Scalable stirred suspension culture for the generation of billions of human induced pluripotent stem cells using single-use bioreactors

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Abstract
The production of human induced pluripotent stem cells (hiPSCs) in quantities that are relevant for cell-based therapies and cell-loaded implants through standard adherent culture is hardly achievable and lacks process scalability. A promising approach to overcoming these hurdles is the use of hiPSCs in suspension. In this study, stirred suspension culture vessels were investigated for their suitability in the expansion of two hiPSC lines inoculated as a single cell suspension, with a free scalability between volumes of 50 and 2400 ml. The simple and robust two-step process reported here first generates hiPSC aggregates of 324 ± 71 μm diameter in 7 days in 125 ml spinner flasks (100 ml volume). These are subsequently dissociated into a single cell suspension for inoculation in 3000 ml bioreactors (1000 ml volume), finally yielding hiPSC aggregates of 198 ± 58 μm after 7 additional days. In both spinner flasks and bioreactors, hiPSCs can be cultured as aggregates for more than 40 days in suspension, maintain an undifferentiated state as confirmed by the expression of pluripotency markers TRA-1-60, TRA-1-81, SSEA-4, OCT4, and SOX2, can differentiate into cells of all three germ layers, and can be directed to differentiate into specific lineages such as cardiomyocytes. Up to a 16-fold increase in hiPSC quantity at the 100 ml volume was achieved, corresponding to a fold increase per day of 2.28; at the 1000 ml scale, an additional 10-fold increase was achieved. Taken together, 16 × 10⁶ hiPSCs were expanded into 2 × 10⁹ hiPSCs in 14 days for a fold increase per day of 8.93. This quantity of hiPSCs readily meets the requirements of cell-based therapies and brings their clinical potential closer to fruition.

KEYWORDS
bioprocessing, human pluripotent stem cells, process optimization, single-use bioreactors, stirred suspension culture, scalable culture system

1 INTRODUCTION

Cell-based therapies have the potential to contribute to disease treatment (Sun, Longaker, & Wu, 2010), whereby living cells can be introduced as therapeutic agents for regenerative medicine (Wu & Hochedlinger, 2011), augmented by tissue engineering technologies (Takebe et al., 2013). For biomedical applications (Das & Pal, 2010) and therapeutic purposes, human pluripotent stem cells represent promising cell sources due to their pluripotency, self-renewing properties, and high proliferation capacities.
Current technologies for pluripotent stem cell expansion hardly fulfill the demands of regulatory boards (Kurtz et al., 2014) and the needs of clinical use (Kamao et al., 2014), as they are associated with low cost-efficiency (Jenkins & Farid, 2015), lack stringent quality control, or do not meet good manufacturing practices (GMP) standards (Elseberg, Salzig, & Czermak, 2015). Therefore, the development of a standardized, scalable method for pluripotent stem cell expansion that is cost-effective and compatible with automation is of great interest. Particularly for those stem cell groups that are used to working at the laboratory scale, the robust and cost-efficient adaptation to the billion-cell scale represents a major challenge and is therefore a significant obstacle to clinical translation.

The landmark study showing reprogramming of adult human fibroblasts to a pluripotent state (Takahashi et al., 2007) is a major contribution to the use of pluripotent stem cells in regenerative medicine. Human induced pluripotent stem cells (hiPSCs) are especially optimal candidates as they can be derived from individual patients to circumvent histocompatibility concerns for cell-based therapies and implants, and can be used for a variety of applications such as personalized drug testing. It is already possible to obtain clinical- and GMP-grade autologous hiPSCs, but this remains a time-consuming and costly procedure. Various banks of allogeneic clinical GMP-grade hiPSCs are being prepared globally (Turner et al., 2013), and disease-specific banking efforts, such as for Parkinson's disease (Holmqvist et al., 2016), are underway.

Ongoing clinical trials with cells derived from pluripotent stem cells for the treatment of spinal cord injury, macular degeneration of the retina, type 1 diabetes and heart failure mostly use human embryonic stem cells (hESC) as the cell source. The first trial thus far using hiPSCs as the cell source was initiated in Japan in 2013 (Ilic, Devito, Miere, & Codognotto, 2015).

For spinal cord injury treatment, the number of cells required ranges from $2 \times 10^4$ to $20 \times 10^4$ per treatment; other general estimates of the cell numbers required for cell-based therapies are $5 \times 10^5$ to $1 \times 10^6$ (Chen, Reuveny, & Oh, 2013; Hartman, Dai, & Laflamme, 2016; Kehoe, Jing, Lock, & Tzanakakis, 2010). This range of cell numbers is also relevant to other applications of hiPSCs such as disease modeling (Mekhoubad et al., 2012; Soldner & Jaenisch, 2012), high-throughput drug screening, or fabrication of bioartificial organs. Currently, recruitment of patients for cell therapy of diabetes with allogeneic hESC-derived cells is in progress (ID: NCT02239354, clinicaltrials.gov), where about 1 billion cells will be needed for a single application. Therefore, to support the aforementioned cell banking efforts of hiPSCs, tissue engineering and cell-based therapies, as well as potential applications in high-throughput drug screening, scalable methods to generate billions of hiPSCs have been in development.

