

A novel culture medium for high-density 3D expansion of human induced pluripotent stem cells as free-floating aggregates

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Title

A novel culture medium for high-density 3D expansion of human induced pluripotent stem cells as free-floating aggregates

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Abstract

Background: Human induced pluripotent stem cells (hiPSCs) are widely regarded as having immense potential for advancing modern medicine. However, their applications remain highly restricted, in part due to the complexity and high costs of pluripotent stem cell culture media (PSC-CM). Further progress in the already long history of PSC-CM development is necessary to overcome these barriers.

Results: We evaluated S8, a novel low-cost PSC-CM, specifically for high-density 3D expansion of pluripotent stem cells as free-floating aggregates. S8 was used to expand hiPSCs in the DASbox[®] Mini Bioreactor System for five days. A final cell density of 4.5×10^6 cells/mL was achieved, corresponding to an expansion of nearly 34-fold. To optimize culture medium consumption throughout culture, a novel dynamic perfusion strategy that matches medium exchange rate to cell oxygen uptake in real time was implemented, leading to a final cell yield of 7.72×10^5 cells/mL of consumed medium and a maximum volumetric productivity of 2.4×10^6 cells/[mL·day], supported by an exponential phase population

doubling time of 17.5 hours for the hiPSC line cultivated in this study. S8 sustained the formation and maintenance of healthy hiPSC aggregates and promoted active glycolytic metabolism. Analysis of the expanded hiPSCs through flow cytometry, qRT-PCR, and serum-stimulated trilineage differentiation confirmed proper pluripotency maintenance when using S8. Despite rarely being directly reported, cell yield and volumetric productivity were employed in this study as improved metrics for comparatively evaluating the performance of distinct hiPSC expansion processes. This showed that the results of the S8-based process here presented are comparable to benchmarks observed for optimized processes based on other well-established PSC-CM formulations, such as bioreactor-adapted E8 and StemFlex™.

Conclusions: The results presented herein showcase the use of S8 coupled with a novel dynamic perfusion strategy for the cost-efficient and translation-ready 3D expansion of hiPSCs in a stirred-tank bioreactor. This constitutes an important step toward decreasing the complexity and exorbitant cost currently associated with the large-scale production of hiPSCs, thus bringing their groundbreaking applications closer to routine clinical practice.

Keywords: S8 culture medium, human induced pluripotent stem cells, stirred-tank bioreactor suspension culture, dynamic perfusion, scalable stem cell manufacturing

Background

Since their discovery in 2007, human induced pluripotent stem cells (hiPSCs) have captivated the imagination of countless scientists [1]. The ability of hiPSCs to self-renew and to differentiate into the various cell lineages of the human body has led to several promising innovations in the fields of drug discovery, disease modeling and regenerative medicine [2, 3]. However, the accelerated pace of these innovations has outstripped the biopharmaceutical industry's capacity to implement them, leading to dissonance between the perceived potential of hiPSCs and their concrete application beyond laboratory or clinical trial settings [4, 5]. Among other reasons, this is due to the complexity and high costs of pluripotent stem cell culture media (PSC-CM) [6], which remain major hurdles despite the remarkable progress achieved since the first isolation of human embryonic stem cells (hESCs) in 1998 [7].

At that time, the lack of knowledge regarding human pluripotency pathways resulted in hESCs originally being co-cultured with mitotically inactivated mouse embryonic fibroblast feeder layers in

Dulbecco's modified Eagle medium (DMEM) supplemented with fetal bovine serum (FBS), following standard practice for ESCs of other species. Although this ensured that hESCs were provided with the necessary cues for maintaining pluripotency, this culture paradigm was untenable due to it being costly, labor-intensive and prone to variability, as well as incorporating xenogeneic components that raised safety concerns. Thus began a quest for chemically-defined and animal-component-free PSC-CM.

A milestone was reached in 2006 with the development of TeSRTM1, a feeder-independent, serum-free and more defined PSC-CM [8]. When hiPSCs were first derived a year later, they still relied on feeder layers since serum-free media had yet to be commonly adopted. However, TeSRTM1 soon found a prominent place among researchers working with both hESCs and hiPSCs, supporting groundbreaking work and remaining widely used, along with its derivative formulations, to this day. Despite this, the complex formulation of TeSRTM1, including a broad range of excipients found in feeder-conditioned media, and its reliance on albumin make it particularly expensive to produce, with routine research often being forced to employ versions that contain bovine serum albumin (e.g., mTeSRTM1) instead of chemically-defined recombinant human albumin (e.g., TeSRTM-AOF) [9]. As such, TeSRTM1 did not fully obviate xenogeneic components and remained not fully chemically defined.

As researchers increasingly came to see the advantages of hiPSCs comparatively to hESCs, namely their patient specificity and their avoidance of the ethical controversies surrounding the destruction of human embryos, PSC-CM development began to focus on hiPSC culture. In this new context, another milestone was reached in 2011 with the development of the E8 formulation, a fully chemically defined PSC-CM containing merely eight essential components, drastically reducing complexity and cost relative to TeSRTM1 [10]. This was made possible by eliminating the need for serum albumin and methodically narrowing down TeSRTM1's formulation to two recombinant growth factors crucial for pluripotency maintenance, i.e., fibroblast growth factor 2 (FGF2) and transforming growth factor beta 1 (TGF- β 1), along with those components which had a significant positive impact on cell viability and growth, i.e., DMEM/F12, L-ascorbic acid, sodium selenite, sodium bicarbonate, transferrin and insulin. Notably, while the original E8 formulation and some of its derivative versions employed recombinant zebrafish FGF2 to reduce costs and were not animal component free due to including bovine plasma

holo-transferrin and pancreas-derived insulin, E8 can be formulated entirely from recombinant human (rh) sources.

A further milestone was reached in 2020 with the development of B8, a more optimized and cost-efficient PSC-CM inspired by E8 that is both chemically-defined and animal-component-free [11]. B8 presents slightly increased concentrations of L-ascorbic acid, sodium selenite and rh-transferrin (*O. sativa*) while foregoing TGF- β 1 in favor of rhTGF- β 3 (*E. coli*), a more potent and selective alternative that can be used at a magnitude lower concentration; employs rhFGF2-G3 (*E. coli*), a thermostable version of FGF2 that at a reduced concentration supports more efficient culture medium consumption; adds rh-neuregulin 1 (*E. coli*), which plays a synergistic role with FGF2 to further facilitate pluripotent cell proliferation; and substitutes in rh-insulin (*P. pastoris*). This set of optimizations make B8 one of the most economically viable PSC-CM, fully defined and animal-origin-free.

It is interesting to note that, for the most part, PSC-CM development has historically relied on comprehensive 2D culture assays guided by statistical methods such as Design of Experiments [12], where pluripotent stem cells proliferate as a monolayer attached to a surface coated with a substrate that promotes cell adhesion, such as Matrigel[®] or recombinant extracellular matrix proteins. While efficient, this methodology has the drawback that the optimal component cocktail for monolayer culture may not translate directly to 3D growth environments, such as stirred-tank bioreactors in which hiPSCs are cultured as free-floating aggregates. This is because bioreactor culture usually sustains higher cell densities than are ever achieved in monolayer culture and the organization of hiPSCs into aggregates leads to cell-cell interactions and concentration gradients that are entirely absent when they attach to a planar surface [13]. Moreover, typical processes associated to bioreactor operation, such as peristaltic pump culture medium circulation, have been observed to compromise the physical stability of insulin leading to its precipitation in low-protein media [14]. The transition of PSC-CM from static 2D to bioreactor culture environments must therefore always be carefully evaluated.

Even so, there are numerous examples of PSC-CM originally developed for 2D adherent culture being successfully employed for the suspension culture of hiPSCs as free-floating aggregates (Table 1). Among these are mTeSRTM1 [15–19], bioreactor-adapted E8 [20, 21], StemFlexTM [22] and a modified version of B8 [23]. In many of these cases, PSC-CM are adapted for suspension culture by

supplementing them with inert additives, such as Pluronic F68, polyvinyl alcohol or dextran sulfate, to increase culture medium viscosity, reduce aggregate sheer stress and/or modulate aggregation [24, 25], or by supplementing them with a protein stabilizing agent, such as albumin [14]. Interestingly, the continued use of the aforementioned PSC-CM for hiPSC aggregate suspension culture is not due to the nonexistence of PSC-CM specifically designed with this purpose in mind, such as mTeSR™3D [19] and StemScale™ [26]. However, their tendency toward more complex formulations makes them more expensive, reducing their economic viability. Additionally, it has been reported that mTeSR™3D does not outperform mTeSR™1 for suspension culture [19], revealing that added complexity does not necessarily result in improved performance.

Table 1 Summary of bioprocess metrics related to the culture of hiPSCs as free-floating aggregates in bioreactors.

Study/ Year	Culture System/ Volume	Culture Medium/ Operation Mode	Initial/Final Cell Density (cells/mL) ⁽¹⁾	Fold Expansion/ Duration	Cell Yield (cells/mL)	Volumetric Productivity (cells/[mL·day])
Nogueira et al. (2019) [19]	VWBR (60 mL)	mTeSR™1 (Batch ⁽²⁾ – 0.30 L)	2.50×10^5 (1.2×10^6)	4.8-fold (7 days)	1.90×10^5	1.36×10^5
	VWBR (60 mL)	mTeSR™3D (Batch ⁽²⁾ – 0.30 L)	2.50×10^5 (8.8×10^5)	3.5-fold (7 days)	1.26×10^5	9.00×10^4
Manstein et al. (2021) [20]	STBR (150 mL)	Modified E8 (Perfusion – 3.53 L)	5.00×10^5 (3.50×10^7)	70-fold (7 days)	1.47×10^6	4.93×10^6
Dang et al. (2021) [23]	VWBR (100 mL)	mTeSR™1 (Batch ⁽²⁾ – 0.25 L)	2.00×10^4 (7.40×10^5)	37-fold (7 days)	2.88×10^5	1.03×10^5
	VWBR (500 mL)	mTeSR™1 (Batch ⁽²⁾ – 1.25 L)	2.00×10^4 (5.40×10^5)	27-fold (7 days)	2.08×10^5	7.43×10^4
	VWBR (100 mL)	Modified B8 (Batch ⁽²⁾ – 0.25 L)	2.00×10^4 (1.06×10^6)	53-fold (7 days)	4.16×10^5	1.49×10^5
	VWBR (500 mL)	Modified B8 (Batch ⁽²⁾ – 1.25 L)	2.00×10^4 (1.24×10^6)	62-fold (7 days)	4.88×10^5	1.74×10^5
	VWBR (100 mL)	StemFlex™ (Batch ⁽²⁾ – 0.15 L)	2.00×10^4 (1.88×10^6)	94-fold (5 days)	1.24×10^6	3.72×10^5
Cuesta-Gomez et al. (2023) [22]	VWBR (100 mL)	StemFlex™ (Batch ⁽²⁾ – 0.25 L)	2.00×10^4 (3.62×10^6)	181-fold (7 days)	1.44×10^6	5.14×10^5
	VWBR (500 mL)	StemFlex™ (Batch ⁽²⁾ – 0.75 L)	2.00×10^4 (1.99×10^6)	100-fold (5 days)	1.33×10^6	3.94×10^5
	STBR (150 mL)	Modified E8 (Perfusion – 0.53 L)	5.00×10^5 (5.2×10^6) ⁽³⁾	10-fold (3 days)	1.34×10^6	1.57×10^6
Ullmann et al. (2024) [21]	STBR (150 mL)	Modified E8 (Perfusion – 0.98 L)	5.00×10^5 (8.10×10^6) ⁽³⁾	16-fold (4 days)	1.17×10^6	1.90×10^6
	STBR (150 mL)	Modified E8 (Perfusion – 0.98 L)	5.00×10^5 (8.10×10^6) ⁽³⁾	16-fold (4 days)	1.17×10^6	1.90×10^6
This study (2026)	STBR (80 mL)	HiDef® S8® (Perfusion – 0.45 L)	1.25×10^5 (4.47×10^6)	36-fold (5 days)	7.72×10^5	8.69×10^5

Only a small selection of studies is shown. Abbreviations: STBR, stirred-tank bioreactor; VWBR, vertical-wheel bioreactor.

