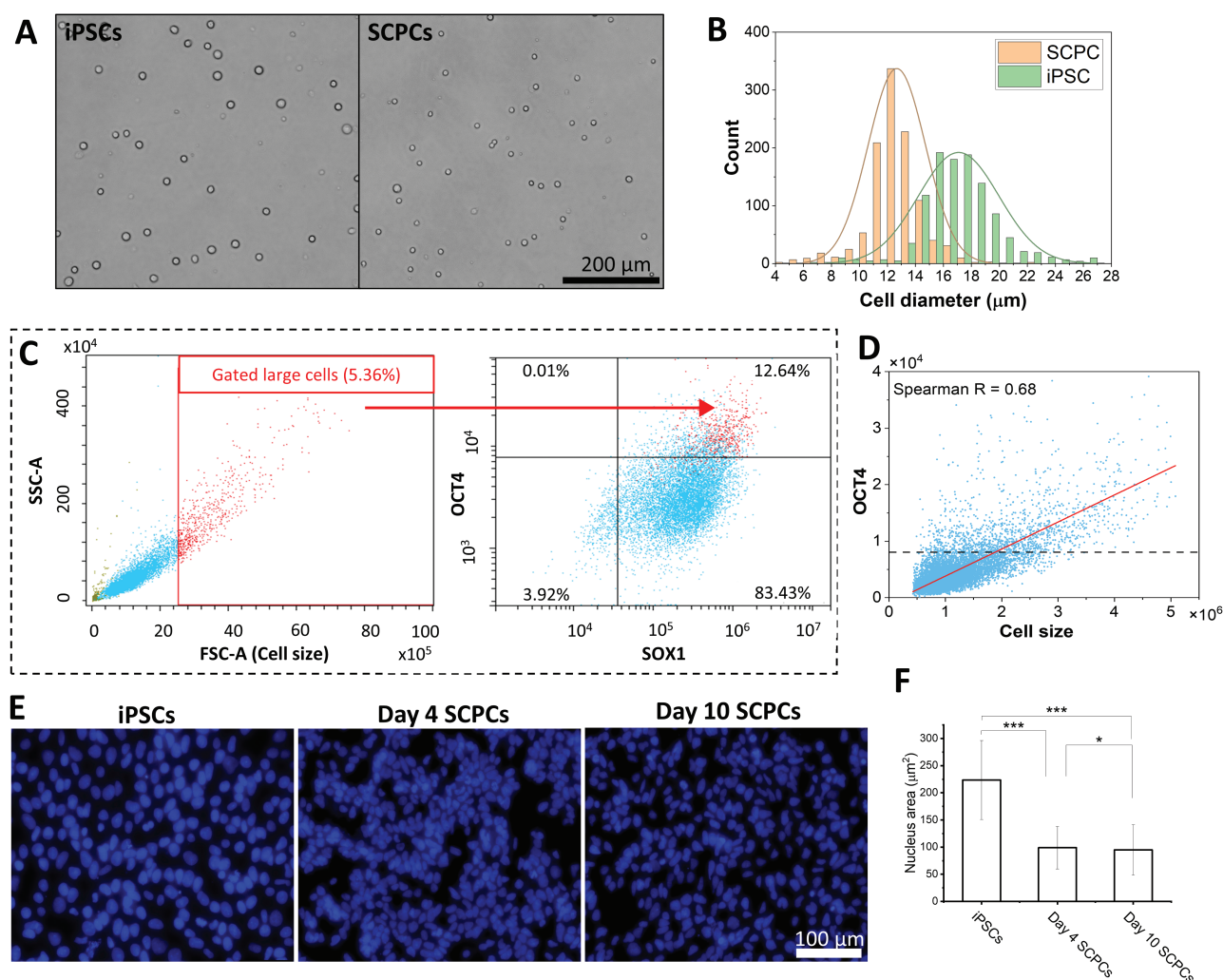


Figure 1. iPSC neural differentiation results in size and nucleus area reduction. **(A)** Microscopy images of suspended CLEC-iPSCs and CLEC-SCPCs. **(B)** Size profiling of the CLEC-iPSC and CLEC-SCPC using our customized MATLAB code. Cell size was significantly different, $P < .001$, Mann-Whitney test ($n = 1326$ cells). **(C)** Flow cytometer analysis of the SCPCs. The large cells gated in the SSC-A/FSC-A plot were mostly OCT4-positive cells. The FSC intensity is proportional to the diameter of the cells in flow cytometry. **(D)** The OCT4 fluorescent intensity is correlated with the size of SCPCs (based on FSC intensity). Rank correlation by Spearman analysis ($R = 0.68$) indicates a moderate/good correlation between cell size and OCT4 level. The dashed line gated OCT4 positive and negative cells. **(E)** Fluorescent images of DAPI staining in CLEC-iPSCs, and CLEC-SCPCs differentiated for 4 and 10 days. The nucleus area of iPSCs was reduced during the differentiation processes. **(F)** Quantification of the nucleus area of the iPSCs, CLEC-SCPCs after 4 and 10 days. The nucleus area was reduced significantly after 4 days ($P < 0.001$), and after 10 days ($P = 0.016$), Kruskal-Wallis test was followed by Dunn post hoc test ($n = 1200$ cells/group).