The stirred suspension culture technique is one promising method for the large-scale bioprocessing of pluripotent stem cells and maintaining their pluripotency (Amit et al., 2011; Badenes et al., 2015; Chen et al., 2015; Elanzev, Sommer, Pusch-Klein, Brüstle, & Haupt, 2015; Zweigerdt, Olmer, Singh, Haerverich, & Martin, 2011). The aim of this study was to demonstrate the scalability of the technique in a single-use stirred bioreactor (Kaiser, Eibl, & Eibl, 2011), and to develop a protocol for the expansion of billions of hiPSCs using stirred suspension culture vessels, while maintaining the undifferentiated, self-renewing state and pluripotency of the hiPSCs.

2 MATERIALS AND METHODS

2.1 Human induced pluripotent stem cells

The experiments were performed using AR1034ZIMA hiPSC clone 1 (hereafter AfIPS, adult fibroblast derived IPS) and FS hiPSC clone 2 (hereafter FsIPS, juvenile foreskin fibroblast derived IPS). AfIPS cells were generated from adult dermal fibroblasts from a 24-year-old male as previously described (Kadari et al., 2014), after obtaining informed consent and ethical clearance by the ethics committee of the University of Würzburg, Germany (ethical report number 96/11, dated 10 June 2011). FsIPS cells were generated by lentiviral reprogramming as described in section 2.2, and were cultivated to select for a line amenable to suspension culture. hiPSCs were cultured at 37°C, 5% CO₂ and maintained in either mTeSR™1 medium (mTeSR; STEMCELL Technologies, Vancouver, Canada) or StemMACS™ iP5-Brew XF medium (StemMACS; Miltenyi Biotec, Bergisch Gladbach, Germany) on six-well cell culture plates (Greiner Bio-One, Kremsmünster, Austria) coated with hESCs-qualified Matrigel® (Corning, New York, NY, USA) according to the manufacturer’s instructions. The media was changed daily. At ~80% confluence, cells were dissociated with StemPro® Accutase® (Thermo Fisher Scientific, Waltham, MA, USA) for 5 min at 37°C and triturated to obtain a single cell suspension. hiPSCs were plated at a cell density of 20,000 cells/cm² in mTeSR or StemMACS medium, both supplemented with 10 μM rho-associated protein kinase inhibitor Y27632 (Miltenyi Biotec) for the first 24 h after passing.

2.2 Reprogramming

FsIPS cells were generated from normal human dermal fibroblasts (NHDF; Promocell, Heidelberg, Germany) by reprogramming using hSTEMCCA-lentiviral construct as described by Somers and colleagues with minor changes (Somers et al., 2010). Briefly, 1 $\times 10^5$ cells were plated on a well of a six-well plate pre-coated with 0.1% gelatin (Sigma–Aldrich, St Louis, MO, USA) in fibroblast medium consisting of Dulbecco’s modified Eagle’s medium (DMEM; Gibco®, Waltham, MA, USA), 10% fetal calf serum (FCS; Biochrom, Berlin, Germany), 1% non-essential amino acids (NEAA; Gibco), and 100 μM β-mercaptoethanol (Gibco). One day after plating, freshly produced, unconcentrated hSTEMCCA-lentivirus was added to the cells in fresh medium in the presence of 5 μg/ml polybrein (Sigma–Aldrich). Medium was changed one day later and from then on daily to reprogramming medium including KnockOut™ DMEM/F12 (Gibco), 20% KnockOut™ Serum Replacement (Gibco), 1 mM L-glutamine (Gibco), 1% NEAA, 100 μM β-mercaptoethanol, 10 ng/ml human fibroblast growth factor 2 (FGF2; Gibco) and 200 μM ascorbic acid (Sigma–Aldrich). On day 6, cells were dissociated with Accutase and replated on a 100-mm tissue culture dish with gamma-irradiated mouse embryonic fibroblasts (Gibco) as a feeder layer on gelatine in reprogramming medium supplemented with 10 μM Y27632. Medium change was continued daily. Putative hiPSC colonies started appearing on day 12 after transduction and were isolated manually by colony picking on day 28. From here on,
putative hiPSC colonies were expanded under feeder-free conditions using mTeSR and hESC-qualified Matrigel.