⁽¹⁾ Initial cell density relative to final bioreactor working volume.

⁽²⁾ Fed-batch and/or partial repeated batch.

⁽³⁾ The study of Ullmann et al. investigates serial bioreactor passaging. Here, only the results of the 1st passage are considered.

Among the hiPSC expansion bioprocesses so far established in the literature, two stand out. The bioprocess developed by Manstein et al. in the DASbox[®] Mini Bioreactor System, which achieved a 70-fold expansion and a final cell density of 35×10^6 hiPSCs/mL in seven days using an in-house formulated version of E8 adapted for bioreactor culture, is currently the most impressive in terms of cell yield (culture medium consumption efficiency) and volumetric productivity (space/time efficiency) [20]. Importantly, these results were the culmination of several years of successive optimizations, which consisted in meticulously finetuning culture medium exchange and establishing tight control over pH, glucose and lactate. While highly efficient, such a high degree of optimization is difficult to replicate when working under cost/resource constraints, since sustaining such high cell densities requires consuming copious amounts of culture medium coupled with glucose supplementation. Furthermore, reaching such a high cell density is also cause for potential quality concerns when the produced hiPSCs are intended for clinical applications. As a contrasting approach, Cuesta-Gomez et al. developed a relatively low-density bioprocess in the vertical-wheel bioreactor system, which achieved a 94-fold expansion in 5 days and a final cell density of around 1.9×10^6 cells/mL with StemFlex[™] [22]. Despite a magnitude lower volumetric productivity, this bioprocess maintained high cell yield by avoiding any slowdown in cell growth due to ample availability of nutrients coupled with minimal culture medium exchange, the latter also making the bioprocess overall much more economically accessible. The same group also developed an extended bioprocess which achieves 181-fold expansion in 7 days and a final cell density of 3.62×10^6 cells/mL, which resulted in improved volumetric productivities in the last days of culture while maintaining cell yield. However, the shorter process was stated to be optimal.

This work validates S8, a novel PSC-CM developed by Defined Bioscience (HiDef[®] S8[®], cat # LSS-604), the current commercial producer of B8 (HiDef[®] B8, cat # LSS-204). S8 consists of components similar to those used to produce B8, with concentrations optimized to support extended suspension culture of pluripotent stem cells as aggregates, retaining the latter's low cost relative to comparable media while minimizing population doubling time (PDT) and preserving pluripotency even at high cell densities. S8 was validated in terms of hiPSC growth kinetics, aggregate dynamics, glycolytic metabolism, pluripotency maintenance and genomic stability within the context of a novel hiPSC

expansion bioprocess, whose main distinguishing feature is a dynamic perfusion strategy that matches medium exchange rate to cell oxygen uptake in real time (Fig. 1). The bioprocess here presented sought to conjugate the high volumetric productivity of the bioprocess developed by Manstein et al. and the economic accessibility of the bioprocess developed by Cuesta-Gomez et al., thus establishing a scalable, simple to implement, consistent and cost-efficient protocol for hiPSC expansion.

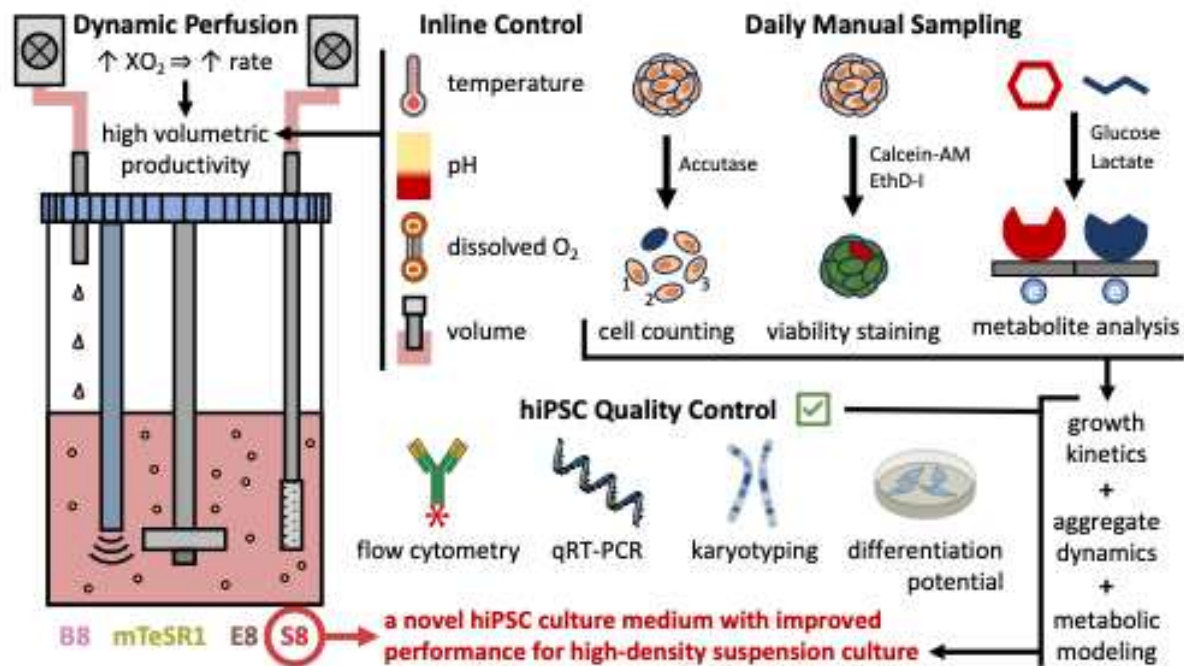


Fig. 1 S8 validation workflow. hiPSCs were cultured as free-floating aggregates in a stirred-tank bioreactor with a tightly controlled environment. Daily manual sampling was performed to glean relevant data regarding growth kinetics, aggregate dynamics and glycolytic metabolism. At the end of culture, quality control assays were performed to verify that hiPSC pluripotency and genomic integrity were preserved. S8 was compared with B8, mTeSR1 and E8 under identical bioreactor operating conditions, revealing improved growth kinetics without compromising hiPSC quality. A novel dynamic perfusion strategy driven by cellular oxygen uptake was implemented to further optimize hiPSC proliferation and increase the bioreactor's volumetric productivity when using S8.

Results

S8 promotes robust hiPSC expansion in an STBR

Following observations by our group that during suspension culture of hiPSCs as free-floating aggregates in a stirred-tank bioreactor (STBR) B8 reliably preserved pluripotency only while cell density remained below 1×10^6 hiPSCs/mL (Additional file 1: Fig. S1). A preliminary comparison of S8 with B8, mTeSRTM1 (STEMCELL Technologies, cat# 85850) and Essential 8TM (Gibco, cat# A1517001) under identical STBR operating conditions showed that S8 noticeably improved cell proliferation comparatively to the other tested media (B8 PDT = 21.4 hours; mTeSRTM1 PDT = 22.4

hours; E8 PDT = 20.9 hours; S8 PDT = 17.6 hours) while preserving pluripotency on a level equivalent to mTeSRTM1 (Additional file 1: Fig. S2).

To further test the effectiveness of S8 as a suspension-oriented PSC-CM, it was used for the development of a novel STBR-based hiPSC expansion bioprocess. After thawing into S8, hiPSCs were expanded in 2D until enough cells were obtained to inoculate a small-scale STBR (DASbox[®] Mini Bioreactor System, Eppendorf) with a working volume of 85 mL and inoculation density of 125,000 cells/mL. hiPSCs were then cultured as free-floating aggregates for five days, with culture medium exchange being performed through dynamic perfusion starting on Day 2.

Post-inoculation hiPSC survival measured on Day 1 was $70 \pm 10\%$, indicating that cell viability during the transition of hiPSCs to suspension culture was not excessively compromised. Following an initial one-day adaptation phase, hiPSCs proliferated exponentially through to the end of culture, achieving a final cell density of $(4.47 \pm 0.36) \times 10^6$ cells/mL in a working volume of 80 mL, which corresponds to a final cell number of $(3.57 \pm 0.29) \times 10^8$ cells and a fold increase of 33.56 ± 2.74 (Fig. 2A). This was made possible by a low mean population doubling time of (17.54 ± 1.31) hours for Days 1 to 5, in line with what can be expected for an hiPSC population in its exponential growth phase (Fig. 2B). No significant increase in population doubling time was observed after Day 2, when cell proliferation was fastest, indicating that exponential growth was maintained up to the last day of culture.

To meet the metabolic needs of hiPSCs, perfusion was implemented with a dynamic exchange rate modulated indirectly by cell oxygen uptake. This was done by determining in real time an optimal exchange rate based on the oxygen fraction in the inflow gas stream supplied by the STBR's controller in order to maintain dissolved oxygen (DO) at the predetermined setpoint of 40%. By employing this novel strategy, the volume of culture medium consumed during culture was automatically adjusted based on the number of cells in the bioreactor, avoiding culture medium depletion while preventing its unnecessary waste. The usefulness of this method is exemplified by the fact that R3, which presented lower cell density throughout culture relative to R1 and R2 due to reduced post-inoculation survival, consumed less culture medium until the end of culture and therefore presented an equivalent cell yield and volumetric productivity to R1 and R2 (Fig. 2C and 2D). In this way, bioprocess metrics were kept

more consistent between distinct batches by adjusting the STBR perfusion rate according to the actual culture status as opposed to defining it a priori.

