Sup. Table 1: Feeding rates for the respective processes during respective culture period.

culture day	Feeding rate [1/d] pUC	Feeding rate [1/d] p7GOS80oF	Feeding rate [1/d] Stg2M
0-1	0	0	0
1-2	1	1	1
2-3	1	1.25	1.5
3-4	1	1.5	3
4-5	1	1.75	4
5-6	1	2	6
6-7	1	2	7

Sup. Table 2: *Stg3M* kinetic model parameters for respective cell lines.

Model parameter	Value hHSC	Value Phoenix	Value GMPDU_8
K_{Glc} [mM]	1.5	1.5	1.5
K_{Lac} [mM]	65	45	50
K_{Gln} [mM]	0.01	0.01	0.01
K_{Agg} [µm]	350/2	275/2	325/2
K_{Osm} [mOsm/kg]	500	500	500
µ [d^{-1}]	1.35	1.30	1.35
q_{Glc} [x 10^{-8} mmol x $cell^{-1}$ x d^{-1}]	1.474	1.924	1.574
q_{Lac} [x 10^{-8} mmol x $cell^{-1}$ x d^{-1}]	2.37	3.27	2.47
q_{Gln} [x 10^{-9} mmol x $cell^{-1}$ x d^{-1}]	1.856	2.556	1.806
agg_f [-]	0.95	0.7	0.93
agg_g [-]	0.25	0.24	0.24

Sup. Table 3: Calculation of media requirements for producing 1 × 10^6 cells by a typical 4 day monolayer cultivation (Monolayer) versus 4 day processes under pH-uncontrolled (pUC), the most controlled process conditions (p7GOS80oF) and *in silico* model-based optimized conditions (Stg2M).

	Monolayer on 525 cm²	**pUC**	**p7GOS80oF**	**Stg2M**
starting cell density [x10^6 cells/mL]	0.125	0.5	0.5	0.5
starting cell number [x10^6 cells]	12.5	75	75	75
working volume [mL]	100	150	150	150
final cell density [x10^6 cells/mL]	0.9	2	7.6	10.6
final cell number [x10^6 cells]	90	300	1140	1590
total media consumption [mL]	300	600	712.5	975
media/cell ratio [mL/1x10^6 cells]	2.58	2.67	0.67	0.64
Productivity increase relative to Monolayer [-]	1	0.97	3.85	4.03

Sup. Table 4: Berkeley Madonna Script for Stg3M.

```
METHOD RK4

STARTTIME = 0
STOPTIME=7

DT = 0.02

{start parameters}

X0 = 500000000          {cells/L; starting cell concentration}
Glc0 = 17.5             {mmol/L; starting glucose concentration}
Lac0 = 0                {mmol/L; starting lactate concentration}
Gln0 = 2.5              {mmol/L; starting glutamine concentration}
V0 = 0.15               {L; used reactor volume}
BFGF0 = 100             {ng/L; starting bFGF concentration}
Osmo0 = 318             {mOsm/kg; starting osmolality}
Agg0 = 17               {µm, size of a single cell}
SFLac = 0               {mM; lactate concentration in the feed medium}

YXG = um/(qGlc*0.75)
YXL = um/(qLac*0.75)
YXGln = um/qGln
mG = qGlc*0.15
mL = qLac*0.15
qGlc = 1.474e-8         {mmol/Zelle*d; cell specific glucose consumption rate}
qGln = 1.856e-9         {mmol/Zelle*d; cell specific glutamine consumption rate }
qLac = 2.37e-8          {mmol/Zelle*d; cell specific lactate production rate}
um = 1.35               {1/d; maximal cell specific growth rate}
Kglc = 1.5              {mmol; Monod constant for glucose}
Kgln = 0.1              {mmol; Monod constant for glutamine}
Klac = 65               {mmol; Monod constant for lactate}
Kagg = 350/2            {µm; Monod-like constant for aggregate size}
KOsmo = 500             {mOsm; Monod-like constant for osmolality}
am = 0.25               {aggregate growth factor}
ad1 = 0.95              {aggregate formation factor}
```

