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Application Note

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Efficient Optimization and Scale-up Production of Human IPSC Aggregates and Organoids for Bioprinting

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Abstract

Culturing human induced pluripotent stem cells (hiPSCs) suitable for bioprinting applications requires billions of cells, which can differentiate into the desired cell types for tissue engineering. It is challenging to select optimal, reproducible, and scalable process conditions, and culture media, for generating high-yield, functional hiPSCs. To optimize process conditions, we used the Ambr® 250 Modular multiparallel bioreactor platform to culture hiPSCs in NutriStem® hPSC XF Medium using a range of process parameter combinations generated by MODDE® Design of Experiments (DOE) software. We determined the optimal parameters "sweet spot" for high cell density and aggregate formation using the software and then assessed for scalability by transferring the process into a 2 L benchtop Univessel® Glass stirred-tank bioreactor. After 5 days, utilizing the optimal combination with NutriStem® hPSC XF Medium in Ambr® 250 Modular multiparallel bioreactors, we generated high cell density hiPSCs aggregates (~4 × 10^6 cells/mL), which maintained high viability, performance, and pluripotency over multiple passages, while maintaining appropriately-sized and shaped aggregates. When the optimized process was transferred to a larger scale, it also produced over 4 × 10^6 cells/mL and functional aggregates that had the potential to differentiate into three different germ layers as well as showing high differentiation marker expression for cardiomyocytes. In summary, using the Sartorius suite of bioreactors that are geometrically conserved across scales with optimized media and guided data analytics offers a streamlined strategy to scale up hiPSCs derived aggregates for bioink applications, bringing tissue and organ bioprinting a step closer to commercial reality.

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Introduction

Bioprinting for clinical-scale tissue and organ manufacturing requires processes that can generate clinically-relevant numbers (~10^8 cells/mL)[1] of hiPSCs. These cells also need to form organoids that can differentiate into a range of cell types, allowing them to be functional bioinks[2]. The aim of this study was to identify the optimal cell culture conditions for achieving high cell density and maintaining high cell viability, while preserving consistent aggregate morphology and pluripotency marker expression.

To achieve this, we employed a multi-disciplinary approach that combined computational software with automated, scalable bioreactor systems. The optimized conditions and specialized media were then evaluated in a larger scale stirred-tank bioreactor to assess if this process could be directly scaled.

Key Materials

- NutriFreez® D5 Cryopreservation Salt-Based Solution
- NutriFreez® D10 Cryopreservation Medium
- Ambr® 250 Modular, 8 way configuration
- MODDE® DOE Software
- NutriStem® hPSC XF Medium
- Univessel® Glass, 2L
- Biostat® B-DCU CC 2L

Methods

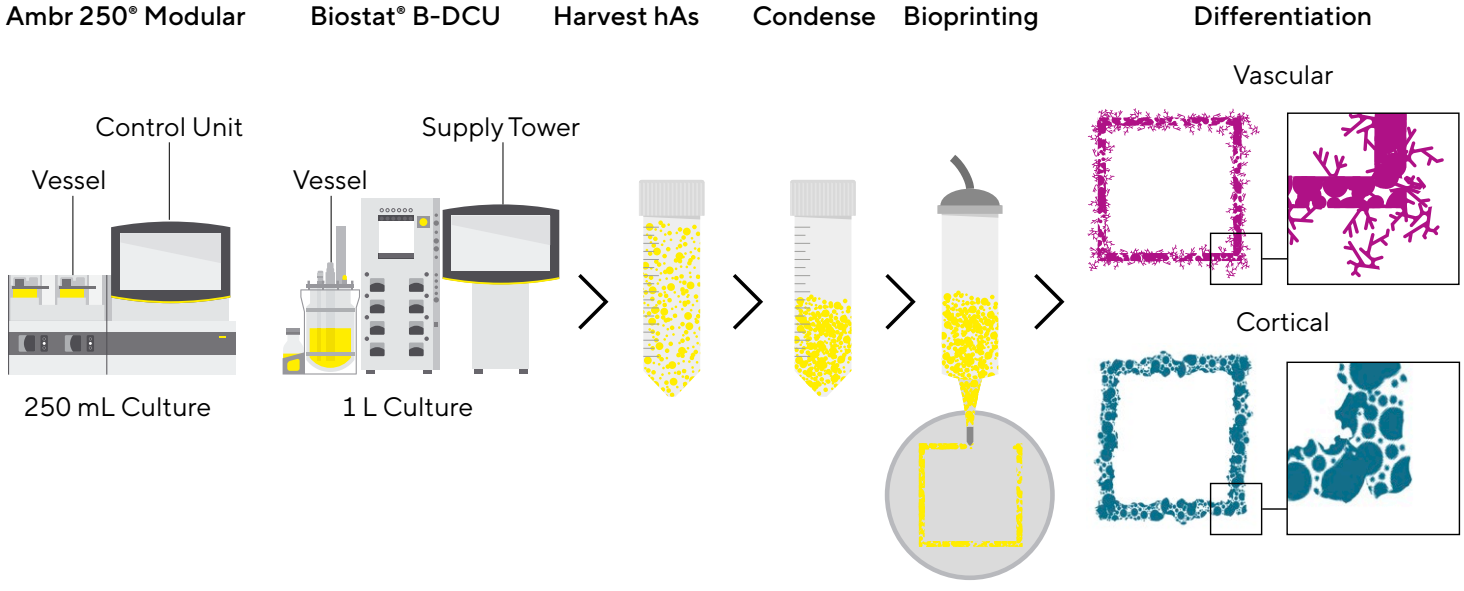
Setting Up a Bioprinting Process

To set up a bioprinting process (figure 1), Ambr® 250 Modular multiparallel bioreactor vessels are connected to a control station , which maintains dissolved oxygen (DO), pH, and temperature at desired setpoints. Mixing rates and human aggregate (hA) suspension are determined by impeller speed, controlled by the workstation. When cells in the aggregates reach the required density (around 1×10^6 cells/mL), they are harvested and centrifuged to produce a concentrated bioink. The hAs bioink is loaded into a syringe and bioprinted in a collagen-Matrigel® matrix to form the desired tissue or organ shape, and the hAs can then be differentiated into cortical or vascular tissue.