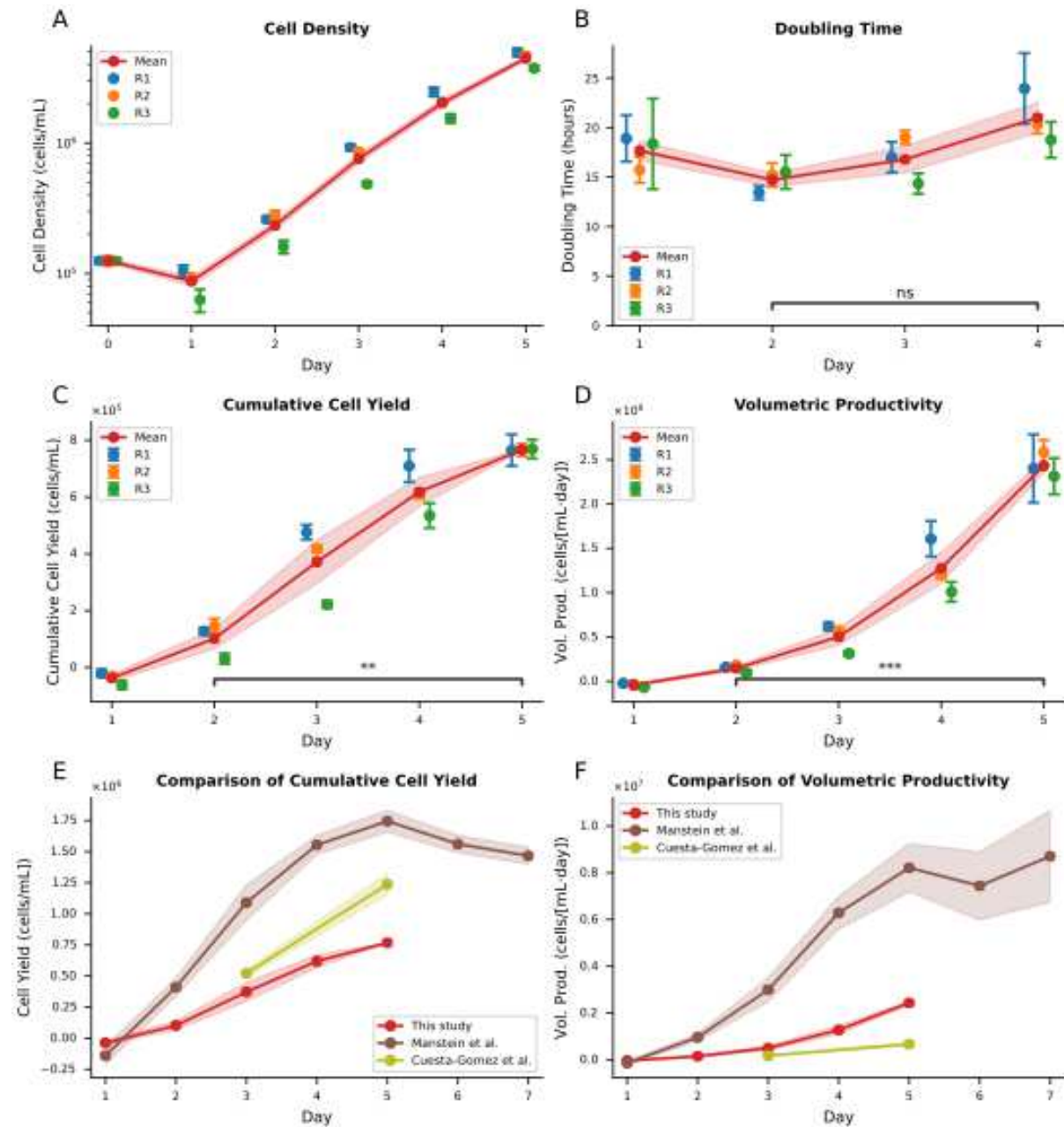


Fig. 2 Bioprocess metrics of hiPSCs cultured in an STBR as free-floating aggregates using S8. **A** Evolution of cell density (log scale). **B** Evolution of population doubling time. **C** Evolution of cumulative cell yield. **D** Evolution of volumetric productivity. **E** Comparison of cumulative cell yield between this study, Manstein et al. and Cuesta-Gomez et al. **F** Comparison of volumetric productivity between this study, Manstein et al. and Cuesta-Gomez et al. Differences were considered statistically significant at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Cell yield and volumetric productivity are two of the most relevant metrics for assessing the performance of STBR culture. The first, defined as the ratio between the number of cells produced and the volume of culture medium consumed up to a given timepoint, delineates how efficient a culture medium and its associated feeding strategy are at supporting cell proliferation. The second, defined as

the number of cells produced between two timepoints normalized by the bioreactor working volume and time elapsed, combines the volumetric and time efficiency of a bioreactor culture. Ideally, both of these metrics should increase and eventually, for prolonged culture, reach a stable plateau. S8 sustained steady increases in both cell yield (Fig. 2C) and volumetric productivity (Fig. 2D) until the end of culture, with these metrics having reached maximum values of $(7.72 \pm 0.01) \times 10^5$ cells/mL of consumed medium and $(2.43 \pm 0.08) \times 10^6$ cells/[mL·day], respectively. The observed trends can be attributed to steadily increasing cell density coupled with efficient culture medium consumption regulated by dynamic perfusion.

To evaluate the performance of the established hiPSC expansion bioprocess herein, these two metrics were compared side-by-side with the standout bioprocesses developed and described above by Manstein et al. [20] and by Cuesta-Gomez et al. [22] (Fig. 2E and 2F). All bioprocesses see an increase in cell yield and volumetric productivity until Day 5, with Manstein et al. exhibiting the highest values for both at this timepoint. Compared to the bioprocess here presented, Manstein et al. reached a 2.3× higher cell yield of $(1.74 \pm 0.08) \times 10^6$ cells/mL and a 3.4× higher volumetric productivity of $(8.21 \pm 1.03) \times 10^6$ cells/[mL·day], whereas Cuesta-Gomez et al. reached a 1.6× higher cell yield of $(1.24 \pm 0.09) \times 10^6$ cells/mL but 3.6× lower volumetric productivity of $(6.67 \pm 0.67) \times 10^6$ cells/[mL·day].

S8 supports the formation and maintenance of healthy hiPSC aggregates

Brightfield imaging showed that S8 supported the formation of spherical aggregates which steadily increased in size over time and presented a characteristic central cavity, with additional smaller cavities sometimes being observed in larger aggregates (Fig. 3A, Bright, DAPI). Cavitation is often associated with naïve, as opposed to primed, hiPSCs since these tend to form structures akin to a pre-implantation blastocyst with a prominent blastocoele [27, 28]. In fact, studies have shown that hiPSCs transition to a more naïve state simply by being cultured in three-dimensional structures [22]. Alternatively, cavities may also result to a greater or lesser extent from hiPSCs organizing themselves to minimize diffusion limitations to the aggregate core. The absence of significant diffusion limitations was corroborated by staining with calcein AM (live cells, green) and ethidium homodimer I (dead cells, red), which showed that hiPSCs within aggregates remained viable throughout culture, with most dead cells floating freely

238 or in small clumps or occasionally being found on the surface of aggregates (Fig. 3A, Live/Dead;
 239 Additional file 1: Fig. S4).

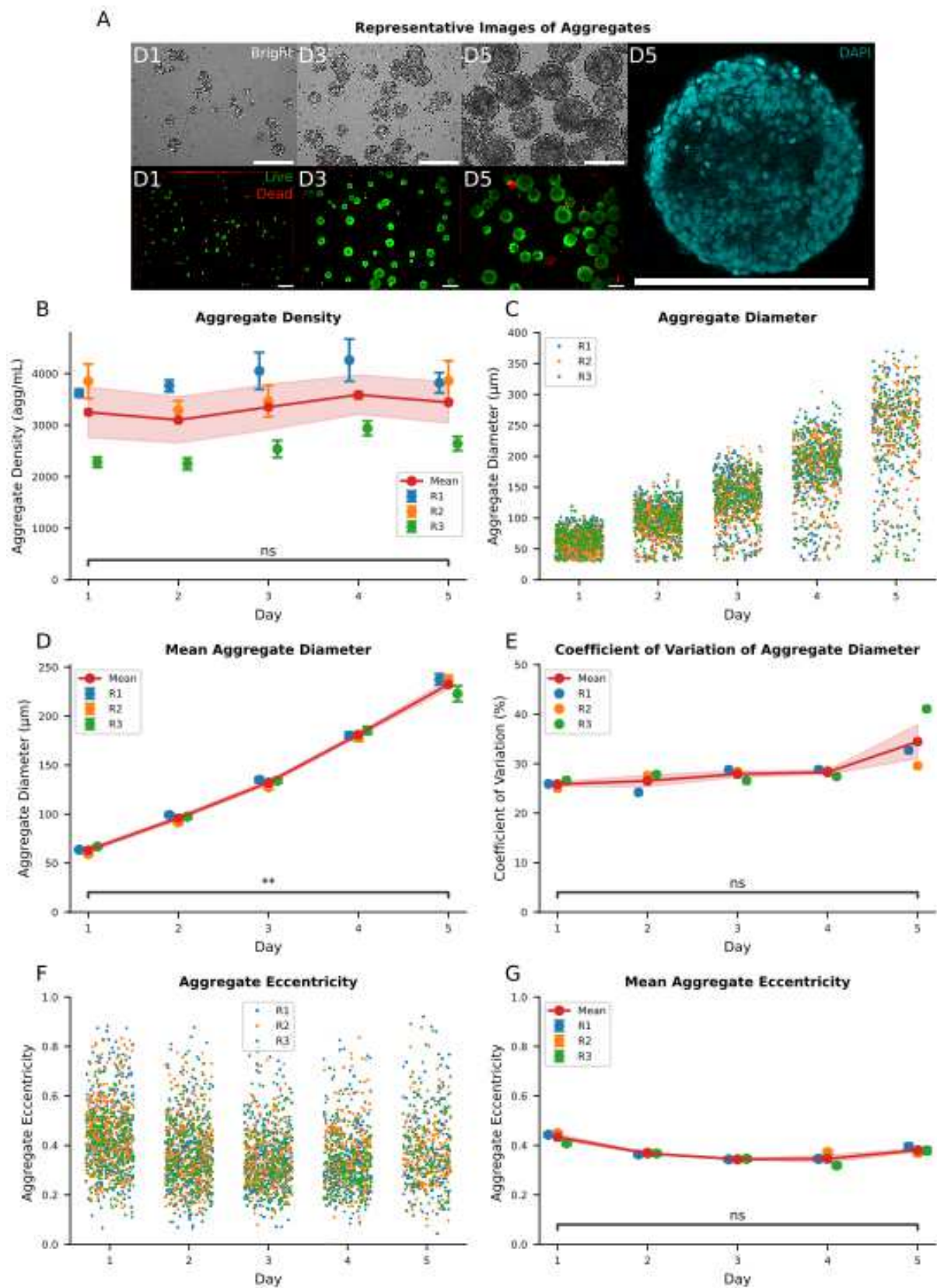


Fig. 3 Aggregate dynamics of hiPSCs cultured in an STBR as free-floating aggregates using S8. **A** Representative images of aggregates during culture obtained through brightfield microscopy, fluorescence microscopy after live/dead staining with

calcein AM and ethidium homodimer I and confocal microscopy after DAPI staining (scale bars = 300 μ m). **B** Evolution of aggregate density. **C** Measured aggregate diameters. **D** Evolution of mean aggregate diameter. **E** Evolution of the coefficient of variation of aggregate diameter. **F** Measured aggregate eccentricities. **G** Evolution of mean aggregate eccentricity. Differences were considered statistically significant at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