sorted cells were subsequently stained with pluripotency marker (OCT4), progenitor marker (SOX1), and spinal cord marker (HOXB4) and quantified through imaging with a fluorescent microscope to determine the cell composition (Fig. 2B). Figure 2C shows the size profiling and the percentage of OCT4⁺, SOX1⁺, and HOXB4⁺ cells in both unsorted and sorted groups. As iPSCs are typically larger than SCPCs, the S4 and S5 groups have a greater number of iPSCs stained with OCT4. The S2 group, which comprises the smallest cells, exhibited the lowest OCT4 percentage at approximately 1%. Additionally, this group demonstrated a significantly higher percentage of HOXB4 expression and a stronger tendency toward SOX1 expression. This finding suggests that a larger proportion of these smaller cells may contain SCPCs. On the other hand, there was no significant difference observed in size and stained markers between the S4 and S5 groups. The difference in cell size between the S3 and S4 groups caused significant differences in the expression

levels of OCT4, SOX1, and HOXB4. This indicates a clear distinction between small and large cells. Therefore, to reflect this observation, in the following sections of the paper, the cells will be separated into only 2 groups—Small (S2) and Large (S4 and S5) instead of the initial 4 groups. The S3 group consists of a mixture of both large and small cells, which could potentially lead to less significant differences in cell size. Hence, the cells from outlet S3 were subsequently resorted to allow for the collection of more cells in either the Large group (from outlets S4 and S5) or the Small group (from outlet S2). This adjustment aimed to enhance the clarity and accuracy of our sorting outcomes.

Optimization of MDDS Sorter

We identified an issue with overlapping cell sizes between sorted groups that resulted in poor separation when using the previous 5-outlet setting at a constant flow rate (ie, 2 mL/minute). To address this issue, we implemented a