Optimizing Factors

To produce a bioink of functional hAs that have sufficiently high cell density requires an optimal combination of process parameters such as impeller speed and cell seeding density. To determine this combination, we generated a DOE that has different impeller speeds (initial and final) and cell seeding densities using MODDE® DOE software (table 1). Then we seeded SCVI-15 hiPSCs (Stanford Cardiovascular Institute Biobank) and WTC-11 hiPSCs (30-year-old Japanese Male, Gladstone Institute) in NutriStem® hPSC XF Medium (240 mL) at a range of seeding cell densities (table 1). NutriStem® hPSC XF Medium was selected as the basal media because this is a xeno-free, serum-free media designed to support culture and expansion of hiPSCs.

Figure 1: *hiPSC Aggregate Production Workflow for Bioprinting and Differentiation.*



The cells were cultured in perfusion culture using a range of impeller speeds (as listed in table 1) for 5 days in Ambr® 250 Modular multiparallel bioreactors, which were controlled by an Ambr® 250 automated bioreactor workstation.

The bioreactor was programmed to maintain a DO level of 25%, a pH of 7.3, and a temperature of 37 °C.

The Ambr® 250 Modular multiparallel bioreactors were selected for this study because they have been demonstrated to be an efficient tool for developing processes for the manufacturing of other human stem cells, such as mesenchymal stem cells (MSCs)[3].

Additionally, they have geometrical height-to-diameter ratios similar to those of larger Sartorius stirred-tank bioreactors such as the Univessel® and Biostat STR®, allowing simpler process transfer across a range of manufacturing scales.

MODDE® DOE software was also used to analyze data from a range of different tests to determine the optimum factor combination for high cell density culture and aggregate formation.

Table 1: *Factor Settings for a Range of Parameters to Be Tested in a DOE.*

Experiment	Initial Speed [RPM]	Final Speed [RPM]	Seed Density [× 10^4 cells/mL]
S_a	150	250	190
S_b	200	250	187
S_b Repeat	200	250	166
S_{low}	100	250	117.5
S_c	150	250	191.5
S_d	200	300	89.76
S_e	200	300	155
S_f	200	400	153.6
S_f Repeat	200	400	110.04
S_g	200	400	217.5
S_h	300	400	119.5
S_{high}	200	500	204.12
S_{mid}	200	200	214.72
S_i	200	275	205.92
S_j	300	300	187

Note. Experiments designated S_a, S_b S_{low}, S_c have either low initial or final stirring speeds (100–250 RPM, initial impeller speed at 100 RPM then increased to 250 RPM over Days 1–5) or seeding densities. Experiments designated S_d, S_e, S_f, S_{mid} have either medium initial (constant speed at 200 RPM) or final stirring speeds or seeding densities. Experiments designated S_g, S_h, S_{high}, S_i, S_j have either high initial or final stirring speeds (200–500 RPM, initial impeller speed at 200 RPM on Day 0–1, then increased to 500 RPM over Days 1–5) or seeding densities.

Assessing Effects of Impeller Speed and Seeding Density

During the 5-day culture in Ambr® 250 Modular multiparallel bioreactors, we sampled multiple aggregate suspensions by centrifuging, followed by washing, and treating them with proteolytic enzymes to produce hiPSC suspensions. Cells were then passaged or assayed. We determined cell density and viability using the Countess™ 3 FL Automated Cell Counter (Thermo Fisher Scientific). We also evaluated cells for the presence of standard hiPSC pluripotency markers (OCT4, NANOG, SSEA-4, TRA-1-60) with immunofluorescence imaging and analyzed cell size and circularity using Brightfield Imaging. Additionally, karyotyping was performed on both cell lines to check the effects of the culture parameters on their chromosomes.

Scaling Up

Optimum impeller and seeding density combinations identified using the MODDE® DOE software were applied to a 2 L scale bioreactor. We seeded SCVI-15 hiPSCs and WTC-11 hiPSCs in NutriStem® hPSC XF Medium (1 L) and cultured them in perfusion culture for 7 days in Univessel® bioreactors (2 L) controlled by a Biostat® B-DCU, which was set to automatically maintain a stirring speed of 113 RPM, 2 L agitation was based on 5.25% DO, pH of 7.3 ±0.2, and temperature at 37 °C. We assessed cell density, viability, cell size and circularity, as well as tested for pluripotency markers (OCT4, NANOG, SSEA-4, TRA-1-60). We used perfusion culture with continuous media exchange in this study because it has been shown to increase yield by preventing nutrient depletion. Additionally, with perfusion culture, we can easily automate it so that no manual handling is required to maintain the culture after the initial set up. NutriFreez® D5 or D10 was used to bank cells using the Sartorius protocols.

Organoid Differentiation

hAs were prepared for differentiation into organoids using a previously described method[5] and the hAs collected from the bioreactor were printed in Matrigel® for maturation. Organoids were fixed with 4% paraformaldehyde on day 15 for analysis of differentiation markers (CD31, VE-cadherin, TUJ1, PAX6, NKX2.5, and cTNT) using immunofluorescence as previously described.