### 2.3 Stirred suspension culture of hiPSC

hiPSC standard adherent cultures were harvested from cultureware by treatment with Accutase for 5 min at 37°C. Dissociated cells were mixed with DMEM/F12 (Gibco), transferred to a 15-ml centrifuge tube (Sarstedt, Nümbrecht, Germany), and centrifuged at 300×g for 5 min at 4°C. The cell pellet was resuspended in an appropriate volume of respective media by gentle pipetting and the cell suspension was strained through a 40-μm cell strainer (VWR, Radnor, PA, USA) to generate a single cell suspension. Cells were counted with a hemocytometer and inoculated at an optimized cell density of 2 × 10^5 cells/ml into a 125 ml spinner flask (henceforth referred to as spinner; Corning) to a final volume of 80 ml (optimized protocol) or 100 ml (regular protocol). Cells were grown in mTeSR or StemMACS medium supplemented with 10 μM Y27632 for the first two days without refreshing the medium. The spinners were incubated in a 37°C, 5% CO2 incubator and the side-arm caps of the spinners were loosened to permit gas exchange. They were stirred at 85 rotations/min (RPM) by the bioMIXdrive 2 (2mag, Munich, Germany). With the regular protocol, medium exchange was performed daily starting on day 2 postinoculation until harvest at 7 days postinoculation. Spinners were allowed to stand for 5–8 min to sediment the hiPSC aggregates by gravity. Then the supernatant was aspirated and fresh prewarmed medium was fed into the spinners up to a total volume of 100 ml. Next, the spinners were transferred back onto the bioMIXdrive 2 in the incubator. With the optimized protocol, 20 ml of fresh prewarmed medium was added at 2 and 3 days postinoculation without medium aspiration. Medium exchange was then performed daily up to a volume of 100 ml, starting from 4 days postinoculation until harvest at 7 days postinoculation. hiPSC aggregates were harvested by transferring to 50 ml centrifuge tubes (Sarstedt) and centrifuged at 200×g for 3 min at 4°C. After carefully aspirating the supernatant, the cell pellet in each tube was dissociated by resuspension in 10 ml Accutase and horizontal incubation at 37°C for 5–10 min with gentle agitation. DMEM/F12 (20 ml) was added to each tube and triturated to obtain a cell suspension. The cells were pelleted by centrifuging at 300×g for 5 min at 4°C, resuspended by pipetting in 10 ml of mTeSR or StemMACS, and strained through a 40-μm cell strainer to obtain a single cell suspension. This single cell suspension was used for flow cytometry, as an inoculum for standard adherent culture, or for further suspension culture in 125 ml spinners.

#### 2.4 Flow cytometry

Between 0.5 × 10^6 and 1 × 10^6 cells were used for each sample. Dead cells were excluded from the analysis by BD Horizon Fixable Viability Stain 450 (BD Biosciences, East Rutherford, NJ, USA). Samples were suspended in staining buffer composed of 5% FCS in Dulbecco’s phosphate-buffered saline (DPBS; Sigma–Aldrich) for 10 min on ice and then pelleted by centrifugation (300×g, 5 min, 4°C). For staining of intracellular targets, cells were fixed in 4% paraformaldehyde for 15 min, then permeabilized with 0.2% Triton X-100 in DPBS for 15 min at room temperature (RT) before continuing with staining. After 15 min incubation at 4°C with appropriate antibodies (Supplementary Table 1) diluted in staining buffer, the cells were centrifuged, washed, and resuspended in DPBS for analysis. Isotype and unstained controls were included to control for nonspecific binding and background fluorescence. The BD FACSDuo™ II (BD Biosciences) flow cytometer was used for data acquisition. For each sample, 50,000 events were captured. Data were analyzed and graphed using FlowJo 10.0.7 software (Tree Star Inc, Ashland, OR, USA).

#### 2.5 Magnetic-activated cell sorting

Magnetic-activated cell sorting (MACS) was only used during initial optimization of the protocol to demonstrate the feasibility of recovering hiPSCs from a cell population that contains a substantial fraction of spontaneously differentiated cells (i.e. as judged by flow cytometry, <80% TRA-1-60-positive cells). The optimized workflow reported here resulted in a hiPSC population with little to no spontaneous differentiation, thus obviating the routine need for routine MACS to sort out hiPSCs from suspension cultures. Cell suspensions comprised a heterogeneous mixture of hiPSCs and differentiated cells in culture medium were supplemented with 10 μM Y27632 and passed through a 30-μm preseparation filter (Miltenyi Biotec) to remove cell clumps. Cells were labeled with anti-TRA-1-60 MicroBeads (Miltenyi Biotec) or anti-SSEA-4 MicroBeads (Miltenyi Biotec) according to the manufacturer’s instructions. Positive labeled cells were sorted and recovered using the MidiMACS Separator (Miltenyi Biotec).

#### 2.6 Germ layer differentiation

hiPSC aggregates were transferred to nontissue culture Petri dishes (Greiner Bio-One) and cultured in static suspension for 7 days with differentiation medium composed of DME medium containing 4 mM L-glutamine, 10% FCS, 1% NEAA, and 100 μM β-mercaptoethanol. Differentiation medium was exchanged every 3 days. Five to seven aggregates were then transferred to 6-well plates precoated with 0.1% gelatine, and further cultured in standard adherent culture in differentiation medium. The medium was changed every 3 days for 3 weeks. Differentiated cells were analyzed for germ layer markers neuron specific class III β-tubulin (TUJ1; ectoderm), smooth muscle actin (SMA; mesoderm), and α-fetoprotein (AFP; endoderm) by immunocytochemistry.

#### 2.7 Immunocytochemistry analysis

Cells were fixed with 4% paraformaldehyde (AppliChem, Darmstadt, Germany) in DPBS for 15 min at RT then blocked with staining buffer
for 1 h at RT, in the presence of 0.1% Triton X-100 (Sigma-Aldrich) for intracellular markers, or without Triton-X for surface markers. Thereafter, cells were incubated with primary antibodies (Supplementary Table 1) in staining buffer overnight at 4°C. The samples were rinsed three times with DPBS, then incubated for 2 h at RT with appropriate fluorophore-conjugated secondary antibodies diluted in staining buffer. Stained cells were imaged with the BioRevo fluorescence microscope (Keyence, Osaka, Japan) or Zeiss Axioskop 2 mot plus microscope (Carl Zeiss, Oberkochen, Germany).