On the first day after inoculation, an aggregate density of $3,250 \pm 489$ aggregates/mL was obtained (Fig. 3B), reflecting the successful aggregation of hiPSCs. Notably, R3, which presented the lowest final cell density, was also the replicate with the lowest aggregate density at the start of culture. This is indicative of a direct relationship between the number of aggregates formed after inoculation and the growth potential of an STBR's hiPSC population, making the former a reasonably good predictor of the latter. The mean ratio between final cell density and initial aggregate density is $(1.41 \pm 0.10) \times 10^3$ cells/aggregate. Using this ratio to predict final cell density as shown in Table 2 results in a mean relative error of $1.4 \pm 7.1\%$ and a normalized root mean square error (nRMSE) of 11.6%, demonstrating its potential as a predictor. Maximizing the initial number of aggregates after inoculation is therefore particularly important.

Table 2 Prediction of final cell density (Day 5) based on initial aggregate density (Day 1).

Replicate	Initial Aggregate Density (agg/mL)	Final Cell Density (cells/mL)	Predictive Ratio (cells/agg)	Predicted Final Cell Density (cells/mL)	Relative Error
R1	3,620	4.95×10^6	1.37×10^3	5.10×10^6	+3.0%
R2	3,850	4.70×10^6	1.22×10^3	5.43×10^6	+15.5%
R3	2,280	3.75×10^6	1.64×10^3	3.21×10^6	-14.4%
Mean $\pm$ SEM	$3,250 \pm 489$	$(4.47 \pm 0.36) \times 10^6$	$(1.41 \pm 0.10) \times 10^3$	$(4.58 \pm 0.56) \times 10^6$	$1.4 \pm 7.1\%$

After initial aggregate formation, aggregate density remained stable throughout culture, with no significant changes being detected (Fig. 3B). However, this does not mean that some formation of new aggregates did not occur, since small aggregates were detected even at later days of culture (Fig. 3C), suggesting that cells shed from already established aggregates can reaggregate. Since aggregate density remained mostly unchanged, it is likely that some aggregate fusion also took place.

When it comes to culturing hiPSCs as aggregates, controlling their size is a crucial aspect. Once aggregates exceed 300 μ m in diameter, diffusion limitations to their core can become a problem, leading to necrosis or spontaneous differentiation [29]. Appropriate STBR agitation kept most aggregates below this threshold even though S8 supported significant aggregate growth, with their mean diameter increasing from 63 ± 2 μ m on Day 1 to 232 ± 5 μ m by the end of culture (Fig. 3D). To determine

aggregate size homogeneity, the coefficient of variation of aggregate diameter was calculated, with it having increased from $26 \pm <1\%$ to $34 \pm 3\%$ during culture, although this change was not deemed to be significant (Fig. 3E). While not ideal, the high heterogeneity in aggregate size is explained in part by the gradual formation of new aggregates as culture progressed.

The shape of aggregates can serve as a good indicator of the state of hiPSCs, with more spherical aggregates (eccentricity closer to 0) being indicative of hiPSCs with typical proliferative capacity and preserved pluripotency. Despite some aggregates with irregular eccentricity being detected (Fig. 3F), mean aggregate eccentricity was 0.38 ± 0.10 by the last day of culture with no significant change since aggregate formation (Fig. 3G), showing that the majority of aggregates remained highly spherical throughout their lifetime.

S8 sustained active glycolytic hiPSC metabolism

Due to being highly proliferative, pluripotent stem cells rely heavily on aerobic glycolysis, i.e., the metabolic conversion of glucose into lactate even when sufficient oxygen is available for performing oxidative phosphorylation, a phenomenon which has been dubbed the Warburg effect [30–32]. Spontaneous deviations from this distinctive metabolism tend to indicate either a loss of viability or pluripotency, thus being a good indicator of hiPSC quality. Because of this, daily samples of STBR culture medium were collected and their glucose and lactate concentrations were measured. These were used to determine cellular glucose uptake (qG) and lactate secretion (qL) rates through a novel recursive multi-step forecasting model, described in detail in the Methods section, that improves upon traditional methodologies when working with dynamic perfusion. The apparent yield (AY) of lactate from glucose, representative of cell reliance on pure glycolysis for energy production, was then calculated.

As expected, bioreactor glucose concentration decreased steadily throughout culture, reaching a final value of 1.04 ± 0.46 mM (Fig. 4A). This was accompanied by a simultaneous increase in bioreactor lactate concentration, which reached a final value of 29.29 ± 1.94 mM (Fig. 4B). While some sources consider such a high lactate concentration to be prejudicial to cell growth and pluripotency maintenance [33, 34], in this case it did not exert a noticeably negative impact. Conversely, lactate has also been shown to itself be a potential energy substrate for hiPSCs subjected to low glucose concentration [35].

Notably, the implemented dynamic perfusion strategy maintained reasonable glucose levels until the very end of culture, proving that it could keep up with cellular energy demands.

The models fitted to the experimental concentration data correctly captured the observed trends, except on the last day of culture, for which the models of the two replicates with the lowest glucose concentration, R1 and R2, show unstable behavior (Fig. 4C and 4D). This instability is indicative of extreme variation in q_G and q_L , which likely dropped abruptly once glucose availability became insufficient for normal uptake during the last day of culture. This is supported by the stability of the glucose and lactate models of R3, which presented a higher final glucose concentration.

Given the close fit of the glucose/lactate models to the experimental data, reliable estimates of daily average q_G and q_L were obtained. q_G remained stable during the first two days of culture, exhibiting a maximum value of 20.47 ± 0.86 pmol/[cell·day], before gradually declining to a significantly lower value of 12.70 ± 0.44 pmol/[cell·day] by the end of culture (Fig. 4E). q_L increased significantly during the first day of culture, reaching a maximum value of 40.47 ± 2.58 pmol/[cell·day], before similarly declining to a significantly lower value of 23.38 ± 1.57 pmol/[cell·day] (Fig. 4F). The decrease of q_G over time suggests that glycolysis slowed as culture progressed. While in part this might be caused by a decrease in bioreactor glucose concentration, an equally plausible reason could be reduced glucose accessibility experienced by hiPSCs within larger aggregates [36].

Apparent yield, calculated by dividing q_L by q_G , remained between 1.8 and 2.0 for a majority of the culture timeframe, presenting a final value of 1.84 ± 0.08 (Fig. 4G). This is a clear sign that hiPSCs maintained a stable and predominantly glycolytic metabolism, as is desirable. The exception to this was the first day of culture, whose reduced q_L led to an AY of 1.45 ± 0.07 . Likely this was a symptom of impaired cell viability and reduced proliferation as hiPSCs adapted to suspension culture, with their metabolism recovering once aggregates had properly formed.

Given the relevance of glycolysis to hiPSC proliferation, it is reasonable to assume that growth rate is highly dependent on glucose availability. An nRMSE of 16% was obtained by assuming growth rate was related to glucose concentration through the traditional Monod equation (Fig. 4H), demonstrating that this indeed seems to be the case.

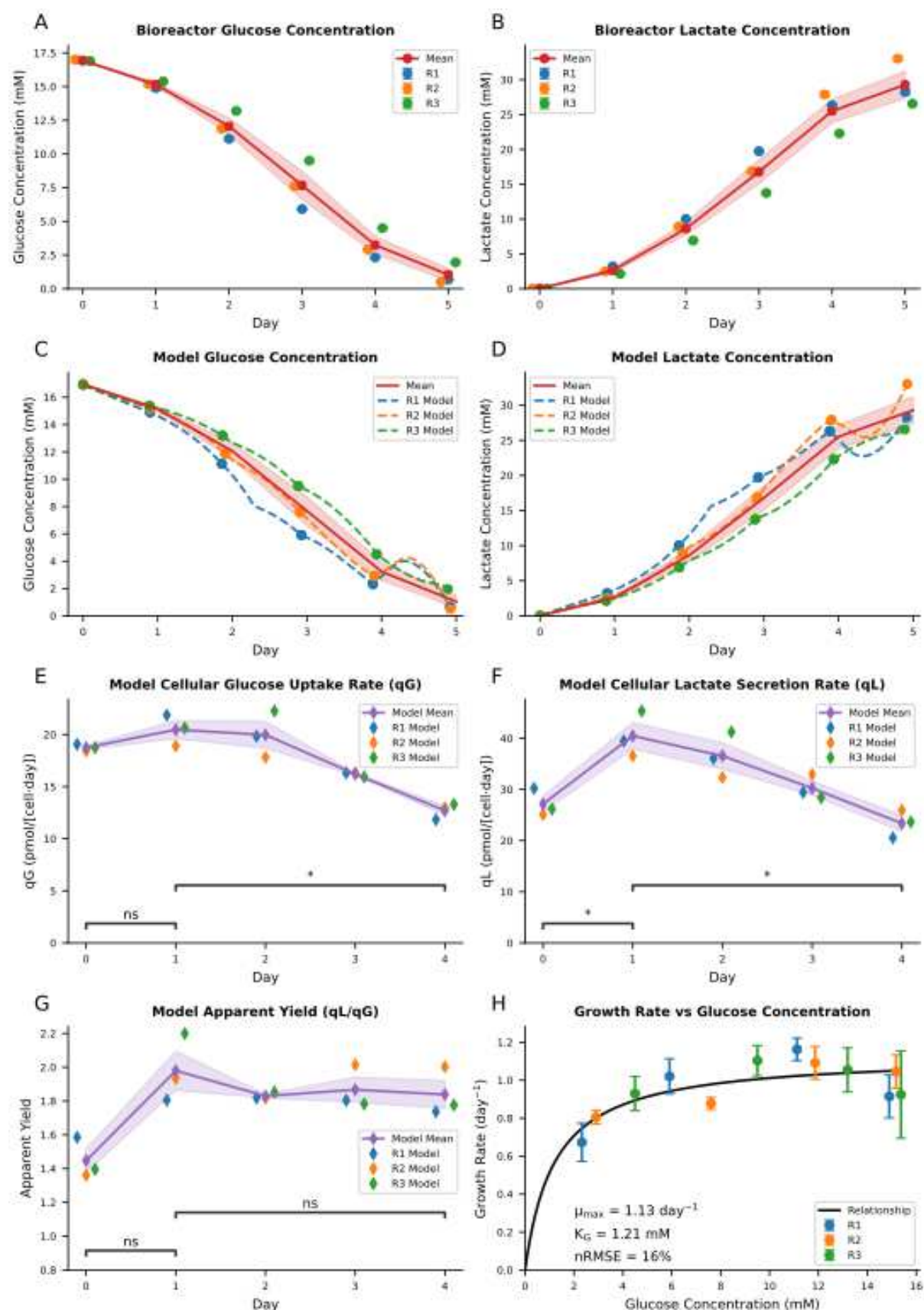


Fig. 4 Glycolytic metabolism of hiPSCs cultured in an STBR as free-floating aggregates using S8. **A** Evolution of glucose concentration in bioreactor culture medium. **B** Evolution of lactate concentration in bioreactor culture medium. **C** Evolution of glucose concentration predicted by best-fitting glucose uptake rate (qG) models. **D** Evolution of lactate concentration predicted by best-fitting lactate secretion rate (qL) models. **E** Evolution of qG values of best-fitting qG models. **F** Evolution