Results

Optimization

The cell density, viability, and circularity data from a 5-day culture using 15 different process parameters (as shown in table 1) were analyzed by MVDA using MODDE® DOE software. The sweet spot plot (figure 2) showed the S_{mid} conditions fulfilled the MVDA criteria and resulted in the highest cell density. The process parameter combination from the S_{mid} experiment (initial and final impeller speed, 200 rpm and ~200 × 10^4 cells/mL seeding density) resulted in a 23-fold expansion of cells, yielding a total of 4.89 million cells/mL in 250 mL of culture by day 5, with 96% viability, as well as aggregates with good circularity scores (~0.9) in the desired size range of 250 – 300 µm for differentiation and 301 – 450 µm for maintenance (figure 3). Additionally, aggregates maintained high cell density, viability, functionality, and normal karyotypes over multiple passages and over 95% of cells expressed pluripotency markers with the S_{mid} parameter combination (figures 4 and 5). Therefore, we selected the S_{mid} factors for all subsequent experiments.

Figure 2: *Sweet Spot Plot for Predicting Optimal Culture Conditions Generated Using MODDE® DOE Software. Yellow Regions Indicate Where the Required Responses Are Met by the Parameter Settings.*

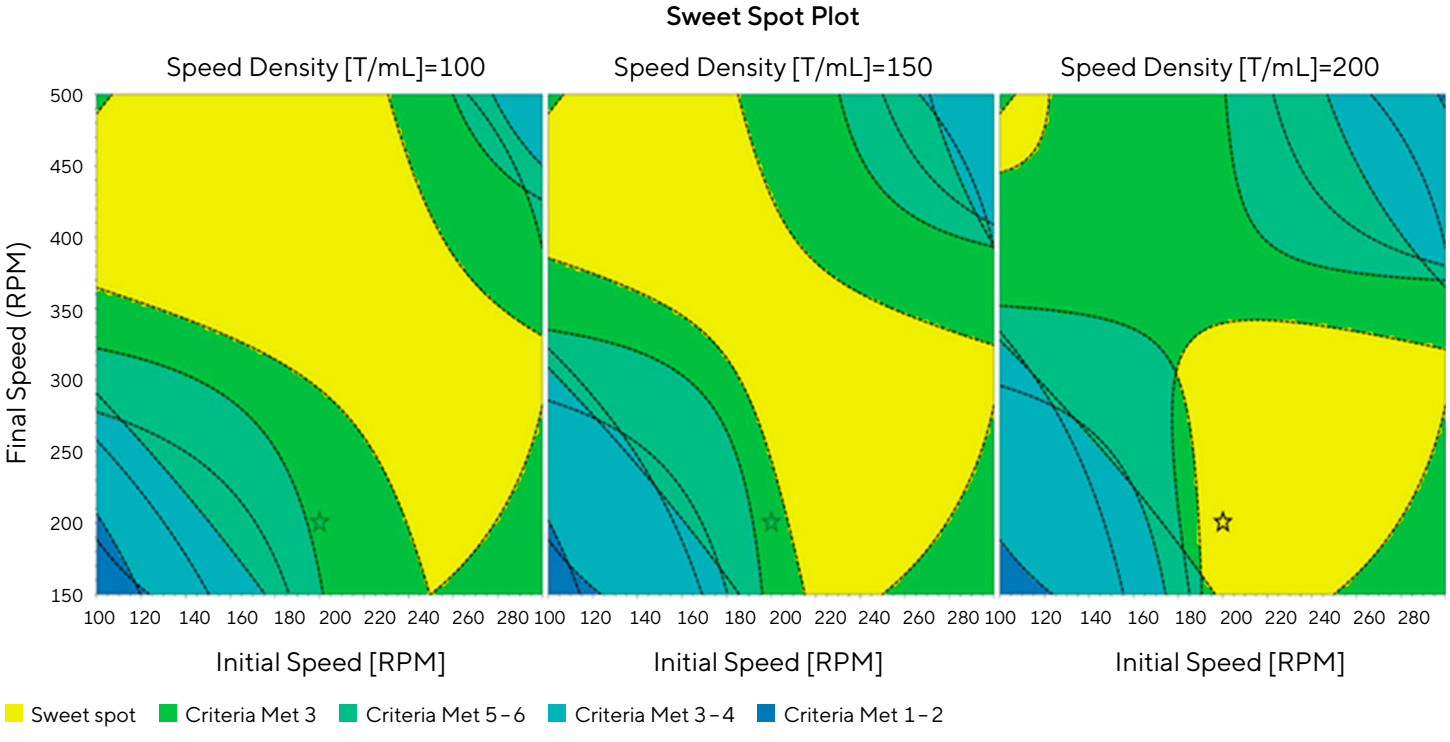


Figure 3: *Optimization of Impeller Speed and Seeding Density Parameters to Produce hiPSC Aggregates With Desired Characteristics for 3D Bioprinting and Differentiation.*