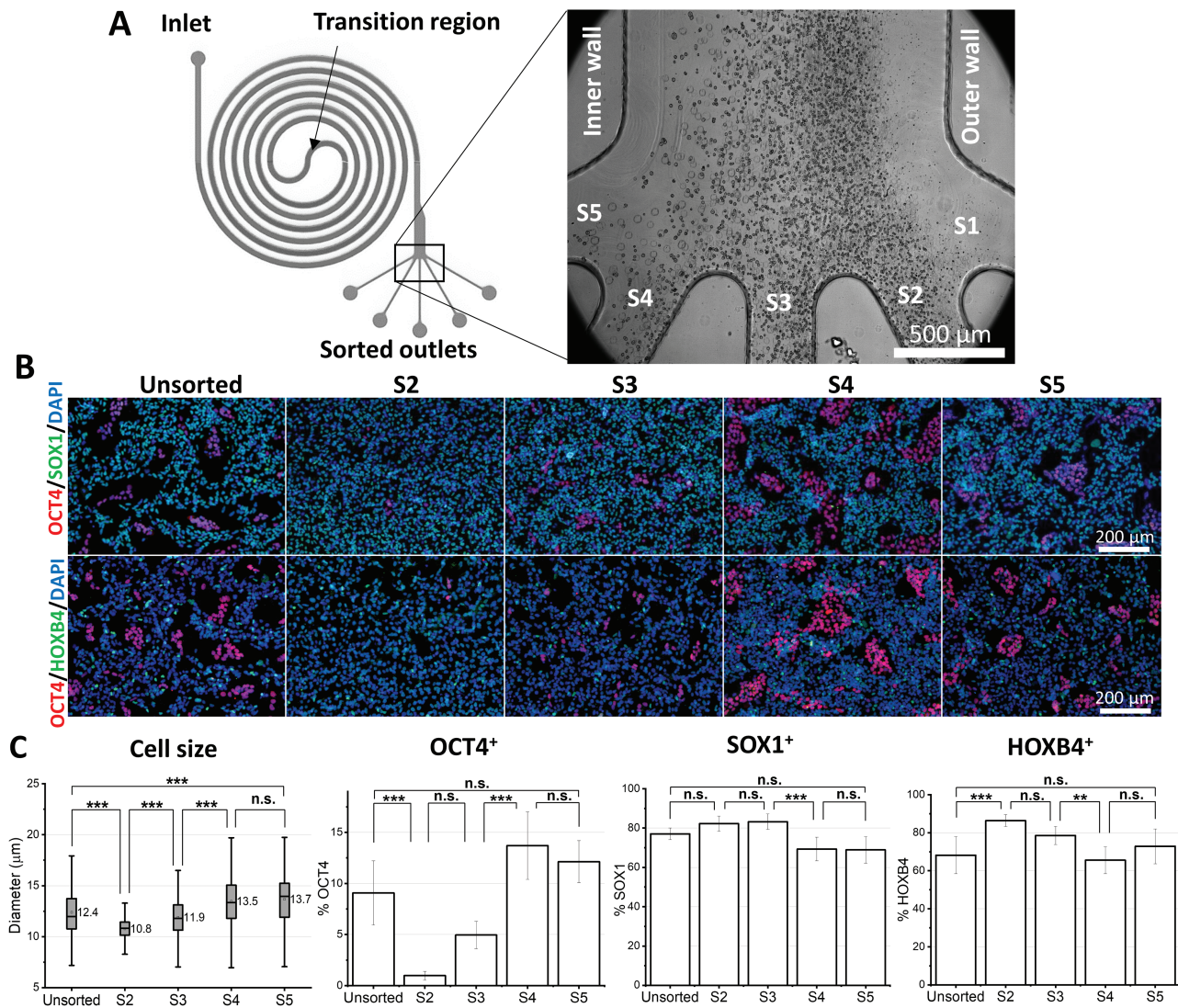


Figure 2. Sorting SCPCs and iPSCs using a microfluidic MDDS sorter. **(A)** Schematic of MDDS sorter (left) and representative bright-field image (right) showing cells were sorted into 5 outlets ranging from the smallest (S1) to the largest (S5). **(B)** SCPCs spiked with iPSCs were sorted using an MDDS sorter. The sorted cells were analyzed by immunofluorescence with OCT4 (red), SOX1 (green), HOXB4 (green), and DAPI (blue). **(C)** Quantification of cell size, OCT4, SOX1, and HOXB4 expression by unsorted group and sorted groups (ie, S2–S4). Cell size was significantly different, $P < .001$, Mann-Whitney test ($n = 340$). Significant difference in the expression of OCT4 and HOXB4 between the unsorted and S2 groups was observed. There was a significant difference in the expression of OCT4, SOX1, and HOXB4 between the S3 and S4 groups. Kruskal-Wallis test was followed by Dunn post hoc test ($n = 15$).

Low-High-speed sorting strategy using the same MDDS sorter. By running the device at low speed (ie, 2 mL/minute), most cells focused on the inner wall except for small cells that were sorted to outlets S1 and S2 (ie, Small group). Conversely, at higher speeds (ie, 3 mL/minute), most of the cells shifted to the outer wall, while very large cells were sorted to outlets S4 and S5 (ie, Large group) (Fig. 3A). The size difference between the cells sorted into the Small and Large groups can be visually recognized from Fig. 3B, while their sizes are shown in the box plot graph in Fig. 3C. We also evaluated the viability of sorted cells and found that they remain highly viable, with no significant difference from the unsorted groups (Fig. 3D). When comparing the results of low-high-speed sorting to constant-speed sorting (ie, 2 mL/minute), as shown in Fig. 3E (microscopy image) and Fig. 3F (box plot graph), the mean difference in cell size between the Small and Large groups increased from 1.5 to 2.9 μ m.

Furthermore, we implemented a recirculation strategy to re-sort uncollected cells, increasing cell recovery for further analyses (Fig. 3G).

Immunofluorescent Staining, Colony Culture Assay, and Flow Cytometry Analysis of Sorted SCPCs: Reduced Number of Residual iPSCs

We used the sorting of CLEC-SCPCs and characterized the removal efficiency of residual iPSCs. SCPCs were generated by following the standard 10-day differentiation protocol. The collected SCPCs were separated using an MDDS sorter with the recirculation strategy (resorted 2 times at low speed and 3 times at high speed). The three groups (Unsorted, Small, and Large) were stained for immunofluorescent imaging (Fig. 4A). The cells that expressed OCT4 but not SOX1 (ie, OCT4⁺SOX1⁻ cells) were analyzed and shown in Fig. 4B. The figure indicates that the Large group had significantly