of qL values of best-fitting qL models. **G** Evolution of apparent yield of lactate from glucose (qL/qG). **H** Relationship between growth rate and qG, described by a nonlinear saturation equation with growth as a function of glucose concentration (Monod equation). Differences were considered statistically significant at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

S8 preserved hiPSC pluripotency even at high cell densities

Although the results presented so far have shown that cells cultured in S8 maintained proliferative capacity, aggregate morphology and metabolic activity typical of hiPSCs, several assays were executed to further corroborate pluripotency maintenance of cells collected at the end of culture. Flow cytometry analysis showed that two intracellular (OCT4 and SOX2) and one surface (SSEA4) pluripotency markers were expressed by most cells (>90%) by the end of culture, with no significant change occurring relative to hiPSCs collected prior to STBR inoculation (Fig. 5A). Quantitative real-time polymerase chain reaction (qRT-PCR) analysis further showed that hiPSCs maintained high expression of key pluripotency genes – *OCT4*, *NANOG* and *SOX2* – by the end of culture, whereas differentiation genes associated to the three germ layers, namely ectoderm (*SOX1*), mesoderm (*TBXT*) and endoderm (*SOX17*), were not significantly upregulated (Fig. 5B). The expression level of all pluripotency genes relative to differentiation genes was at least 1,000× greater.

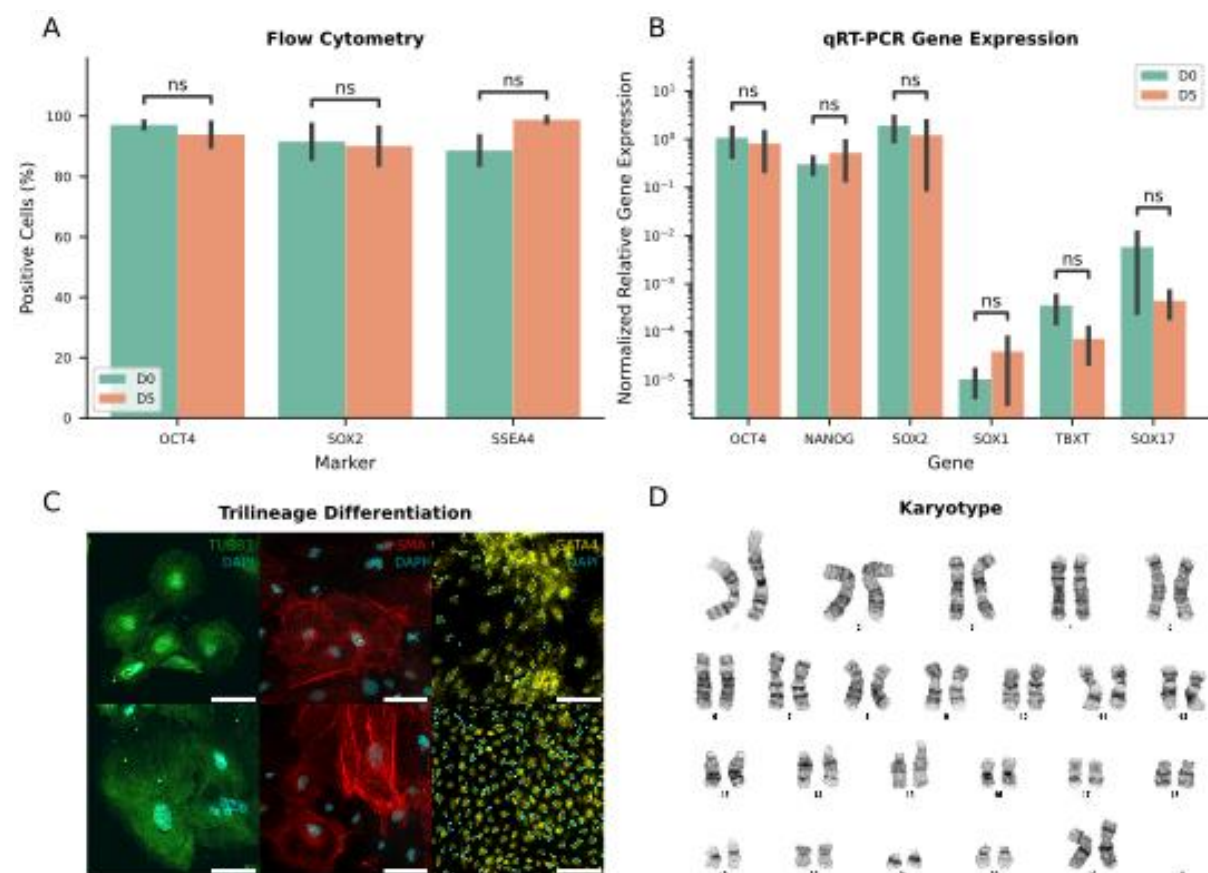


Fig. 5 Pluripotency and genomic stability of hiPSCs cultured in an STBR as free-floating aggregates using S8. **A** Flow cytometry analysis of two intracellular (OCT4 and SOX2) and one surface (SSEA4) pluripotency markers. **B** Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of three pluripotency genes (*OCT4*, *NANOG* and *SOX2*) and genes associated to each of the three germ layers, namely ectoderm (*SOX1*), mesoderm (*TBXT*) and endoderm (*SOX17*) (log scale). **C** Illustrative images of hiPSC aggregates which were collected on the last day of culture and allowed to spontaneously differentiate for 10 days after plating. Cells were immunostained for markers associated to each of the three germ layers, namely class III β -tubulin (TUBB3), alpha smooth muscle actin (α -SMA) and GATA4, representative of ectoderm, mesoderm and endoderm, respectively (scale bars = 100 μ m). **D** Karyotypic analysis confirming that no chromosomal aberrations occurred and cells maintained a normal karyotype after STBR culture with S8. Differences were considered statistically significant at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

To further confirm hiPSC pluripotency maintenance, aggregates at the end of culture were transferred to Matrigel[®]-coated cell culture plates. Once cells had attached and spread out, a spontaneous trilineage differentiation protocol commenced. After 10 days, cells were immunostained for markers characteristic of the three germ layers, namely class III β -tubulin (TUBB3), alpha smooth muscle actin (α -SMA) and GATA4, representative of ectoderm, mesoderm and endoderm, respectively. Several cell populations expressing each one of these markers were found after staining, demonstrating that hiPSCs could originate cells belonging to all three germ layers (Fig. 5C). Finally, a karyotypic analysis conducted by an accredited clinical laboratory confirmed that no chromosomal aberrations occurred, and cells maintained a normal karyotype after STBR culture with S8. Altogether these assays corroborate the ability of S8 to preserve hiPSC pluripotency and genetic stability at cell densities of at least 5×10^6 cells/mL, much higher than the 1×10^6 cells/mL threshold our group observed for the original B8 formulation.

Discussion

The purpose of this study was to evaluate S8, a novel low-cost PSC-CM designed specifically for hiPSC suspension culture as free-floating aggregates, and to establish a cost-efficient STBR protocol for hiPSC expansion using S8. Simultaneously, it sought to present a methodology for systematically assessing bioreactor hiPSC expansion, identifying key metrics for comparing distinct bioprocesses. This is of particular importance given that the studies found in the literature concerning this topic differ significantly in terms of inoculation density, timeframe, bioreactor platform, operating parameters and culture medium, among many other factors. Table 3 summarizes the identified metrics, their optimal trends and their current benchmarks, which represent either best values achieved so far or values expected for healthy hiPSCs. In accordance with the quality by design framework [37, 38], these metrics

can be classified as critical process parameters (CPPs) or critical quality attributes (CQAs), constituting the minimum operational and quality criteria of a bioreactor hiPSC expansion bioprocess.

Table 3 Summary of key metrics for bioreactor hiPSC expansion along with their respective current benchmarks.

Category	Metric (CPP/CQA)	Optimal Trend	Benchmark	Source
Growth Kinetics	PDT / growth rate	no change	15-18 hours / 0.9-1.1 day ⁻¹	TS, [20, 22]
	cell yield	increase until stable	1.47×10^6 cells/mL (overall)	[20]
	volumetric productivity	increase until stable	4.93×10^6 cells/[mL·day] (overall)	[20]
Aggregate Dynamics	aggregate density	no decrease	$3-4 \times 10^3$ aggregates/mL	TS
	aggregate diameter	increase	majority < 300 μ m	TS, [20]
	aggregate eccentricity	no increase	majority < 0.5	TS
Metabolic Activity	glucose uptake rate	no decrease	15-20 pmol/[cell·day]	TS, [20]
	lactate secretion rate	no decrease	24-40 pmol/[cell·day]	TS, [20]
	apparent yield	no decrease	1.6-2.0	TS, [20]
Pluripotency Maintenance	protein expression	no change	PMs (+) > 90% and DMs (+) < 10%	cGMP
	gene expression	no change	PGs expression >> DGs expression	cGMP
	differentiation potential	no change	differentiation into 3 germ layers	cGMP
Genomic Stability	karyotype	no change	normal karyotype, no mosaicism	cGMP

Abbreviations: cGMP, current good manufacturing practices; CPP, critical process parameter; CQA, critical quality attribute; DGs, differentiation genes; DMs, differentiation markers; PDT, population doubling time; PGs, pluripotency genes; PMs, pluripotency markers; TS, this study.