{initiators}

init X = X0
init Glc = Glc0
init Lac = Lac0
init Gln = Gln0
init Glc2 = Glc0
init Lac2 = Lac0
init Agg = Agg0
init Osmo = Osmo0

{balances}

d/dt(X) = u * X-1.9*u*o*X
d/dt(Glc) = -u/YXG * X - F / V0 * (Glc - SFGlc) - mG * X
d/dt(Lac) = u/YXL * X - F / V0 * (Lac - SFLac) + mL * X
d/dt(Gln) = -u/YXGln * X - F / V0 * (Gln - SFGln)
d/dt(Glc2) =- F / V0 * (Glc2 - SFGlc)
d/dt(Lac2) = u/YXL * X + mL * X
Base = 0.1873 * Lac2 -1.5767
d/dt(Agg) = ag * Agg * 1.7

{kinetics}

u = um * (Glc/(Kglc + (1+ (Lac/Klac)) * Glc)) * (Gln/(Kgln+Gln)) * (Kagg^9/((Agg/2)^9+Kagg^9)) * (KOsmo^9/(Osmo^9+KOsmo^9))
d/dt(Osmo) =if time <= 1.5 then 0 else 0.06 * (F / V0 * SFGlc * 9.25 - (u/YXL * X + mL * X) * 3.5 - (u/YXG * X - F / V0 * (Glc - SFGlc) - mG * X)) / (F/V0) - F / V0 * (Glc2 - SFGlc)
ag = h * (((am * u))-0.03)   + d * ad1

{Feeding}
F = a*(F2a+F3a+F4a+F5a+F6a+F7a)   {L/d}

a = 1

b= if TIME >= 2 then 0 else 1

d= if TIME >= 1 then 0 else 1
h= if TIME <= 1 then 0 else 1
m= if TIME >= 6 then 0 else 1
n= if TIME <= 6 then 0 else 1
o= if TIME >= 0.5 then 0 else 1
p= if TIME <= 0.5 then 0 else 1

SFGlc = a*(SFGlcd1a+ SFGlcd2a+ SFGlcd3a+ SFGlcd4a+ SFGlcd5a+ SFGlcd6a) {mM; glucose concentration in the feed medium}
SFGln = a*(SFGlnd1a+ SFGlnd2a+ SFGlnd3a+ SFGlnd4a+ SFGlnd5a+ SFGlnd6a) {mM; glutamine concentration in the feed medium }

tf2 = 1
tf3 = 2
tf4 = 3
tf5 = 4
tf6 = 5
tf7 = 6
tf8 = 7

F2 = V0 * 1 {L}
F3 = V0 * 1.5 {L}
F4 = V0 * 3 {L}
F5 = V0 * 4 {L}
F6 = V0 * 6 {L}
F7 = V0 * 7 {L}

F2a = if time <= tf2 then 0 else if time >= tf3 then 0 else F2
F3a = if time <= tf3 then 0 else if time >= tf4 then 0 else F3
F4a = if time <= tf4 then 0 else if time >= tf5 then 0 else F4
F5a = if time <= tf5 then 0 else if time >= tf6 then 0 else F5
F6a = if time <= tf6 then 0 else if time >= tf7 then 0 else F6
F7a = if time <= tf7 then 0 else F7

SFGlcd1 = 34.15{mM}
SFGlcd2 = 34.15{mM}
SFGlcd3 = 34.15{mM}
SFGlcd4 = 42.5 {mM}
SFGlcd5 =42.5 {mM}
SFGlcd6 = 42.5 {mM}