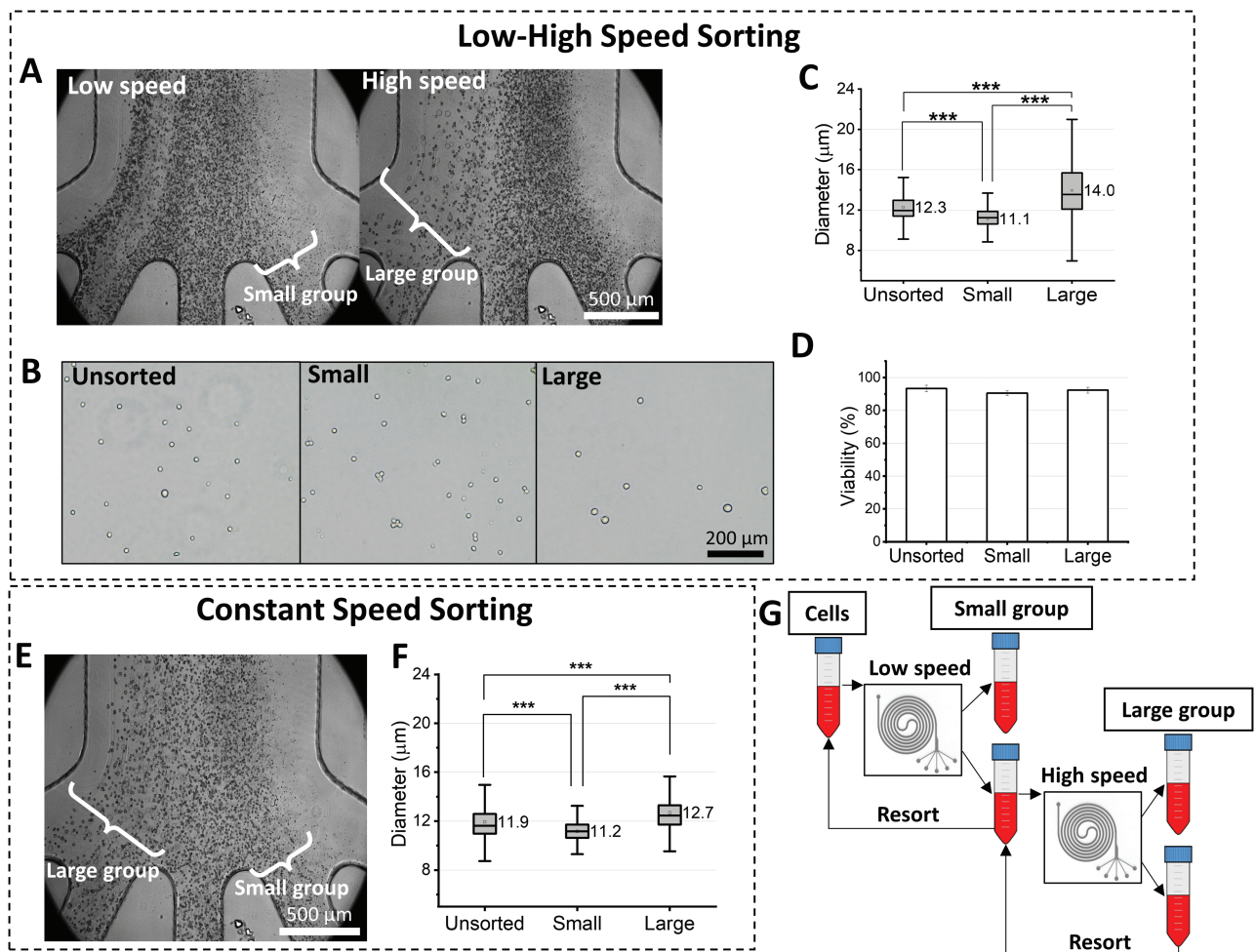


Figure 3. Optimization of SCPC sorting using a microfluidic MDDS sorter, transitioning from a constant-speed sorting (E, F) to a low-high-speed sorting (A-D). **(A)** Representative bright-field images showing cell separation under low speed (ie, 2 mL/minute) and high speed (ie, 3 mL/minute), enabling the collection of smaller and larger cells, respectively. **(B)** Representative microscopy images of Unsorted, Small, and Large groups using low-high-speed setting. **(C)** Box plot graph of cell size for the Small and Large groups (with a mean difference of 2.9 μ m). The cell size varies significantly among the groups ($P < .001$), as determined by the Kruskal-Wallis test, followed by a Dunn post hoc test ($n = 324$ cells/group). **(D)** Viability of the Unsorted, Small, and Large groups using low-high-speed sorting ($n = 3$). Cell size is significantly different among groups, $P < .001$, Kruskal-Wallis followed by a Dunn post hoc test ($n = 324$ cells/group). **(E)** Representative bright-field image showing cells sorted into Small (S2) and Large groups (S4 and S5) at a constant flow rate of 2 mL/minute. **(F)** Box plot graph shows the size distribution of the Unsorted, Small, and Large groups at a constant flow rate of 2 mL/minute (mean difference of 1.5 μ m). **(G)** Schematic diagram depicting the recirculation strategy to re-sort cells for low-high-speed setting.

more OCT4⁺SOX1⁻ cells compared with the Unsorted group. However, it is important to note that the Small group did not exhibit any statistically significant difference when compared to the Unsorted group. In addition, there was a trend indicating that the cells in the Large group had a larger nucleus area compared to the cells in the Unsorted and Small groups (Fig. 4C). To assess whether the proliferation rate is correlated to cell size, EdU incorporation assays and Ki67 immunostaining were performed (Supplementary Fig. S3). The results showed that iPSCs exhibited higher proliferation levels due to their capacity for unlimited self-renewal, while SCPCs displayed lower proliferation rates. Within the SCPCs population, smaller SCPCs showed a slower growth rate compared to larger SCPCs.

Next, colony culture assay was performed by culturing SCPCs in a culture system that promotes the growth of iPSCs. Four separated cell populations consisting of SCPCs spiked with 13% iPSCs (positive control group), the Unsorted, Small, and Large SCPCs groups were cultured in the 12-well

plate. The cells were cultured in iPSC maintenance media for 6 days. Figure 4D shows the colony formation in the positive control group (SCPCs spiked iPSCs) and unsorted SCPCs group. The positive control group had well-defined colonies with inner packed cells and sharp edges (pointed by white arrow 1), which are the typical morphologies of iPSC colonies in culture. These colonies can also be identified in the DAPI staining, while the OCT4⁺ cells are visible in the OCT4 staining. Besides the defined colonies, some colonies are in their early stages of colony formation with the large and elongated nuclei arranged in a circle (pointed by white arrow 2). For the unsorted SCPC group, similar immature colonies could also be found with the morphology of large nuclei arranged in circles (pointed by white arrow 3). Although immature colonies can be recognized by morphology, some colonies only express OCT4 marker (OCT4⁺ aggregates) but do not have clear morphologies. Moreover, the distribution of OCT4⁺ cells in the SCPC group was not always clustered in the form of colonies. Therefore, a density-based