Among these metrics, cell yield and volumetric productivity are often overlooked and not directly reported, despite being excellent metrics for direct bioprocess comparison, more so than the frequently mentioned cell fold increase. Taking as examples this study and those of Manstein et al. [20] and Cuesta-Gomez et al. [22], we find that the fold increases of 34× in 5 days, 70× in 7 days and 94× in 5 days which were obtained, respectively, provide scant information regarding the underlying differences in cell density and culture medium exchange strategies. Conversely, cell yield directly informs on how efficient the employed culture medium and its associated feeding strategy are at sustaining hiPSC proliferation, while volumetric productivity informs on how efficient a bioreactor platform and its associated culture parameters are at producing hiPSCs within a certain culture volume and process duration. Together, these two metrics provide a concise picture of bioprocess cost-efficiency, especially if complemented further by consideration of relative PSC-CM price per volume. Despite not reaching the highest fold increase and presenting the longest culture duration, the bioprocess developed by Manstein et al. achieved the highest overall cell yield and volumetric productivity. The high cell yield can be attributed to meticulous finetuning of culture medium exchange through an in silico metabolic

model coupled with glucose supplementation which delayed culture medium depletion. The high volumetric productivity can be attributed to the high inoculation density (500,000 cells/mL) along with the preservation of ideal hiPSC growth kinetics through tight control of bioreactor culture parameters.

Notably, as seen from the three hiPSC expansion bioprocesses being compared, higher inoculation density leads directly to higher volumetric productivity, with the S8-based bioprocess shown here (125,000 cells/mL) presenting the second highest value for this metric and the bioprocess of Cuesta-Gomez et al. (20,000 cells/mL) presenting the lowest. Of course, this is only true as long as high inoculation density is accompanied by a suitable increase in the exchange of culture medium, meaning that a bioprocess with high volumetric productivity tends to be more costly overall despite being more cost-efficient. The pursuit of high volumetric productivity should therefore be dependent on the amount of hiPSCs required by the end of a bioprocess. The amount of hiPSCs required to inoculate a bioreactor at high cell densities is also a factor that needs to be considered, with lower cell densities eschewing prolonged and potentially laborious hiPSC expansion prior to bioreactor inoculation. A sufficiently low inoculation density, such as the one used by Cuesta-Gomez et al., also allows for minimal culture medium exchange, which reduces overall cost and promotes high cell yield if near total medium depletion is allowed during batch/fed-batch operation.

An effective strategy for increasing hiPSC volumetric productivity without necessarily increasing inoculation density is to maximize the number of aggregates formed at the start of culture, which was here shown to be correlated with final cell density. This requires determining the ideal initial bioreactor agitation profile during aggregate formation, which can be guided by computational fluid dynamic studies. Existing work in this field has focused mostly on the impact of agitation on aggregate size and usually analyzes static agitation profiles [17, 39], leaving room for more comprehensive studies specifically targeted toward optimizing aggregate formation. Supplementing culture media with polysulfated compounds (e.g., dextran sulfate) that modulate cell surface charge and thus reduce aggregation is also an interesting option to consider [19, 24], although these compounds require further vetting before they can be employed for hiPSC bioprocesses envisaging clinical applications. It should also be noted that hiPSCs do not necessarily need to be cultured as free-floating aggregates, with the use of microcarriers having also been explored in the past [40, 41]. However, they entail additional

costs and hinder final cell recovery [42], making them an unappealing alternative compared to spontaneously forming hiPSC aggregates.

Optimal culture medium exchange is crucial for maximizing volumetric productivity while maintaining a high cell yield. This entails consuming the least amount of medium possible while ensuring that hiPSC proliferation and pluripotency are not negatively affected. Perfusion is indubitably the most advantageous bioreactor operation mode in this regard, despite batch and fed-batch operation remaining prominent in the context of hiPSC expansion [43]. Conspicuously, in a majority of cases perfusion is implemented in such a way as to have either a constant flow rate or one that varies in a discrete stepwise manner [15, 20, 21, 44], which can limit its associated benefits to a certain degree. Ideally, perfusion flow rate should be adjusted continuously and dynamically based on the inline monitoring of key bioprocess parameters (e.g. glucose concentration, oxygen uptake, viable cell density) or at the very least it should follow an expected evolution predicted from process knowhow or in silico modeling. Although dynamic perfusion entails added complexity, this is compensated by its potential to eliminate wasteful medium consumption. The current work showed an innovative strategy for implementing dynamic perfusion successfully in an STBR system, even though future improvement is still possible.

Dynamic perfusion constitutes a challenge when it comes to estimating cellular metabolite uptake and secretion rates. Traditionally, this is done according to Equation 1:

$$q_t = \left[\frac{C_{t+\Delta t} - C_t}{\Delta t} + \frac{r}{V} \cdot (\bar{C}_{\Delta t} - I) \right] \cdot \frac{1}{\bar{X}_{\Delta t}} \quad (1)$$

where q is the uptake/secretion rate, C is the metabolite concentration, Δt is the time elapsed between two consecutive data points, r is the perfusion flow rate, V is the bioreactor volume, $\bar{C}$ is the logarithmic mean of metabolite concentration, I is its concentration in fresh inflow medium, $\bar{X}$ is the logarithmic mean of cell density and t indicates the earlier data point. Unlike constant or stepwise perfusion, dynamic perfusion presents a continuously changing r . While an average r could also be calculated, the compounded error of averaging perfusion flow rate, metabolite concentration and cell density over a prolonged period, usually a day, means that Equation 1 is unlikely to yield accurate results. As a better alternative, a recursive multi-step forecasting metabolic model integrated into a binary search algorithm

was developed. Instead of performing a simple calculation, the model interpolates the evolution of a metabolite's concentration between two consecutive data points and checks if its prediction matches the original measurements. Despite being more computationally intensive, this type of modeling can provide a more reliable and more informative estimate of uptake/secretion rates, as long as its assumptions remain valid.

One of the main reasons for accurately characterizing hiPSC metabolism is understanding how it can be related to proliferative capacity. The Monod equation is perhaps the most well-known model for relating cell growth rate with the concentration of a limiting nutrient [45]. Even though it was originally derived for bacterial cultures it is also commonly employed for describing mammalian cell growth. Here it was found to do so with reasonable accuracy when considering glucose alone as a limiting nutrient. As per the expression derived from the data obtained when culturing hiPSCs with S8, a minimum glucose concentration of 3.63 mM is required for keeping growth rate above 75% of its maximum value. This means that between day 4 and 5 of culture glucose became a limiting factor. Increasing the maximum flow rate of the implemented dynamic perfusion strategy would help to overcome this limitation, but a more efficient approach could be to simply increase the glucose concentration in the inflow medium, as long as excess glucose does not prove to be detrimental to hiPSC health. Although osmolarity above physiological levels can impair cell health once a critical threshold is exceeded, Manstein et al. demonstrated that glucose concentration could be increased from the typical 3.15 g/L (used in this study) up to 7.65 g/L without noticeable adverse effects, a 2.4× increase which was essential for achieving the highest possible cell yield and volumetric productivity. In fact, it is interesting to note that this increase in glucose concentration almost perfectly coincides with the ratio between maximum cell yield achieved by Manstein et al. and that achieved in this study (2.3×). It is reasonable to assume that adapting the same supplementation regime would proportionally increase the cell yield obtained with S8.

Overall, the cell yield and volumetric productivity of the established S8-based hiPSC expansion bioprocess proved to be within reach of the best values found in the literature. With further optimization, namely by adopting glucose supplementation and reducing the amount of culture medium dynamically

perfused through the STBR during the early phases of culture when glucose concentration is still well above the threshold at which it becomes restrictive to hiPSC proliferation, the S8-based bioprocess would likely attain or surpass these benchmark values. As it stands, the S8-based bioprocess presents itself as an economically accessible alternative to the bioprocess developed by Manstein et al., which requires the consumption of a roughly 7× greater volume of culture medium (3.5 L as opposed to 0.4-0.5 L). At the same time, it provides a higher volumetric productivity than the bioprocess developed by Cuesta-Gomez et al., allowing for the efficient production of a greater amount of hiPSCs per batch. As such, the S8-based bioprocess here shown constitutes a relevant middle ground between the Manstein et al. and Cuesta-Gomez et al. bioprocesses.

Within a relevant hiPSC bioprocessing context, S8 proved capable of sustaining rapid cell growth, supporting the formation of aggregates with ideal size and morphology, promoting active glycolytic metabolism and fully preserving pluripotency and genomic stability, all of which were systematically assessed. When coupled with an optimal STBR agitation and inoculation regime, it led to the highest initial aggregate density found in the literature, although this is difficult to fully ascertain due to many studies not reporting this metric. Moreover, preliminary tests showed that S8 (PDT = 17.6 hours) improved cell proliferation comparatively to well-established PSC-CM such as B8 (PDT = 21.4 hours), mTeSR™1 (PDT = 22.4 hours) and E8 (PDT = 20.9 hours) when culturing hiPSCs in an STBR operated under equivalent conditions (Additional file 1: Fig. S2). Given the reduced cost of S8 relative to comparable media and the very promising results obtained so far, S8 has revealed itself to be a novel alternative with potential to outperform the PSC-CM currently employed for hiPSC bioreactor culture.

On a final note, although the CQAs analyzed in this work are useful for monitoring hiPSC quality in a bioprocessing context, the cursory characterization they provide is insufficient for clinical scenarios, which demand more comprehensive evaluation of genomic stability [46]. When subjected to prolonged culture, hiPSCs are at risk of developing genomic abnormalities such as aneuploidies, submicroscopic copy number variations, oncogenic mutations and single nucleotide variants. Screening for these is indispensable and entails, aside from karyotyping, techniques such as array comparative genomic hybridization, targeted next-generation sequencing and whole-genome/exome sequencing. Only through these is it possible to categorically ensure that cells derived from a given hiPSC line do not

present an added risk of tumorigenicity or aberrant behavior. Full-fledged validation of S8 would thus require the application of these techniques, with this remaining a relevant avenue for future research.