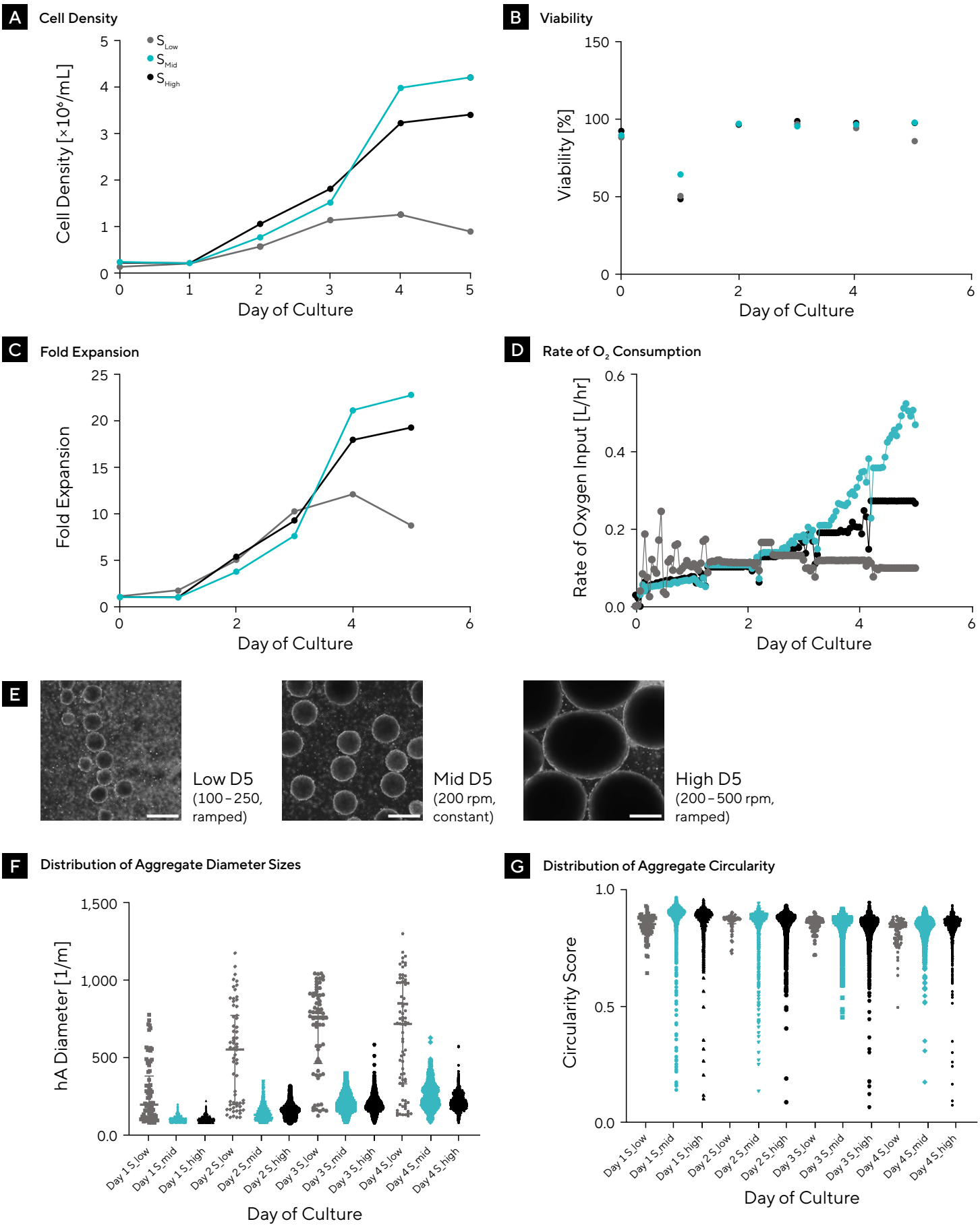
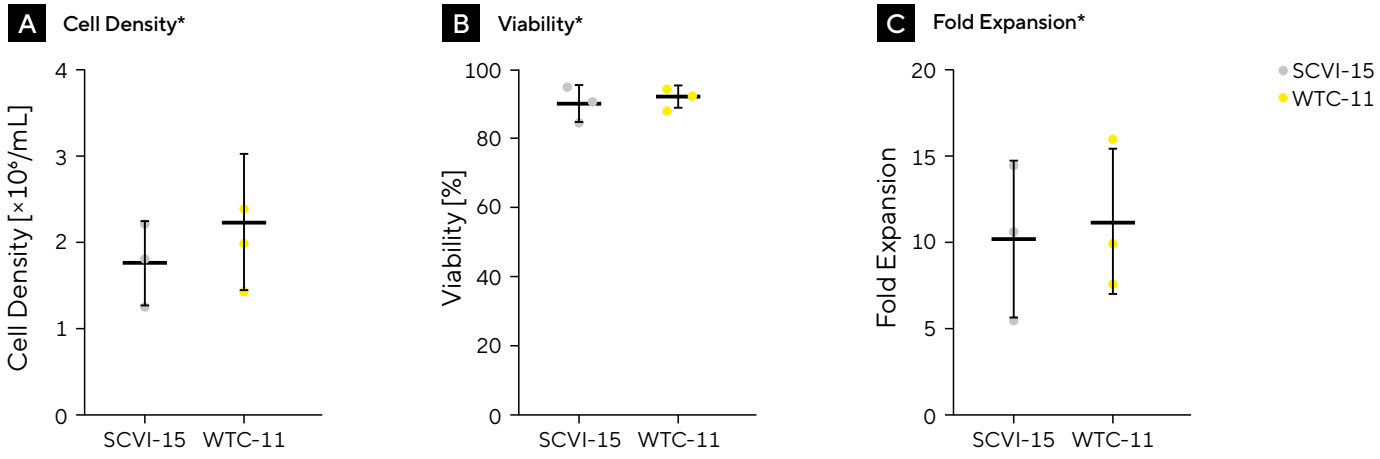
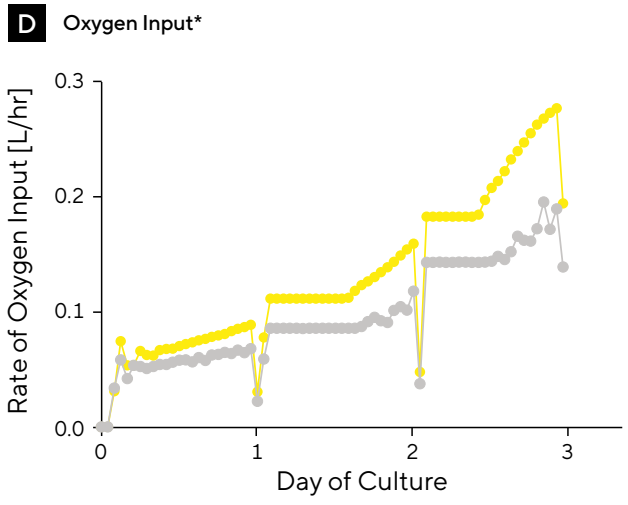


Figure 4: *hiPSC Aggregates Maintain Consistent Growth Rates and Morphologies When Cultured for Multiple Passages in Bioreactors.*





Note.
*n = 3 serial passages. Error bars show mean ± s.d.
**P1, P2, P3 = Passage 1, 2, 3. Median (IQR) shown. SCVI-15: P1, n = 3,084 hAs; P2, n = 2,828 hAs; P3, n = 3,911 hAs; WTC-11: P1, n = 5,674 hAs; P2, n = 6,333 hAs; P3, n = 10,488 hAs.