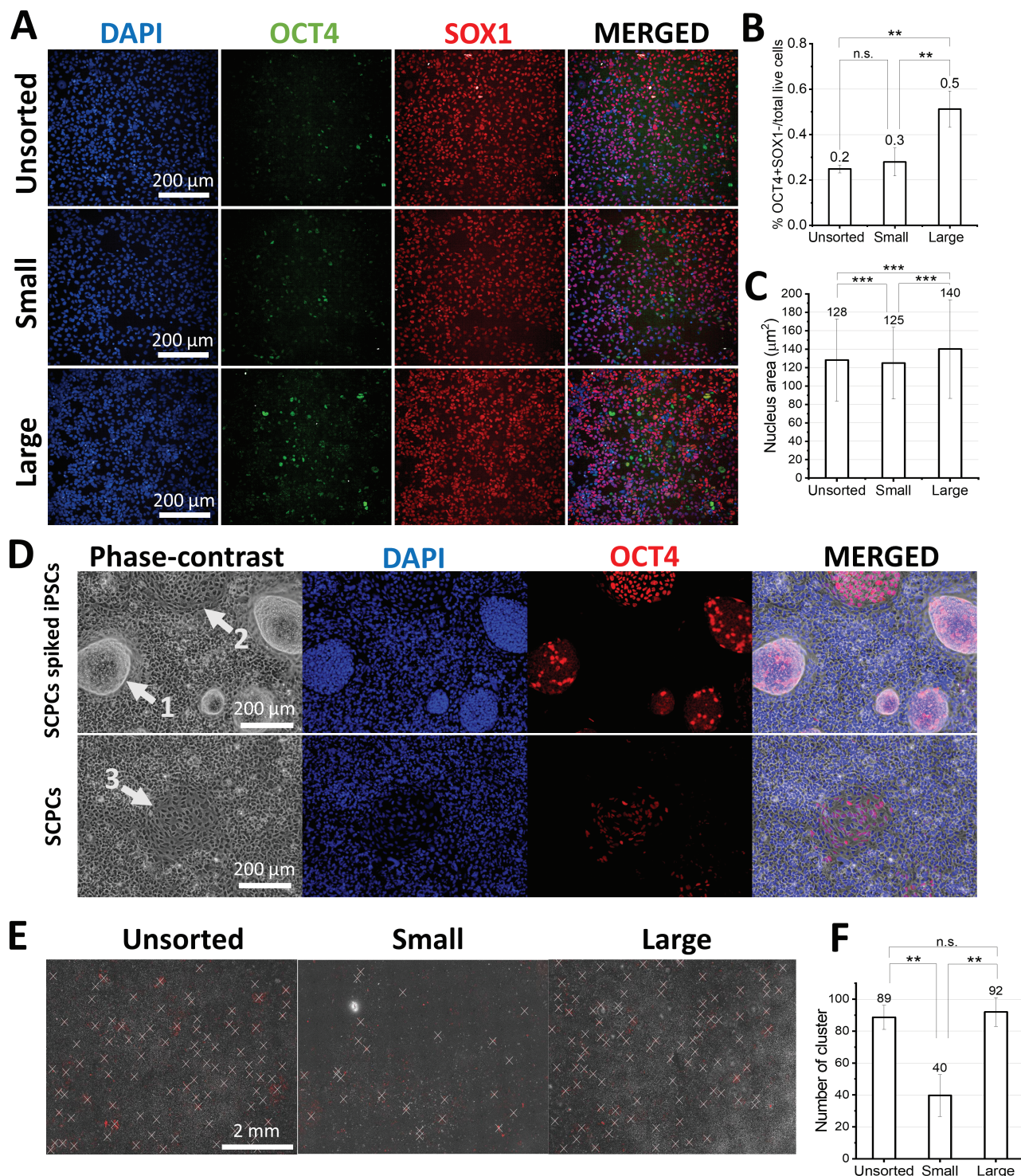


Figure 4. Immunostaining and colony culture assay of sorted CLEC-SCPCs. The SCPCs were separated into Small and Large groups by the recirculation strategy. **(A)** Immunofluorescence images of the Unsorted group and the sorted (Small and Large) groups with OCT4, SOX1, and DAPI, along with the merged images. **(B)** Percentage of OCT4⁺SOX1⁺ cells in the Unsorted, Small, and Large groups (one-way ANOVA followed by the Tukey post hoc test, $n = 3$). **(C)** Comparison of cell nucleus area of the Unsorted, Small, and Large groups (Kruskal-Wallis test was followed by Dunn post hoc test, $n = 5000$ cells/group). **(D)** Colony culture assay. SCPCs and SCPCs spiked with iPSCs were cultured in an iPSC growth medium on Matrigel coating for 6 days before immunostaining and imaging. The SCPCs spiked with iPSCs were used as a positive control group to observe colony morphology formed from spiked iPSCs. Colonies were observed in phase-contrast images of both SCPCs, and SCPCs spiked with iPSCs. Two types of colony morphology were observed: defined colony (with a sharp edge, compact cells pointed by number 1) and immature colony (with a defined edge, large nucleus pointed by number 2 for SCPCs spiked iPSCs group and number 3 for SCPCs groups). These colonies were verified by immunofluorescence images with OCT4 (red), DAPI (blue), and the merged image overlaid with phase contrast. **(E)** The map of identified colonies based on colocalization of phase contrast and OCT4 staining maps. The map consisted of 10×10 images, and identified colonies were marked with white "X." **(F)** The number of quantified colonies in the Unsorted, Small, and Large groups (one-way ANOVA followed by the Tukey post hoc test, $n = 3$).