Conclusions

Despite a myriad of promising pharmacological and therapeutic applications, the potential impact of hiPSCs remains constrained by manufacturing challenges. Current production methods struggle to deliver the billions of hiPSCs required in a cost-efficient and quality-assured manner. The solution to this is improving the culture of hiPSCs in bioreactors, which can achieve high volumetric productivity and allow continuous monitoring and control of critical process parameters, ensuring that hiPSCs conform to critical quality attributes and facilitating bioprocess optimization. Central to bioreactor operation is the culture medium employed, which must favorably balance rapid hiPSC proliferation and pluripotency maintenance with a streamlined formulation that minimizes cost and batch-to-batch variability. The work here described validates HiDef® S8®, a novel suspension 3D aggregate PSC-CM that successfully achieves this balance. For validation, an S8-based STBR expansion bioprocess for hiPSCs was developed, which was then compared with two hiPSC expansion bioprocesses which stood out amidst the literature for their high cell yield and/or volumetric productivity. The S8-based bioprocess resulted in a cell yield and volumetric productivity similar to these best-performing bioprocesses. S8 led to the formation of healthy aggregates, promoted active glycolytic metabolism, typical of hiPSCs, and did not compromise hiPSC pluripotency or genomic stability even at relatively high cell densities (5×10^6 cells/mL). Moreover, it outperformed HiDef® B8, mTeSR™1 and Essential 8™ in a preliminary head-to-head comparison. Concomitantly, it preserved the cost reductions and animal-origin-free componentry introduced by the B8 formulation, from which it was adapted. Of note are its simple, fully-defined and animal-component free nature, unlike that of most other suspension-oriented PSC-CM, making it an ideal candidate for potential clinical applications. Overall, S8 represents a significant advancement in suspension-oriented PSC-CM and hiPSC cost-efficient bioprocess development. Future work should focus on further improving the cell yield and volumetric productivity achievable with S8 by optimizing STBR operation and implementing already proven strategies such as

glucose supplementation and in silico metabolic modelling. Given the results already obtained, S8 will play an important role in bringing the envisaged applications of hiPSCs closer to reality.

Methods

hiPSC planar culture

Throughout this work, the Gibco human episomal induced pluripotent stem cell line (cat# A18945) was employed. This hiPSC line was derived from CD34⁺ cord blood through EBNA-based episomal transfection of factors SOX2, OCT4, KLF4, C-MYC, NANOG, LIN28 and SV40 T antigen. Before STBR inoculation, hiPSCs were expanded for 2 passages on 6-well cell culture plates coated with Matrigel[®] (Corning) diluted by a factor of 1:500. hiPSCs were stored in a humidified incubator at 37 °C and 5% CO₂, being maintained in S8 culture medium, exchanged daily or every other day, according to cell confluence. For planar culture, S8 was composed of 99.5% DMEM/F12 (Corning) plus S8 supplement (Defined Bioscience) and 0.5% penicillin/streptomycin (Gibco). Once hiPSCs reached around 80% confluence, they were passaged at a split ratio of 1:6 using 0.5 mM EDTA (Thermo Scientific). hiPSCs were transferred to suspension culture once 24 wells of 6-well cell culture plates presented roughly 80% confluence.

hiPSC suspension culture

The STBRs used in this work belonged to the DASbox[®] Mini Bioreactor System (Eppendorf) and were operated through the DASware[®] Control software. STBRs were equipped with a 60° pitch 8-blade impeller, temperature, pH and DO sensors, as well as ports for liquid and gas transfer. Temperature was kept at 37 °C, pH was kept at 7.2 through CO₂ gassing and base addition and DO was kept at 40% relative to air. Headspace aeration rate was kept constant at 5 sL/h (approximately 1 vvm). Agitation rate was increased gradually throughout culture, starting at 20 rpm, rising in a linear fashion to 60 rpm by the 1st hour, 80 rpm by the 1st day and 120 rpm by the end of culture. S8 culture medium, composed of 98.5% DMEM/F12 plus S8 supplement, 1% 0.1 g/mL polyvinyl alcohol (Sigma) and 0.5% penicillin/streptomycin was used throughout culture. Starting on day 2, medium was exchanged through perfusion using the bioreactor system's integrated peristaltic pumps, whose flow rate (r) was defined dynamically according to Equation 2:

$$r = r_{min} + (r_{max} - r_{min}) \cdot \left(\frac{s^{\omega} - 1}{s - 1} \right) \quad (2)$$

where r_{min} is the minimum flow rate, r_{max} is the maximum flow rate, s is the flow rate scaling factor and ω is the oxygen offset ratio, defined by the difference of the O_2 fraction in the inflow gas stream (XO_2) relative to a baseline, as per Equation 3:

$$\omega = \frac{XO_{2,PV} - XO_{2,BL}}{XO_{2,max} - XO_{2,BL}} \quad (3)$$

where PV signifies present value, BL baseline value, and max the maximum XO_2 the bioreactor system is capable of providing (i.e., 0.21 when using air, 1 when using pure O_2). Here, $XO_{2,BL}$ was set to 0.084, r_{min} to 0.5 bioreactor volumes per day, r_{max} to 3 bioreactor volumes per day and s to 2. A sintered metal filter with 10-20 μm pore size was used for retaining hiPSCs within the STBR during perfusion.

One hour before inoculation, 10 μM of ROCK inhibitor Y-27632 (STEMCELL Technologies) was added to the medium of ready hiPSCs in planar culture. After this, hiPSCs were collected by incubating them with Accutase (Sigma) at 37 °C for 7 min and mechanically dissociating them with a micropipette. Next, 10.625×10^6 hiPSCs were resuspended in 10 mL of S8 and inoculated into an STBR, already filled with 75 mL of S8, amounting to an overall inoculation density of 1.25×10^5 cells/mL. For inoculation, S8 was supplemented with 10 μM Y-27632. Remaining cells were either fixed in 2% paraformaldehyde (PFA) (Sigma) or frozen at -80 °C as dry pellets.

STBR culture was fully automated, with human intervention being limited to daily sample extraction. Roughly 20% of aggregates per sample were reserved for imaging, with the remaining aggregates being separated from their supernatant, which was stored at -20 °C, before being dissociated with Accutase at 37 °C for 9 min and employed for cell counting. On the last day of culture, additional samples were extracted, with some aggregates being fixed in 4% PFA and some dissociated cells being fixed in 2% PFA or being frozen at -80 °C as dry pellets.

580 **Growth kinetics and bioprocess metrics analysis**

Growth kinetics were determined by manually counting viable hiPSCs obtained from dissociated aggregates with a hemocytometer, using the trypan blue dye exclusion test. Cell growth was considered to be exponential when calculating population doubling time (PDT), which was done with Equation 4:

$$PDT_d = \frac{\ln(2)}{\mu_d} \quad (4)$$

where μ_d is the cell growth rate of day d , calculated with Equation 5:

$$\mu_d = \ln\left(\frac{N_{d+1}}{N_d}\right) \quad (5)$$

where N_d and N_{d+1} are the cell number on day d and its subsequent day, respectively. PDT cannot be calculated for negative μ (i.e., day 0 which saw a decrease in cell number).

Cumulative cell yield (CY) was determined based on Equation 6:

$$CY_d = \frac{N_d - N_{t_0}}{\sum_{t_0}^d V} \quad (6)$$

where t_0 is the moment of inoculation and $\sum_{t_0}^d V$ is the total volume of medium consumed up to day d .

Volumetric productivity (VP) was here defined according to Equation 7:

$$VP_d = \frac{N_{d+t} - N_d}{WV \cdot t} \quad (7)$$

where WV is the working volume of the STBR, and t is the number of days elapsed.

Aggregate dynamics analysis

Aggregates imaged through brightfield microscopy were imaged immediately after collection, while those imaged with fluorescence microscopy were first submitted to live/dead staining. This consisted in incubating aggregates in their original medium supplemented with 2 μ M of calcein AM (Sigma) and 2 μ M ethidium homodimer I (Sigma) at 37 °C for at least 20 min. In both instances, images were acquired using a DMI3000 B microscope (Leica) paired with a DFC7000 T camera (Leica). For visualizing internal aggregate morphology, aggregates previously fixed in 4% PFA were permeabilized by incubating them in 0.1% Triton X-100 (Sigma) for 1 hour, before staining their cell nuclei by incubating them in a 1 μ g/mL DAPI (Invitrogen) solution for 1 hour. DAPI images were acquired using an LSM 900 with Airyscan 2 confocal microscope (ZEISS).

Live/dead images were used to determine aggregate density, diameter and eccentricity. The FIJI software was used to manually binarize images, after which they were segmented and measured using the scikit-image Python library [47]. Aggregate diameter (D) was calculated by assuming aggregates presented an approximately circular cross section, as per Equation 8:

$$D = \sqrt{4A/\pi} \quad (8)$$

where A is an aggregate's cross-sectional area.

The coefficient of variation (CV_D) of aggregate diameter was computed through Equation 9:

$$CV_D = \frac{STD_D}{M_D} \cdot 100\% \quad (9)$$

where STD_D and M_D are the standard deviation and mean of aggregate diameters, respectively.

Aggregate eccentricity (E), which ranges from 0 (circle) to 1 (very elongated shape), was determined according to Equation 10:

$$E = \frac{f}{a} \quad (10)$$

where f is the focal points distance of an aggregate's equivalent ellipse and a is its major axis length.

Metabolic analysis

Supernatants had their glucose and lactate concentrations measured using a 2950D Biochemistry Analyzer (YSI). Given that the implemented perfusion strategy was dynamic in nature and that supernatants were only sampled daily, qG/qL could not be directly computed from them. To overcome this, a recursive multi-step forecasting model was developed. The model interpolates intermediate metabolite concentration values between each pair of consecutive days by simulating continuous cellular glucose uptake and cellular lactate secretion coupled with continuous medium exchange. It assumes that cell growth follows an exponential trend and that between two successive days qG/qL remains constant. Daily best-fitting qG/qL were determined by incorporating the model into a binary search algorithm (Fig. 6). Best-fitting was defined as the qG/qL that resulted in a final model glucose/lactate concentration ($[M]$) with an absolute relative error (E) less than 0.1% comparatively to the respective offline glucose/lactate concentration measurement ($[R]$).

Apparent yield of lactate from glucose was defined as per Equation 11:

$$AY_d = \frac{qL_d}{qG_d} \quad (11)$$

The relationship between μ and glucose concentration ($[G]$) was considered to be described by the Monod equation (Equation 12):

$$\mu = \frac{\mu_{max} \cdot [G]}{K_G + [G]} \quad (12)$$

where μ_{max} is the maximum growth rate and K_G is the half-saturation constant.