2.8 Metabolite analysis
Medium samples were collected from culture vessels immediately prior to medium addition or exchange. Samples were centrifuged at 900×g for 5 min at RT to pellet cells and debris, and the supernatant glucose and lactate concentrations were analyzed with the Stat Profile® PRIME™ CCS Analyzer (Nova Biomedical, Waltham, MA, USA).

2.9 Quantification of cell aggregates sizes
Aliquots of aggregates from the culture vessels were placed into 12-well plates and spread evenly for clear distinction of aggregate boundaries. Aggregates were imaged with a DM IL LED inverted microscope (Leica, Wetzlar, Germany) using Leica Application Suite X software. Images were processed in Fiji v1.49 m (Schindelin et al., 2012) to measure cell aggregate diameters. A minimum of 100 aggregates were measured at each data point. Statistical analysis and plotting were performed in GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA).

2.10 Karyotype analysis
hiPSCs were plated and cultured in T25 flasks pre-coated with hESC-qualified Matrigel. At ~70% confluency, hiPSCs were incubated at 37°C with 100 ng/ml KaryoMAX Colcemid (Gibco) for 3 h. After dissociation with Accutase, cells were pelleted by centrifugation at 300×g for 8 min at 4°C, followed by resuspension in 1 ml of prewarmed 75 mM KCl solution. An additional 14 ml of 75 mM KCl was added, and the tube was gently inverted to mix, before incubation for 16 min at 37°C. Cells were pelleted by centrifugation and resuspended in an ice-cold, freshly-prepared mixture of 1:3 acetic acid and methanol for fixation at ~20°C overnight. These were spread onto cleaned glass slides by dropping, briefly air-dried, aged and stained with Giemsa (Sigma-Aldrich). A minimum of 10 metaphases were analyzed.

2.11 Reverse transcription and quantitative polymerase chain reaction
Total ribonucleic acid (RNA) was extracted from pelleted cell samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) per manufacturer instructions. RNA samples were treated with DNase I (Invitrogen) to remove contaminating genomic deoxyribonucleic acid. 1 μg of RNA was reverse transcribed into complementary deoxyribonucleic acid using GoScript™ Reverse Transcriptase (Promega, Madison, WI, USA). Quantitative polymerase chain reaction (PCR) was carried out using Luna® Universal qPCR Master Mix (New England Biolabs, Ipswich, MA, USA) on a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific) using gene-specific primers (Supplementary Table 2). In particular, primers for OCT4 were designed to amplify only endogenous OCT4 transcripts and not transgenic OCT4 originating from the hSTEMCCA lentivirus. Quantification was carried out with the ΔΔCt method.

2.12 Directed differentiation into cardiomyocyte-like cells
Aggregates harvested from bioreactors were dissociated into a single cell suspension using Accutase, and cells were plated onto hES-qualified Matrigel-coated plates in mTeSR supplemented with 10 μM Y27632. At 80–90% confluency, cardiac differentiation was initiated as described by Kadari et al. (2015). For the 1st day, cells were cultured with 25 ng/ml BMP4 (Gibco) and 5 μM CHIR99021 (Axon Medchem, Groningen, Netherlands) added to a cardiac basal medium comprising RPMI1640 (Gibco), 1× B27 (Gibco), 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol (Gibco), and 50 μg/ml ascorbic acid (Sigma-Aldrich). For the 2nd day, the medium was changed to cardiac basal medium supplemented with 5 μM CHIR99021. For the 3rd day, the medium was changed to cardiac specification medium comprising RPMI1640, 1× B27 without insulin (Gibco), 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, and 50 μg/ml ascorbic acid. From the 4th day, medium was switched to cardiac specification medium supplemented with 10 μM IWR1 (Sigma-Aldrich) and refreshed daily for the next 5 days or until beating cells could be observed. For the following 4 days, cells were cultivated with cardiac basal medium. To enrich the culture for cardiomyocyte-like cells, for the next five days the cells were cultivated in cardiac enrichment medium comprising RPMI1640 without glucose (Gibco) and 4 mM sodium lactate (Sigma-Aldrich) (Tohyama et al., 2013). After enrichment, cells were maintained in cardiac basal medium with medium change every other day. Cells were dissociated with Accutase and plated onto Matrigel-coated glass coverslips for further analysis.