method of identifying OCT4⁺ cells was developed to quantify the number of colonies formed among unsorted and sorted SCPCs (Supplementary Fig. S4). Quantified colonies in a map of 100 images (10 × 10 images) are shown in Fig. 4E. Figure 4F shows a summary of the counted colonies obtained from the map of different groups. The figure indicates that the Unsorted and Large groups had a trend of a higher number of colonies compared to the Small group. These results indicate that the Small group has a much lower chance of forming colonies than the Unsorted and Large groups.

Figure 5A shows the dot plot with gated 4-quadrant (SOX1 and OCT4) of unsorted and sorted cells from three batches of day 10 CLEC-SCPCs differentiated separately. To demonstrate that this strategy also works for other iPSC lines, we also used the same sorting approach to sort the day 10 SCPCs derived from BJ-iPSCs. As a starting point, both iPSC lines show a high level of pluripotency (>98% expressed OCT4, Supplementary Fig. S2B). The OCT4⁺ percentage of Unsorted, Small, and Large groups for the SCPCs are presented in Fig. 5B. The Large group exhibited a 2- to 5-fold increase in OCT4⁺ cells compared to the Unsorted group across different batches and cell lines. Similar to immunostaining results, some of the cells among OCT4⁺ SCPCs also express SOX1. For both OCT4⁺ and OCT4⁺SOX1⁻ quantifications, all Small groups showed a lower percentage than the Unsorted group. Figure 5D provides an estimate of the removal efficiency of OCT4⁺ and OCT4⁺SOX1⁻ cells by comparing their cell count in the Large group to the initial cell number. Across different batches and cell lines, the results show that more than 40% of OCT4⁺ cells and 30% of OCT4⁺SOX1⁻ cells can be removed, except for batch 1 of CLEC-SCPC, which had a high removal rate of OCT4⁺ cells but a low removal rate of OCT4⁺SOX1⁻ cells. On the other hand, sorting of BJ-SCPCs showed an impressive elimination of 98% of OCT4⁺SOX1⁻ cells.

Discussion

Due to its self-renewal and tumorigenic traits, residual iPSCs pose a high risk of forming tumors after transplantation. Much evidence in the past decade has demonstrated tumor formation from residual iPSCs in preclinical studies. Therefore, the removal of residual iPSCs is critical for safe cell therapy. While several markers such as Nanog, SSEA-4, Tra-1-60, and Tra-1-81 have been considered pluripotent markers, OCT4 remains one of the most popular markers used for assessing pluripotency and quantifying residual iPSCs in differentiated cells. This includes its application in clinical trials involving the transplantation of iPSC-derived NPCs/NSCs.³⁵ In our study, we also conducted a series of experiments involving immunostaining, FACS, and qPCR for the other pluripotency markers, including Nanog, Tra-1-60, and SSEA-4 (Supplementary Fig. S5). Our results reveal low expression levels (<1%) of these markers in the SCPCs, while OCT4⁺ cells were more frequently spotted in the differentiated cells.

Our study suggests that the large-sized cell group possesses more OCT4⁺ cells. These cell characteristics indicate their similarity to residual iPSCs and imply that residual undifferentiated cells tend to retain their biophysical properties, such as size, deformability, or shape, during differentiation. By exploiting the size difference between undifferentiated and differentiated cells, we developed a microfluidic size-based sorter for the removal of residual iPSCs in a label-free and high-throughput manner. Sorted cells were examined with immunofluorescence staining, a culture system for colony formation, and flow cytometry. Through both immunofluorescence staining and flow cytometry analysis, we found that a subset of OCT4⁺ cells also expressed the SOX1 marker, suggesting that these cells were in the transition stage of differentiation into SCPCs. The minority population that expresses OCT4 (a