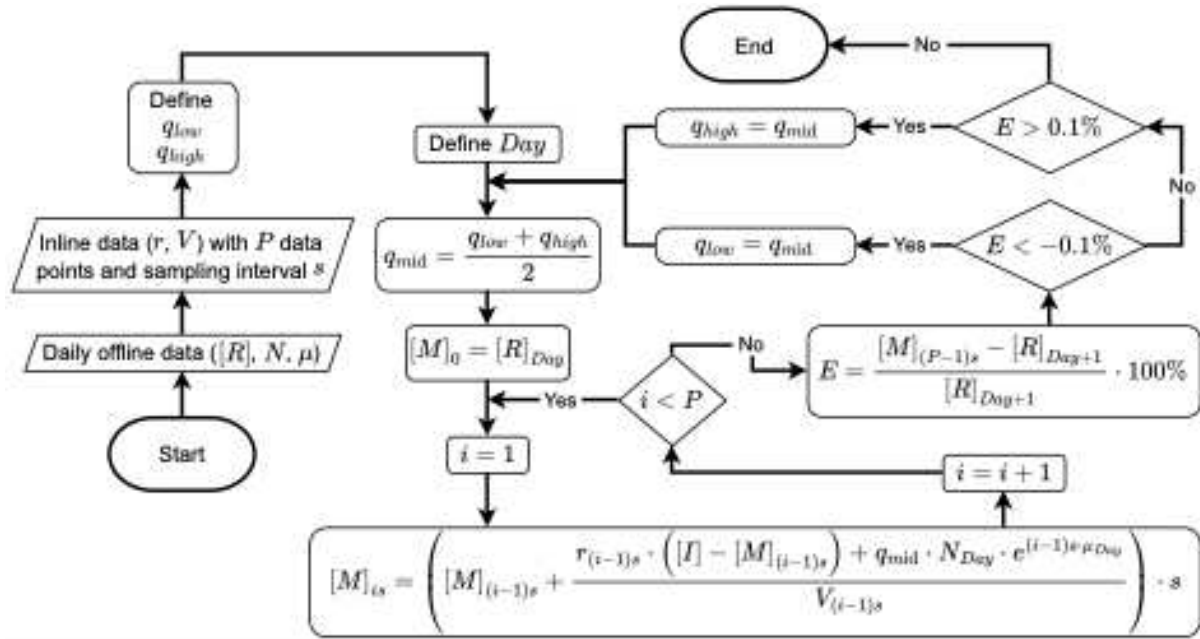


Fig. 6 Binary search algorithm for determining qG/qL through a recursive multi-step forecasting model. The algorithm finds the qG/qL that best fit glucose/lactate offline concentration measurements ($[R]$) for two consecutive days along with offline data, namely cell number (N) and growth rate (μ), inline bioreactor data, namely perfusion flow rate (r) and volume (V), user-defined constraints, namely minimum (q_{low}) and maximum (q_{high}) qG/qL values, and the metabolite concentration of fresh inflow medium ($[I]$). For its first solution attempt, the algorithm considers the midpoint (q_{mid}) between q_{low} and q_{high} . Starting from the offline concentration measured on the earlier day, the model uses its inputs and q_{mid} to recursively predict concentration ($[M]$) for each data point (i) of inline bioreactor data. If the prediction of the final data point does not match the offline concentration measured on the latter day, the algorithm redefines q_{low} or q_{high} as being equal to q_{mid} , depending on whether the prediction was an underestimation or overestimation, respectively. The algorithm then recalculates q_{mid} and begins a new solution attempt. This process continues until a nearly perfectly matching prediction is found, with the q_{mid} of this last attempt being selected as the best-fitting qG/qL. The model assumes that cellular growth is exponential and that qG/qL do not change in the timespan of each offline concentration measurement pair.

Flow cytometry

Cells previously fixed with 2% PFA were stained for the expression of intracellular (OCT4 and SOX2) and surface (SSEA4) proteins. After each of the washing and incubation steps described below, cells were centrifuged at $300 \times g$ for 3 min preceding supernatant removal.

For intracellular staining, a washing solution (WS) composed of 1% fetal bovine serum (FBS) (Gibco) and an incubation solution (IS) composed of 3% FBS were used. Cells were first washed with WS and permeabilized by incubation in a 1:1 mixture of IS and 1% saponin (Sigma) for 15 min. Following this, cells were incubated in IS for 15 min, before being incubated in IS with primary antibodies for 90 min. A dilution of 1:300 was used for OCT4 (mouse IgG anti-OCT4, Merck Millipore, MAB4419) and 1:150 for SOX2 (mouse IgG anti-SOX2, R&D Systems, MAB2018). Cells were then washed twice with WS and incubated in IS with secondary antibody for 45 min. The secondary antibody used was anti-mouse

IgG Alexa Fluor 488 (Invitrogen, A-11001), with a dilution of 1:300. After two more washes with WS, cells were resuspended in phosphate-buffered saline (PBS) (Gibco) and subjected to flow cytometry. Negative controls were processed equally, with the sole exception that primary antibody was not added.

For surface staining, cells were first washed with PBS, followed by incubation in PBS with a 1:50 dilution of SSEA4 (PE-conjugated human IgG anti-SSEA4, Miltenyi, 130-128-254) for 10 min. Cells were then washed twice with PBS before being resuspended in PBS and subjected to flow cytometry. Negative controls were processed equally, with the sole exception that antibody was not added.

Flow cytometry was performed using a BD FACSCanto flow cytometer (BD Biosciences) and its associated BD FACSDiva software. Acquired data was analyzed with the Flowing Software (University of Turku), with a minimum of 10,000 events being considered per sample. The positive event gate was adjusted to contain between 1% and 2% of false positives, according to the negative control peak. An illustrative subset of flow cytometry data can be found in Additional file 1: Fig. S3.

Quantitative real-time polymerase chain reaction

Cell pellets stored at -80 °C had their total RNA extracted using a High Pure RNA Isolation Kit (Roche). RNA purity and concentration were then assessed using a NanoVue Plus Spectrophotometer (GE HealthCare), after which 1 µg of RNA from each sample was converted to first-strand cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Conversion took place in a Thermal Cycler XT⁹⁶ (VWR) with an incubation protocol of 25 °C for 10 min, 37 °C for 2 h and 85 °C for 5 min. Following this, qRT-PCR reactions were prepared by mixing 12.5 ng of cDNA with NZYSpeedy qPCR Green Master Mix (2×), ROX plus (NZYtech) and forward and reverse primers specific to *GAPDH*, *OCT4*, *NANOG*, *SOX2*, *SOX1*, *TBXT* and *SOX17*. Each reaction was performed in triplicate. Reactions took place in a StepOnePlus Real-Time PCR System (Applied Biosystems) with a cycling protocol of 95 °C for 10 min followed by 40 cycles intercalating between 95 °C for 15 sec and 60 °C for 1 min. Cycle threshold (CT) values of each sample's pluripotency and differentiation genes were normalized by the corresponding expression of the housekeeping gene *GAPDH*, generating ΔCT . Normalized relative gene expression was computed as $2^{-\Delta CT}$. The employed primer sequences can be found in Additional file 1: Table S1.

Trilineage differentiation

hiPSC aggregates were extracted at the end of STBR culture and resuspended in fresh S8. Around 10 aggregates were placed per well of an 8-well μ -slide (Ibidi), previously coated with 1:500 Matrigel[®]. On day 1, after cells had attached and spread out, S8 was exchanged for trilineage differentiation medium (TDM). TDM was composed of 77.5% DMEM/F12, 20% FBS, 1% MEM non-essential amino acids, 1% 0.1 mM 2-mercaptoethanol and 0.5% penicillin/streptomycin (all from Gibco). TDM was replaced every other day. On day 11, after 10 days of differentiation, cells were fixed in 4% PFA.

Immunostaining

For intracellular staining, a blocking solution (BS) composed of 10% FBS and 0.1% Triton X-100 and an incubation solution (IS) composed of 5% FBS and 0.1% Triton X-100 were prepared. Spontaneously differentiated cells, previously fixed in 4% PFA, were washed with PBS and permeabilized by incubating them in BS for 1 hour. Following this, cells were incubated in IS supplemented with primary antibodies for 2 hours. A dilution of 1:200 was used for TUBB3 (mouse IgG anti-TUBB3, Merck Millipore, MAB1637), 1:200 for α -SMA (mouse IgG anti-SMA, Dako, M0851) and 1:500 for GATA4 (mouse IgG anti-GATA4, Santa Cruz Biotechnology, sc-25310). Cells were then washed twice with PBS before being incubated in IS with secondary antibody for 1 hour. The secondary antibody used was anti-mouse IgG Alexa Fluor 488, with a dilution of 1:500. Cells were once more washed twice with PBS, after which they had their nuclei stained by incubating them in a 0.1 μ g/mL DAPI solution for 5 min. Finally, cells were washed three times with PBS. Negative controls were processed equally, with the sole exception that primary antibody was not added (Additional file 1: Fig. S5). Trilineage differentiation images were acquired using an Eclipse Ti microscope (Nikon) paired with a Zyla 4.2 PLUS camera (Andor).

Karyotyping

Karyotyping was conducted by SYNLAB, an accredited (NP EN ISO 9001) clinical laboratory. Cytogenetic analysis was performed on 30 metaphase spreads using GTL banding, with an average resolution of 500 bands per haploid set. The analysis excluded with a high degree of probability (>95%)

the occurrence of mosaicism at a frequency greater than 10% of the total cell population. Low-level mosaicism below this threshold cannot be excluded.

Statistical analysis

The results presented in this work were obtained from three independent STBR replicates. Daily culture performance metrics were derived from two independent aggregate samples extracted and processed for each STBR replicate. Quality analysis assays were executed for a single sample from the start and end of culture for each STBR replicate. For data reported in the text, values are expressed as experimental mean $\pm$ standard error of the mean (SEM). For data shown in line plots, error bars represent SEM of individual replicate measurements and shaded area bands represent SEM of combined replicate measurements. For data shown in bar plots, error bars represent SEM of combined replicate measurements. Plots were prepared using the matplotlib Python library [48]. Statistical analysis was performed using the NumPy, pandas and SciPy Python libraries [49–51]. To assess if differences between two days of culture were significant, STBR replicates were compared using a paired t-test. Differences were considered statistically significant at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Supplementary Information

Additional files

Additional file 1 (.docx): Fig. S1 hiPSCs cultured in a stirred-tank bioreactor as free-floating aggregates with B8 and mTeSR1. **Fig. S2** hiPSCs cultured in a stirred-tank bioreactor as free-floating aggregates with B8, mTeSR1, E8 and S8. **Fig. S3** Flow cytometry population gating and pluripotency marker histograms. **Fig. S4** Live/dead staining control (scale bar = 500 μm). **Fig. S5** Negative controls for antibody staining during characterization of trilineage differentiation (scale bars = 100 μm). **Table 1** Forward and reverse sequences of the primers used for quantitative real-time polymerase chain reaction.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

Author GAC is an employee of Defined Bioscience. Authors JVK and SDR are co-founders and VP of R&D and CEO, respectively, of Defined Bioscience. These authors led the development of HiDef[®] S8[®], the novel hiPSC culture medium evaluated in this work. Author KG is CEO of Phoenestra GmbH. This does not alter the authors' adherence to all the policies of the journal on sharing data and materials. All other authors declare no conflict of interest.

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Author's contributions

WOSS participated in the conceptualization and design of the work here presented, performed all relevant experiments, analyzed the resulting data, prepared the original draft and coordinated the internal review process that resulted in the final draft. DESN, GAC, JVK, SDR, JMSC, KG and CAVR participated in the conceptualization and design of the work here presented and provided substantive revisions for the final draft. GAC, JVK and SDR provided B8 and S8 for the execution of experiments. JMSC, KG and CAVR provided the remaining funding. All authors have read and approved this version of the manuscript.

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