3 RESULTS AND DISCUSSION

3.1 Starter culture of hiPSCs as aggregates in stirred suspension culture
First, a regular protocol was developed to transition hiPSCs from standard adherent culture to stirred suspension culture (Figure 1, Regular protocol). Morphological analysis was accomplished by phase contrast microscopy of standard adherent hiPSCs and suspension hiPSCs aggregates. In monolayer, typical hiPSC morphology was observed, exhibiting sharp colony edges and high nucleus to cytoplasm ratio. At 70–80% confluency, AFiPS cells were dissociated with Accutase, triturated into a cell suspension, and inoculated into a 125 ml spinner with Y27632-supplemented medium. Medium was changed daily after day 2. For that, aggregates were allowed to sediment by gravity, and the supernatant was aspirated before adding fresh medium. After 7 days of stirred suspension culture in spinners at 65 RPM, aggregates of irregular size were obtained (Figure 2A upper panel), which were associated with a substantial loss of pluripotency-associated marker (TRA-1-60; Figure 2A, lower panel). With the regular medium
exchange protocol for hiPSC in suspension, aggregates at day 2 (104 ± 25 μm diameter) required >10 min to sediment by gravity to perform a medium exchange. This duration of sedimentation led to the agglomeration of aggregates, resulting in irregularly sized and shaped masses. The survival and maintenance of pluripotency of hiPSC culture relies on the access of hiPSCs to oxygen and medium components, an important factor especially in stirred suspension culture. In aggregates >400 μm in diameter, oxygen distribution to cells closer to the core of the aggregate may be impaired (Wu, Rostami, Cadavid Olaya, & Tzanakakis, 2014), and access to medium components for the maintenance of pluripotency may be similarly affected.

Even with the use of well-established matrices and protocols, unwanted differentiation of pluripotent cells can occur. In our initial attempts to culture AFiPS cells in suspension, irregularly shaped and sized agglomerates appeared to be associated with significant spontaneous differentiation, which it was reasoned could be due to inaccessibility of the cells to culture medium components responsible for maintenance of pluripotency (e.g. FGF2). Thus, this study set out to explore whether MACS can be employed to isolate TRA-1-60-positive or SSEA-4-positive cells from the heterogeneously differentiated population of cells (Figure S1). This presents a capable method for recovering pluripotent cells from a partially differentiated population in the case of unexpected differentiation. Such sorting steps ultimately proved to be unnecessary after further refinement of the protocol to avoid agglomeration. However, it provides proof of principle that cell sorting can be used to rescue a hiPSC population exhibiting signs of spontaneous differentiation. To avoid agglomeration, a shorter sedimentation duration was used. However, this meant that some aggregates remained suspended in the medium and were aspirated during medium removal, leading to lower process yields. Nevertheless, with the regular suspension protocol with refined physical parameters, spherical aggregates could be generated containing a very high proportion of cells exhibiting pluripotency-associated markers, but process yields were slightly lower compared to standard adherent methods (Figure 2B).

Refinement of the physical parameters of inoculation density, impeller rotation rate, and sedimentation duration of aggregates during
the medium exchange are critical determinants to obtain homogeneous spherical aggregates with the preservation pluripotency-associated marker expression. hiPSC inoculation density and impeller rotation rate were first determined for the 125 ml spinners. Inoculation density ranged from $2 \times 10^5$ to $5 \times 10^5$ hiPSCs/ml. Employing $5 \times 10^5$ hiPSCs/ml, a high propensity of formation of irregularly shaped aggregates was observed that was less apparent with $2 \times 10^5$ cells/ml (Figure S2). In terms of stirring speed, the range was varied from 65 to 85 RPM. At 65 RPM, it was possible to generate aggregates, but these aggregates tended to form larger agglomerates (Figure 2A, upper panel). Cells dissociated from spinner with large agglomerates exhibited loss of pluripotency marker TRA-1-60 expression (lower panel), suggesting undirected differentiation of cells, highlighting the importance of optimization of vessel process parameters. (B) medium processing using the regular protocol involved the significant loss of aggregates during medium exchange from day 2 onwards, resulting in slightly lower yields than standard adherent culture. Expansion of hiPSCs in stirred suspension as aggregates using an optimized medium feeding strategy achieves higher process yield compared to standard adherent or regular (nonoptimized) medium processing when normalized to medium consumption ($n = 5$ per condition). (C) hiPSC suspensions after Accutase treatment of hiPSC aggregates can contain small cell clumps (arrowhead), which may result in a large variation in aggregate diameter. Passing the hiPSC suspensions through a cell strainer to remove cell clumps results in reduced variation in aggregate diameter (day 2, after straining). (D) two hiPSC lines can be expanded in consecutive spinner passages for at least 7 passages (49 days), while retaining high viability of $>90\%$, with $>93\%$ of viable cells double positive for pluripotency markers TRA-1-60 and SSEA-4. Cumulative cell number is calculated based on fold-increase for each passage. (E) Representative images of hiPSCs in standard adherent culture before inoculation into spinners, in spinners (day 7), and in the bioreactor (day 7) using the optimized suspension protocol. Scale bars = 200 μm.

**FIGURE 2** Process optimization of stirred suspension culture protocol for expansion of hiPSCs as aggregates. (A) Representative image of large agglomerated aggregates associated with low impeller rotation rates or excessive sedimentation duration in spinners (upper panel). Cells dissociated from spinner with large agglomerates exhibited loss of pluripotency marker TRA-1-60 expression (lower panel), suggesting undirected differentiation of cells, highlighting the importance of optimization of vessel process parameters. (B) medium processing using the regular protocol involved the significant loss of aggregates during medium exchange from day 2 onwards, resulting in slightly lower yields than standard adherent culture. Expansion of hiPSCs in stirred suspension as aggregates using an optimized medium feeding strategy achieves higher process yield compared to standard adherent or regular (nonoptimized) medium processing when normalized to medium consumption ($n = 5$ per condition). (C) hiPSC suspensions after Accutase treatment of hiPSC aggregates can contain small cell clumps (arrowhead), which may result in a large variation in aggregate diameter. Passing the hiPSC suspensions through a cell strainer to remove cell clumps results in reduced variation in aggregate diameter (day 2, after straining). (D) two hiPSC lines can be expanded in consecutive spinner passages for at least 7 passages (49 days), while retaining high viability of $>90\%$, with $>93\%$ of viable cells double positive for pluripotency markers TRA-1-60 and SSEA-4. Cumulative cell number is calculated based on fold-increase for each passage. (E) Representative images of hiPSCs in standard adherent culture before inoculation into spinners, in spinners (day 7), and in the bioreactor (day 7) using the optimized suspension protocol. Scale bars = 200 μm.
3.2 Optimization to increase process yield of hiPSCs