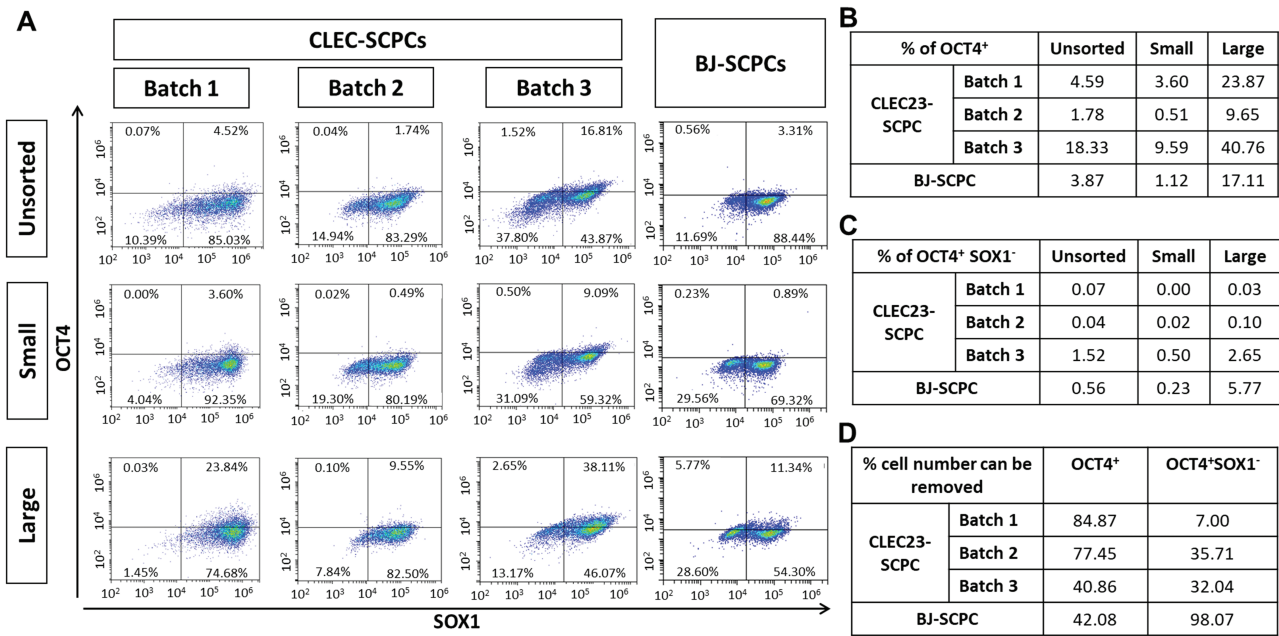


Figure 5. Flow cytometry assay shows that the percentage of OCT4⁺ cells is consistently lower in the sorted Small group regardless of cell culture batches and cell lines. (A) Flow cytometry analysis of the Unsorted group and the Small and Large groups from 3 batches of day 10 CLEC-SCPCs generated separately and day 10 BJ-SCPC. (B, C) The percentage of OCT4⁺ and OCT4⁺SOX1⁻ cells, respectively, of the Unsorted, Small, and Large groups. (D) The removal rate of OCT4⁺ and OCT4⁺SOX1⁻ cells by removing the Large group (method for calculating removal rate is described in Methods section).

pluripotent marker) but not SOX1 (a differentiation marker) would pose a higher risk of forming tumors. We observed a significantly higher percentage of OCT4⁺SOX1⁻ cells in the Large group than in the Small and Unsorted groups with no statistically significant differences between the Small and Unsorted groups. However, immunostaining analysis of OCT4⁺ cells can be only qualitative, not quantitative, due to non-uniform penetration, weak expression levels, susceptibility to background noise, and image-based quantification errors. On the other hand, flow cytometry is a more common method for the detection of residual undifferentiated cells due to high-speed analysis of a large number of cells (eg, 100 000 cells/second) and the capability of identifying even the minor populations (ie, OCT4⁺SOX1⁻ cell population).^{6,12,36} In this study, forward scatter intensity in the flow cytometer can also give us information about the cell size and its relation to other parameters. The OCT4⁺ cell population consists of cells expressing SOX1, forming the double-positive population (OCT4⁺SOX1⁺), while cells not expressing SOX1 constitute the OCT4⁺SOX1⁻ fraction. Double-positive cells are likely progressing toward progenitor stages, whereas SOX1-negative cells may remain in the pluripotent state. In batch 1 of CLEC-SCPCs, we observed a higher percentage of OCT4⁺ cells compared to BJ-SCPCs. The lower percentage of OCT4⁺SOX1⁻ cells in batch 1 of CLEC-SCPCs can be attributed to the higher proportion of double-positive cells (OCT4⁺SOX1⁺), resulting in an overall higher count of OCT4⁺ cells.

Other than cell size, the nucleus area of the cells was significantly decreased during the differentiation process. By analyzing the nucleus morphology, we found that iPSCs appeared to have a larger nucleus area than SCPCs on days 4 and 10 (Fig. 1E). This result is consistent with Fig. 4C, where Large group cells had a larger nucleus area than Small group cells. The nucleus area could be a good-quality attribute for differentiating residual iPSCs in SCPCs or other progenitor cells. This and all the other analyses collectively corroborate our central hypothesis that size-based sorting of SCPCs can eliminate undifferentiated cells, thereby reducing risk.

It has previously been suggested that the slow-dividing neural progenitor cells in culture might be better for clinical applications.⁴⁴ Meanwhile, hyperproliferative cells (ie, possibly tumorigenic cells) may be caused by the genetic and/or epigenetic transformation of neural progenitor cells, not by the contamination of undifferentiated iPSCs.^{45,46} We currently do not have a detailed understanding of the molecular pathways or mechanisms behind the cell size differences that were seen during the phenotype changes, which we observed in iPSCs and their differentiated progenitors. Regardless of the source of the fast-growing residual cells, the high proliferation of the Large group could be associated with higher tumorigenic potential and, therefore, should be removed to reduce such a risk. At the same time, as suggested by some earlier studies, subpopulations with different sizes could represent cells with different phenotypes in therapeutic cells, which we previously addressed with the same cell sorting technology that we demonstrated in this work.^{24,25}

The primary focus of discussing stem cell quality control should revolve around its suitability for manufacturing the end products, particularly those derived from iPSCs.⁴⁷ Our MDDS sorter will contribute to the high-throughput removal of residual iPSC contaminations. Previously, we demonstrated a sorting throughput of 1 L/minute corresponding to ~500 million cells/minute by using a multiplexed plastic spiral

unit containing 100 MDDS sorters.⁴⁸ Therefore, scale-up to manufacturing scale cell production processes is feasible and straightforward.