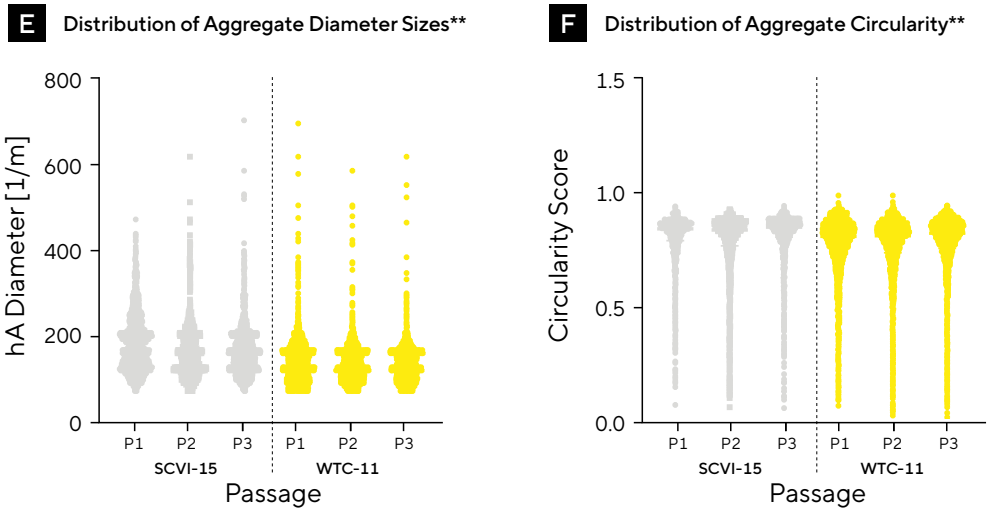
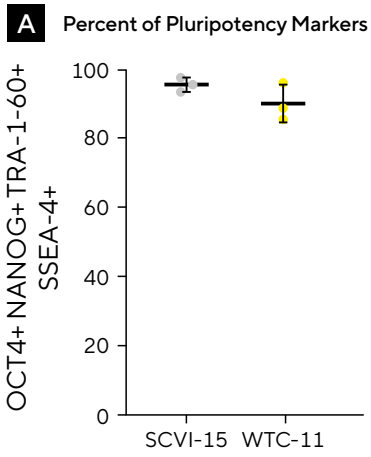
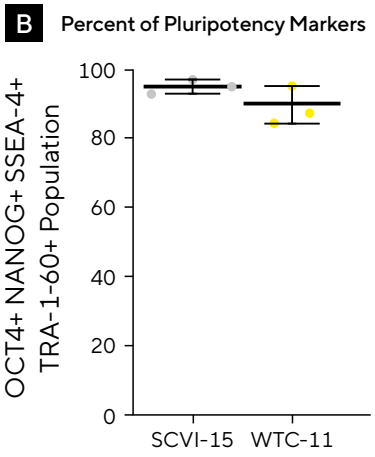


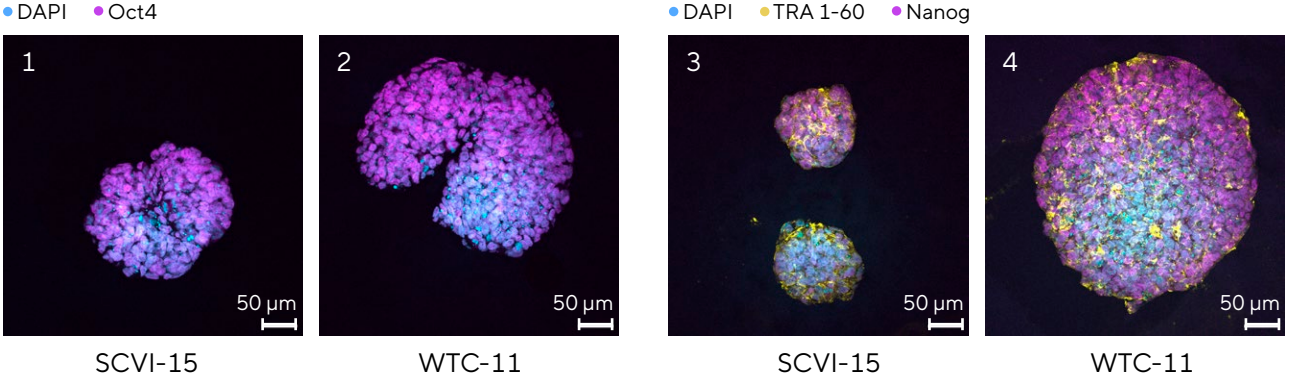
Figure 5: *hiPSC Aggregates Maintain High Pluripotency and Normal Karyotypes When Cultured in Bioreactors.*





SCVI-15
WTC-11

Note.
(A) and (B) Showing percentage of SCVI-15 hiPSCs and WTC-11 hiPSCs that have cell surface pluripotency markers (OCT4, NANOG, SSEA-4 and TRA-1-60), n = 3. Error bars show mean ± s.d.
(C) Qualitative images of SCVI-15 hiPSCs and WTC-11 hiPSCs immunostained to show pluripotency markers (OCT4, TRA-1-60, NANOG, SSEA-4) Sampled on Day 3.