Since sedimentation duration was reduced to prevent agglomeration in the regular suspension protocol, a resulting problem was the loss of small aggregates during the first medium exchange. This meant that the process yield using the regular suspension protocol was less efficient compared to standard adherent culture when normalized to total medium consumed (Figure 2B). Standard adherent culture yielded 636 ± 24 × 10^3 hiPSCs/ml of medium, while the regular suspension protocol yielded 516 ± 46 × 10^3 hiPSCs/ml of medium (n = 5 per condition). While suspension cultures are known to result in higher volumetric yields than standard adherent cultures (Chen, Mallon, McKay, & Robey, 2014), the lower values here could be explained by the loss of aggregates during medium exchange on day 2 of the regular suspension protocol. However, these small aggregates are prone to agglomeration at this stage and their recovery while preventing their agglomeration is challenging. To circumvent the loss of these small aggregates, it was hypothesized that leaving aggregates to grow for a longer period before the first medium exchange would result in larger aggregates, requiring less time for sedimentation and therefore avoiding both the problems of agglomeration and loss of small aggregates. Elanzew et al. (2015) employed a medium supplementation strategy without medium removal and harvested hiPSCs after 4 days of culture, and such a strategy was adapted for the current protocol.

It was established that, in the absence of medium supplementation, hiPSC culture in spinners could only proceed up to day 4 postinoculation, with a loss of viable cells thereafter (Figure S3). Taking these observations together, an optimized suspension cultivation protocol was developed, with medium addition at day 2 and day 3 postinoculation, to supply nutrients and growth factors such as FGF2 to maintain an undifferentiated state and support aggregate growth. This was then followed by daily medium exchange from day 4 onward (Figure 1, Optimized protocol). To demonstrate the robustness of the optimized protocol, it was applied to two different cell lines, AFiPS and FSiPS.

With the optimized suspension protocol, FSiPS aggregates of diameter 179 ± 33 μm and AFiPS aggregates of diameter 185 ± 35 μm (Figure 3A) were generated at day 4. These aggregates required a sedimentation duration of <8 min and the previously observed agglomeration of aggregates was greatly reduced within this period. This avoided the problem of agglomeration, and since small aggregates were not aspirated on day 2, they developed into larger aggregates, resulting in an improved process yield compared to the regular suspension protocol. At 7 days postinoculation, 204 ± 17 × 10^6 FSiPS cells and 252 ± 8 × 10^6 AFiPS cells were harvested. With the AFiPS cells, the optimized suspension protocol yielded 894 ± 90 × 10^3 hiPSCs/ml of medium. This represents a 40.6% increase in process yield compared to standard adherent culture (Figure 2B, p < 0.05), and a 73.3% increase compared to the regular suspension protocol (p < 0.01; Figure 2B).

In initial attempts to dissociate aggregates into a cell suspension for use as inoculum for further expansion in stirred suspension culture in spinners or bioreactors, unusually large aggregates were observed in the resultant cultures (Figure 2C, upper right). It was hypothesized that these large aggregates originated from nondissociated cell clusters in

**FIGURE 3** hiPSC aggregate diameter measurements and growth curves in stirred suspension culture. (A) aggregates from an inoculated single cell suspension (2 × 10^5 hiPSCs/ml) in spinners. FSiPS aggregates measuring 95 ± 17 μm diameter at 2 days grew to 343 ± 76 μm diameter at 7 days, corresponding to a total cell number of 21 ± 3 × 10^6 cells at 2 days and 204 ± 17 × 10^6 cells at 7 days. AFiPS aggregates measuring 112 ± 22 μm diameter at 2 days grew to 305 ± 61 μm diameter at 7 days, corresponding to a total cell number of 21 ± 0.3 × 10^6 cells at 2 days and 267 ± 7 × 10^6 cells at 7 days (three biological replicates per cell line per day; minimum of 100 aggregates measured per cell line per day).