In order to demonstrate general applicability, we tested our idea on 2 different iPSC cell lines and multiple batches. Based on the calculation of OCT4⁺SOX1⁻ percentage from flow cytometry assay and sorted cell numbers, we can eliminate approximately 7%-35.71% of OCT4⁺SOX1⁻ cells from day 10 CLEC-SCPC population and approximately 98.07% OCT4⁺SOX1⁻ cells from day 10 BJ-SCPC population by removing the Large group (Fig. 5D). While the method works for different iPSC lines, the differences in size distribution and differentiation efficiency also validated that the differentiation might not be fully efficient and can be cell origin dependent.⁴⁹⁻⁵¹ We observe significant variations in the relative amount of undifferentiated residual cells, not only for cells from different cell lines but also in different batches from the same cell lines.^{52,53} The sources of such variation are not understood currently and could result from cell source, donor, culture conditions, quality of raw materials used in culture, and operator, to name a few. While it may be feasible to minimize or eliminate such variation by controlling cell culture and production conditions accurately,^{54,55} the availability of an easy-to-use “downstream” purification for cell products would still be highly valuable and desirable in large-scale cell manufacturing. On the other hand, we are also confident that the method holds significant potential for translation to other spinal cord-type NPCs generated from hiPSCs using similar methodologies. Nonetheless, the exploration of other types of NPCs remains a future direction that warrants further investigation.

It is worth noting that the primary objective of this technology is not to ensure absolute cell safety but rather to systematically reduce associated risks, thereby enhancing safety outcomes compared to earlier methodologies. A complete baseline separation between SCPCs and residual cells is not likely to be possible because of the overlap in size between fully differentiated SCPCs and undifferentiated residual cells. Further optimization of the sorting devices, combined with multiple rounds of removal separation, could lead to a satisfactory and acceptable level of residual cell removal, which would still be quantifiable in terms of enhancing the safety of the cells. At the same time, the cost of the device (injection-molded, mass-produced plastic chips) is expected to be minimal, and there are no reagents needed for the removal operation. Therefore, our device would provide a highly economic yet tangible benefit to any iPSC-derived cell products for regenerative medicine. In contrast, conventional fluorescence-activated cell sorting (FACS) would be limited not only in terms of its processing rates and throughput (if based on size sorting by scattering) but also in terms of its prohibitively high cost, if the sorting is based on specific cell-surface marker labels.

In conclusion, we used the size differences between the undifferentiated and differentiated cells to enrich the SCPCs population while reducing the percentage of iPSCs. The technology is label free, is non-contact, and has high throughput without affecting cell viability and functions. While we demonstrate our technique using a specific cell manufacturing scenario (transplantable SCPCs derived from iPSC cell line), we argue that the methodologies demonstrated here will find broad use cases in many clinical indications where cell purity and quality become an important issue. Further development of this technology will be promising for the cell manufacturing workflow, contributing to better quality and

safety of the transplanted cells by reducing the number of potentially tumor-forming cells.

Acknowledgments

We would like to express our sincere gratitude to professor Lim Kah Leong and Dr Chai Chou, SingHealth, Singapore and associate professor Phan Toan Thang, CellResearch Corporation, Singapore for providing us with their proprietary CLiPS cells. Graphical abstract was created using “BioRender.com.”

Funding

This research was supported by the National Research Foundation, Prime Minister's Office, Singapore under its Campus for Research Excellence and Technological Enterprise (CREATE) program (IntraCREATE grant award number: NRF2019-THE002-0001) and Singapore MIT Alliance for Research and Technology (SMART): Critical Analytics for Manufacturing Personalised-Medicine (CAMP) Inter-Disciplinary Research Group.

Conflict of Interest

J.H. declared that he received an honorarium from TGS management for giving a seminar on an unrelated topic (desalination). All the other authors declared no potential conflicts of interests.

Author Contributions

T.D.N.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript. W.H.C.: collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript. H.J.: conception and design, manuscript writing, final approval of manuscript. J.C., D.N.R., J.T.Z.Y., C.Y.P.L.: collection and/or assembly of data, final approval of manuscript. S.Y.N.: provision of study material or patients, manuscript writing, final approval of manuscript. S.Y.C.: financial support, administrative support, provision of study material or patients, data analysis and interpretation, manuscript writing, final approval of manuscript. J.H.: conception and design, financial support, administrative support, data analysis and interpretation, manuscript writing, final approval of manuscript.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Supplementary Material

Supplementary material is available at *Stem Cells Translational Medicine* online.

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