Scale-up

In a scaled-up perfusion culture in a Univessel® 2 L benchtop bioreactor (figure 6), we used similar process parameters to those used in the Ambr® 250 Modular multiparallel bioreactors (pH 7.3 ± 0.2, temperature: 37°C, DO: 5.25%, NutriStem® hPSC XF Medium, and basic fibroblast growth factor (bFGF): 40 ng/mL. The SCVI-15 hiPSCs and WTC-11 hiPSCs, which were cultured in NutriStem® hPSC XF Medium using the S_{mid} factor combination (initial and final impeller speed, 200 rpm and ~200 × 10^4 cells/mL seeding density) showed comparable high cell density, viability, and aggregate formation compared to being cultured in Ambr® 250 Modular multiparallel bioreactors. The S_{mid} factors produced ~ 4 × 10^6 cells/mL (~20-fold expansion), with >95 % viability, as well as aggregate with good circularity scores (~0.9) (figure 7) and high expression of pluripotency markers with both cell lines on day 0 and day 4. These results indicate that the process optimized in the Ambr® 250 Modular with NutriStem® hPSC XF Medium can be transferred to larger scale to generate high cell density, functional hiPSCs.

WTC-11 cells had a normal karyotype (Data not shown). In contrast, a proportion of the SCVI-15 cells tested (8 out of 20) had portions of the long (q) arm of chromosome 1 that were duplicated (Data not shown). Given that a proportion of the SCVI-15 hiPSCs cultured in the Ambr® 250 Modular and Univessel® bioreactors are biologically independent cultures which originated from the same stocks, and yet contain identical 1q duplications, it is possible that the karyotypic abnormalities found in the SCVI-15 hiPSCs may be a result of a preferential expansion of a pre-existing subpopulation of cells bearing the 1q duplication in the stocks. Taken together, our results show that hA culture can be robustly scaled up to 1 L with consistent growth rates and morphologies while maintaining high rates of pluripotency.

Figure 6: *Scaled-up hiPSC Aggregate Production Workflow for Bioprinting and Differentiation.*

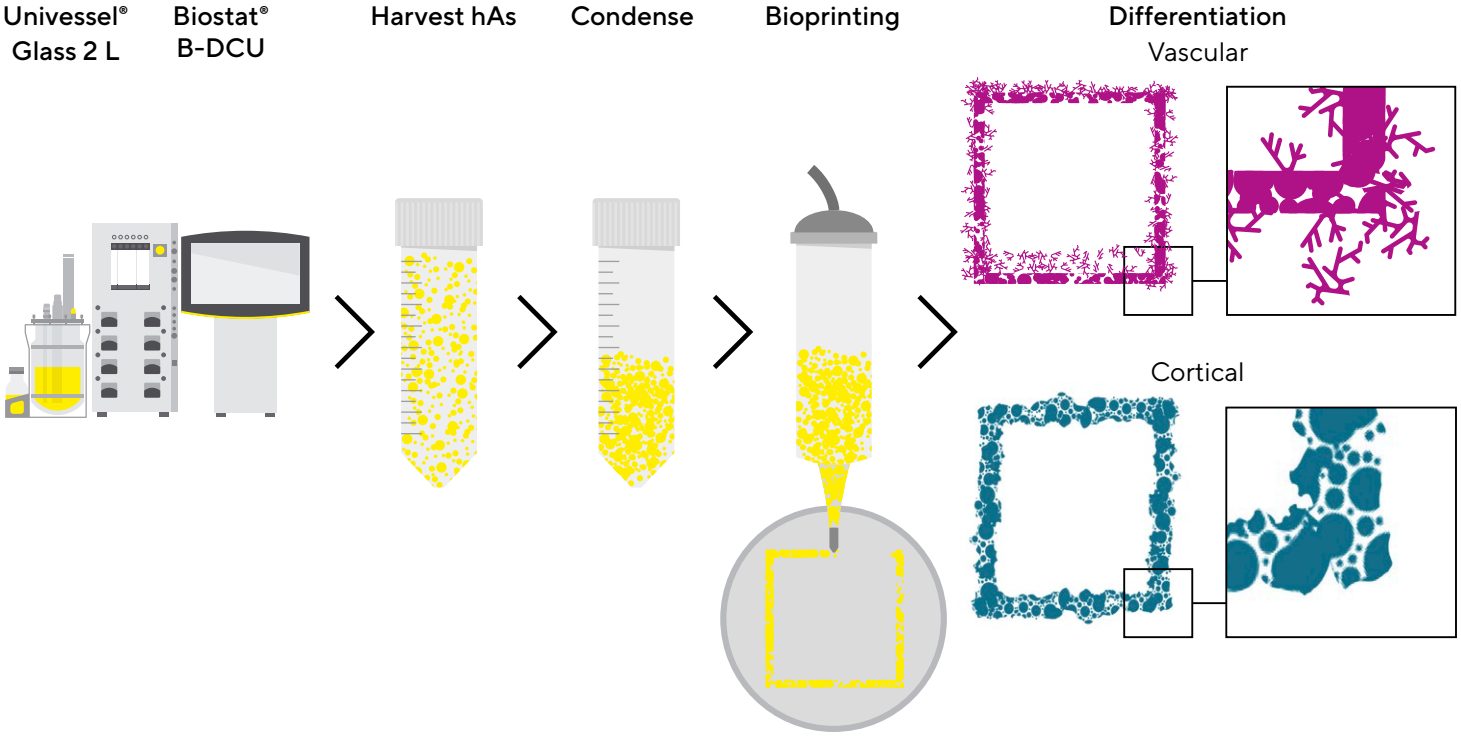
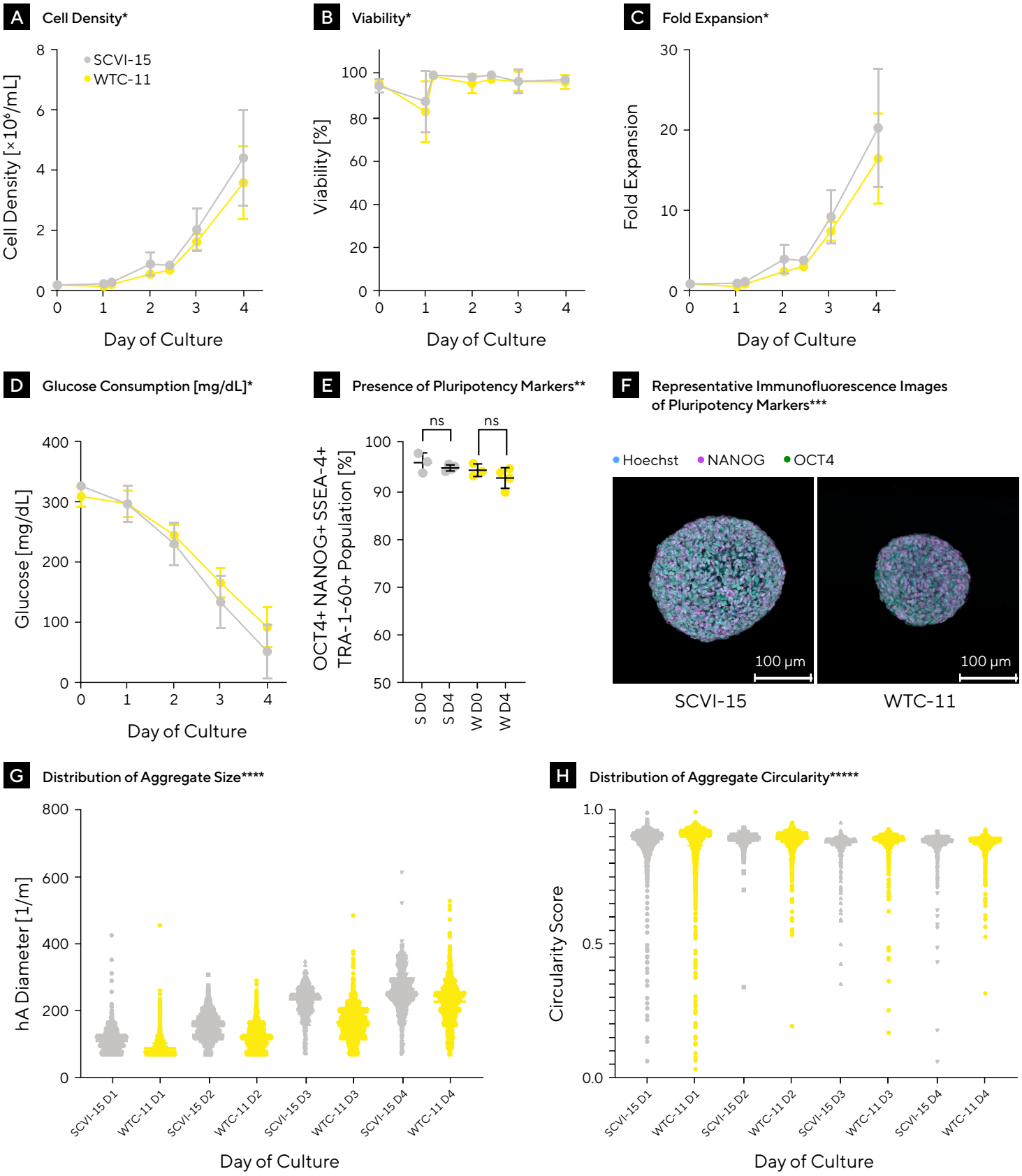