(B) aggregates from an inoculated single cell suspension (2 × 10^5 hiPSCs/ml) in the bioreactor. FSiPS aggregates measuring 44 ± 8 μm diameter at 2 days grew to 197 ± 55 μm diameter at 7 days, corresponding to a total cell number of 243 ± 31 × 10^6 cells at 2 days and 1587 ± 99 × 10^6 cells at 7 days. AFiPS aggregates measuring 54 ± 9 μm diameter at 2 days grew to 198 ± 62 μm diameter at 7 days, corresponding to a total cell number of 267 ± 7 × 10^6 cells at 2 days and 1887 ± 86 × 10^6 cells at 7 days (four technical replicates per cell line per day; minimum of 100 aggregates measured per cell line per day).
the inoculum generated from aggregates. While the inoculum was intended to be a single cell suspension, microscopic examination revealed the presence of cell clusters (Figure 2C, upper left, arrowhead). This could be due to insufficient dissociation of the aggregates into a true single cell suspension by both enzyme treatment as well as trituration, possibly due to the inaccessibility of all cells in an aggregate to Accutase. To standardize this step of the protocol, the cell suspension was passed through a cell strainer of 40-μm pore size before subsequent cell counting and inoculation into culture vessels to remove cell clumps from the inoculum. This step led to the negligible loss of cell numbers in the form of cell clusters, but was beneficial to maintain viability >90% (Figure 2D) by minimizing trituration and the contact duration of the hiPSCs to Accutase. hiPSC aggregates expanded in both spinners and bioreactors from inoculum that was passed through a cell strainer had even and spherical morphologies (Figure 2C, lower right). For future automated systems aimed at cell expansion, cell strainers could be easily implemented to ensure a single cell suspension.

The next step was to ascertain whether hiPSCs could be cultured over longer periods of time in stirred suspension. To this end, seven serial passages were performed in spinners for both FSiPS and AFiPS cell lines (Figure 2D). Each passage lasted 7 days per our optimized protocol, for a total of 49 days in stirred suspension. At the end of each passage, aggregates were treated with Accutase into a single cell suspension and then strained through cell strainers. Pluripotency-associated cell surface markers and cell viability were assessed by flow cytometry, and cell numbers were counted. Based on the fold increase of each passage, the cumulative cell number was calculated if all the cells from the previous passage were grown in spinners. As illustrated in Figure 2D, >90% of the cells remained viable even after Accutase treatment and straining, and >93% of these were double positive for the pluripotency markers TRA-1-60 and SSEA-4. This suggests that hiPSCs can be cultured in stirred suspension for at least 49 days, possibly even longer, while remaining viable and pluripotent.

To demonstrate scalability to larger volumes and higher cell numbers, the protocol was extended for scale-up to a larger bioreactor. Both FSiPS and AFiPS growing in standard adherent culture with typical morphology (Figure 2E, Standard adherent) were introduced into spinners for cultivation for 7 days (Figure 2E, Spinner, Day 7). Following culture in spinners, aggregates were processed into a single cell suspension via Accutase treatment and passed through cell strainers. This single cell suspension was then inoculated into bioreactors and cultured for an additional 7 days in stirred suspension culture (Figure 2E, Bioreactor, Day 7). Thus, the expansion from standard adherent culture to the end of culture in a bioreactor takes 14 days, which remains well below the 49 days of culture demonstrated in spinners.

3.3 Quantification of spinner and bioreactor aggregates

After 7 days of stirred suspension culture in spinners using the optimized protocol, FSiPS aggregates measuring 342 ± 76 μm in diameter and AFiPS aggregates measuring 305 ± 61 μm in diameter were harvested (Figure 3A). The number of harvested hiPSCs from a spinner was up to 252 ± 8 × 10⁶ hiPSCs (Figure 3A, Growth curve). This represents a 16-fold increase in 7 days for a fold increase per day (FIPD; Chen et al., 2014) of 2.28. Cells harvested from spinners were then used as an inoculum in the bioreactor. Based on the 85 RPM in the 125 ml spinner, 164 RPM was derived for the bioreactor, using the energy dissipation rate formula as described by Mollet, Godoy-Silva, Berdugo, and Chalmes (2007). After 7 days of stirred suspension culture in the bioreactor, FSiPS aggregates measuring 197 ± 55 μm in diameter and AFiPS aggregates measuring 198 ± 61 μm in diameter were harvested (Figure 3B). The number of harvested hiPSCs from the bioreactor was up to approximately 2 × 10⁹ hiPSCs at the end of 7 days.

Fold increase, FIPD and volumetric yield are parameters that have been used as indicators of growth rates of cells across different culture platforms (reviewed in Chen et al., 2014 and Serra, Brito, Correia, & Alves, 2012). Our workflow has an FIPD of 2.28 in a 125 ml spinner and an FIPD of 1.43 in the bioreactor when analyzed independently of each other. However, given that our complete culture process involves a starting cell population of 16 × 10⁶ hiPSCs that is expanded into 2 × 10⁹ hiPSCs (125-fold) over 14 days, an overall FIPD of 8.93 is achieved. A recent report comparing fed batch and perfusion feeding strategies in hiPSC suspension culture showed an FIPD of 0.66 for fed batch and 0.96 for perfusion (Kropp et al., 2016) in a 125 ml stirred suspension vessel. Although there is great diversity in the approaches that groups have adopted towards large-scale culture of hiPSCs and its quantification, our FIPD of 8.93 suggests appreciably higher efficiency of our optimized protocol.