Figure 7: *hA Culture Can Be Robustly Scaled Up With Consistent Growth Rates, Morphologies, and High Rates of Pluripotency.*



Note.

*Error bars show mean ± s.d., n = 3 biological replicates per condition.

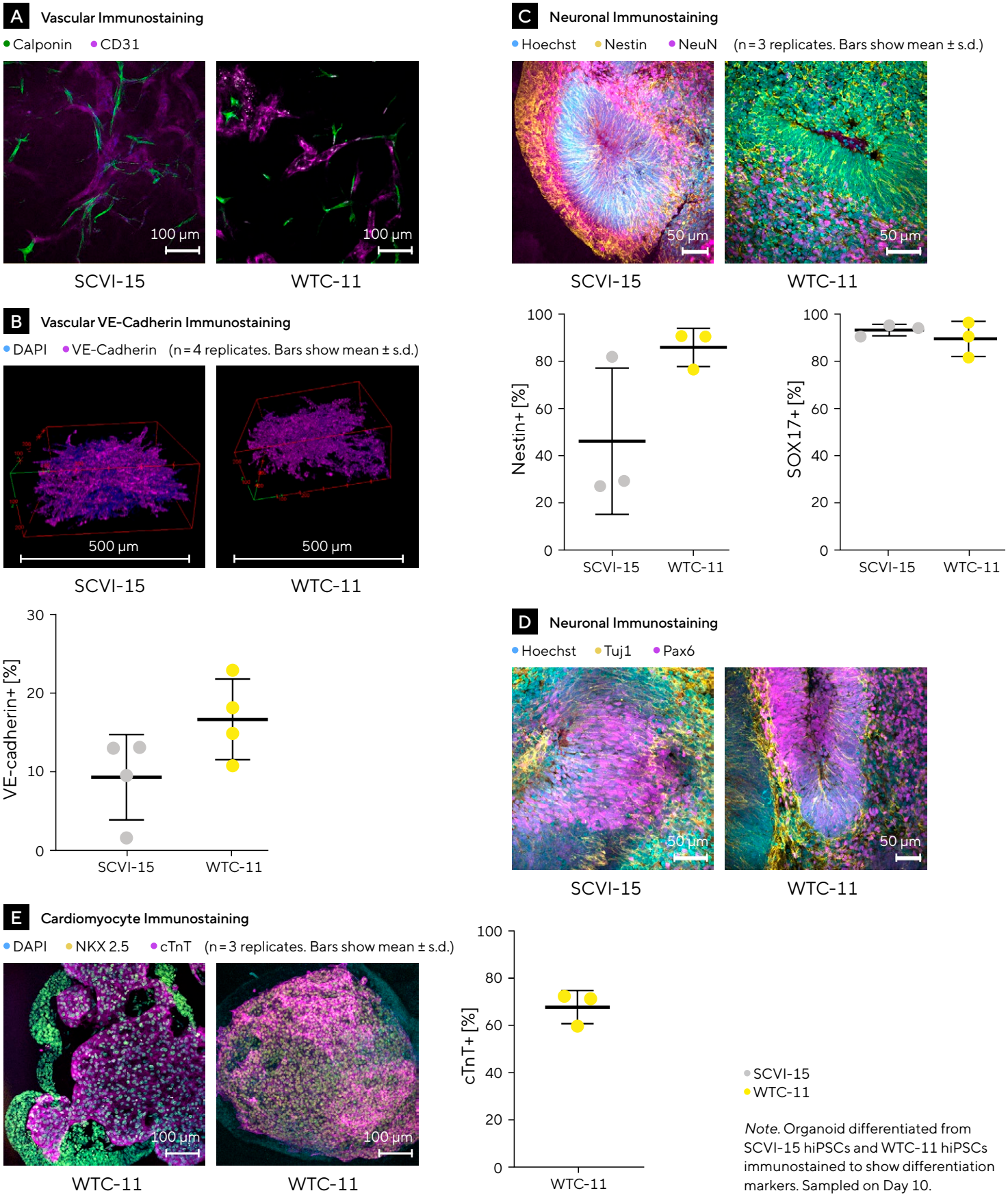
**Oct4, TRA-1-60, SSEA, and Nanog. On Day 0 vs day 4 n = 3 – 4 biological replicates per condition. | ns: not significant, unpaired two-tailed | Student's t-test. Error bars show mean ± s.d. Sampled on Day 4.

***Oct4 and Nanog. Sampled on Day 4.

****Error bars show median, IQR. n = 660 – 4,164 hAs per condition, aggregated from n = 4 replicates for SCVI-15, and n = 3 replicates for WTC-11. ****p <0.0001, Mann–Whitney U-test.

*****Bars show median, IQR. n = 660 – 4,164 hAs per condition aggregated from n = 3 replicates per cell line.

Figure 8: *hiPSC Aggregates Differentiate into Organoids of Ectodermal, Mesodermal, or Endodermal Origins.*



Note. Organoid differentiated from SCVI-15 hiPSCs and WTC-11 hiPSCs immunostained to show differentiation markers. Sampled on Day 10.

Differentiation

Cells cultured in Univessel® Glass CC 2 L SW automated bioreactor system using a process developed in the Ambr® 250 Modular multiparallel bioreactors formed aggregates (figure 8), which showed differentiation into organoids (figure 8) derived from all three germ layers (ectoderm, mesoderm, and endoderm) indicating they could be used to produce bioink for printing cortical, vascular, and intestinal tissue.

Additionally, a high percentage (>60%) of WTC-11 hiPSCs were positive for cTnT, cardiomyocyte markers when cultured in the Univessel® Glass bioreactors using the optimized S_{mid} factors. This indicates that the process developed in the Ambr® 250 Modular and Univessel® Glass bioreactors could be used to generate aggregates for any downstream stem cell processes.

Conclusion

Using the optimum parameter combination (identified by sweet spot analysis using MODDE® DOE software) in Ambr® 250 Modular multiparallelbioreactors with NutriStem® hPSC XF Medium generates high cell density hiPSCs of around 4×10^6 cells/mL and formation of appropriately sized aggerates in just 5 days. These aggregates can also maintain high viability and performance, as well as express high percentage of multiple pluripotency markers over multiple passages when cultured in NutriStem® hPSC XF Medium. This indicates the Ambr® 250 Modular system provides a good small-scale model for developing a robust hiPSCs culture process that can be directly transferred to 2 L stirred tank Univessel® Glass bioreactors to generate comparable cell densities and aggregates.

Cells cultured in the Univessel® bioreactors formed aggregates, which expressed differentiation markers from all three germ layers after directed differentiation, showing that these could be used to generate cortical, vascular, and intestinal tissue. WTC-11 hiPSCs cultured in the Univessel® Glass bioreactors also produced high levels of cardiomyocyte markers, indicating that the process developed in the Ambr® 250 Modular and Univessel® Glass bioreactors could be used to generate aggregates for printing cardiac tissue.

Since Ambr® 250 Modular and Univessel® Glass bioreactors have geometrical consistency into larger volume Biostat STR® bioreactors, which scale from 50 L and beyond, the optimized process developed in this study has the potential to be seamlessly scaled up to simplify production of clinically-relevant numbers of hiPSCs. In summary, combining this suite of bioreactors, which has geometrical consistency across scales with NutriStem® hPSC XF medium and MODDE® DOE data analytics, could optimize and scale up hiPSCs production to make bioprinting of therapeutic tissues a reality.

Order Information

Item	Order No.
NutriFreez® D5 Cryopreservation Salt-Based Solution	05-715-1D
NutriFreez® D10 Cryopreservation Medium	05-713-1D
Ambr® 250 Cell Culture system, 8 way configuration	001-8A67
MODDE® DOE Software	UT-210069-M
NutriStem® hPSC XF Medium	05-100-1A
Univessel® Glass, 2 L	2LUNIVESSEL-00002
Biostat® B-DCU CC 2 L	1708-FAC-5089

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