3.4 hiPSCs cultured as aggregates exhibit pluripotency markers, and maintain their differentiation potential and normal karyotype

While it was possible to achieve high quantities of hiPSCs, the quality of these hiPSCs after stirred suspension culture also needed to be assessed. It was verified that the hiPSC population obtained was maintained in an undifferentiated state as these cells were positive for nuclear (OCT4 and SOX2) and cell surface (SSEA-4 and TRA-1-81) pluripotency markers (Figure 4A). Before, during, and after suspension culture, the fraction of pluripotent cells was also quantified in these cell populations by flow cytometry using the cell surface pluripotency markers SSEA-4 and TRA-1-60. Before inoculating into suspension culture, >95% of hiPSCs grown in standard adherent culture were double-positive for SSEA-4 and TRA-1-60 (Figure 5, top panel). During suspension culture, samples of aggregates were dissociated into a single cell suspension and examined for the presence of TRA-1-60 and SSEA-4 at day 4 and day 6 postinoculation for each 7-day procedure. This made it possible to potentially identify instances of hiPSCs differentiating or losing pluripotency during suspension culture. Using our regular or optimized stirred suspension protocols, >95% of the cells growing in aggregates in suspension culture were consistently double-positive for SSEA-4 and TRA-1-60 at day 6 postinoculation (Figure 5, middle panels). hiPSCs grown in suspension culture for several consecutive passages could also be brought back to standard adherent culture conditions with the preservation of OCT4, SOX2, SSEA-4, TRA-1-81 (Figure 4A) and TRA-1-60 expression (Figure 5, bottom panel) even after more than five passages in standard adherent culture.

An important property of pluripotent stem cells is the ability to differentiate into cells of all three germ layers. The potency of hiPSCs
cultured in stirred suspension was confirmed by allowing them to spontaneously differentiate in differentiation medium. As shown in Figure 4B, this heterogeneous population of differentiated cells was positive for TUJ1 (ectoderm), SMA (mesoderm) and AFP (endoderm). For numerous biomedical applications, it is necessary for hiPSCs to be differentiated into defined cell lineages. Thus, whether the hiPSCs grown in bioreactors could be directed to differentiate into cardiomyocytes were assessed following a previously published protocol (Kadari et al., 2015). After 8 days of differentiation, the cells changed morphology and started to beat spontaneously; after an additional 3 days, beating sheets of cells could be seen (Video S1). After cardiac enrichment of the cells and replating on Matrigel-coated standard adherent culture plasticware for an additional 3 weeks to allow spontaneous differentiation. Differentiated cells were positive for AFP (endodermal marker), SMA (mesodermal marker) or TUJ1 (ectodermal marker) in both hiPSC lines. (C) Representative karyogram of FSiPS is shown, with 10 out of 10 metaphases analyzed exhibiting a normal human male karyotype. Scale bars = 100 μm.

Quantitative reverse transcription-PCR was used to assess the pluripotency state of bioreactor-derived hiPSCs. hiPSCs cultivated in stirred suspension over 14 days exhibit pluripotency markers OCT4 and NANOG at levels comparable to hiPSCs grown in standard adherent culture, and both standard adherent and bioreactor hiPSCs showed
minimal to no expression of AFP, T and SOX1, markers indicative of differentiation (Figure S5). The bioreactor hiPSCs also maintained a normal human male karyotype with no apparent chromosomal aberrations (Figure 4C). Taken together, these data demonstrate that hiPSCs grown in stirred suspension culture maintain their undifferentiated state, express pluripotency markers, exhibit no chromosomal aberrations and are of similar quality to hiPSCs grown in standard adherent culture.

3.5 | hiPSCs glucose metabolism in stirred suspension culture

Energy metabolism plays a highly important role for maintaining pluripotency of pluripotent stem cells and their further differentiation (Harvey et al., 2016; Teslaa & Teitell, 2015). Therefore, monitoring metabolites such as glucose and lactate should be performed throughout the culture process. The glucose consumption and lactate production were evaluated in the culture medium during hiPSC expansion as aggregates in the bioreactor, with measurements of the spent medium performed shortly before medium refreshment. Using the optimized suspension protocol, glucose concentration remained above 167 ± 31 mg/dl at day 7. This was coupled with a corresponding increase in lactate as a metabolite, reaching 15 ± 2 mM at day 7 (Figure S6). If glucose and lactate levels remain relatively constant at each measurement during culture, this could suggest that the cells were viable and respirating, but not increasing in number. In our system, glucose concentration decreases day after day, with a corresponding
increase in lactate concentration, consistent with the observed increase in cell numbers. This increasing number of hiPSCs in culture resulted in higher bulk glucose consumption and higher lactate production.

4 | CONCLUSION

A hiPSC stirred suspension culture system has been developed with a focus on free scalability between 50 ml and 3 l, process yield, maintenance of cell quality, and ease of use by incorporating the use of a well-established single-use stirred tank bioreactor. It is flexible and fits into existing standard adherent culture workflow, and is scalable from a 125-ml spinner to a 3-l bioreactor. The hiPSCs cultured over 14 days with our workflow maintain an undifferentiated state and pluripotency is preserved. A yield of up to 2 × 10^9 cells was obtained from the 3-l bioreactor, a quantity sufficient for cell transplantations, tissue patches, high-throughput drug screening and disease modelling. It is planned to leverage this newly developed approach to explore the possibility of directing differentiation of hiPSCs in stirred suspension culture vessels towards various lineages, as well as adapt this culture system to the large-scale production of progenitor/precursor cells. This brings us closer to the goal to use hiPSCs and/or their derivatives for disease modeling and cell therapy.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the supporting information tab for this article.

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