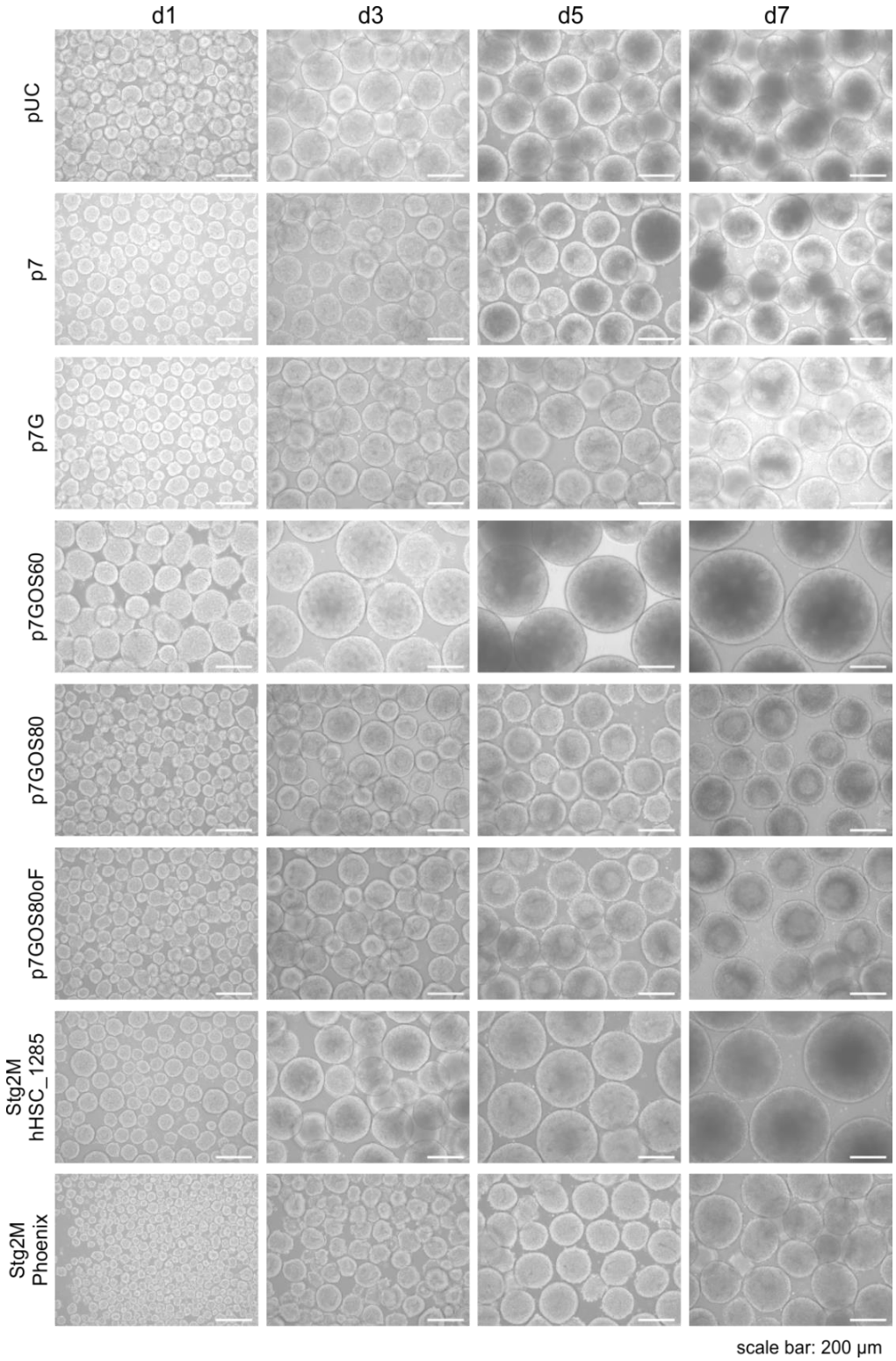
SFGlcd1a = if time <= tf2 then 0 else if time >= tf3 then 0 else SFGlcd1
SFGlcd2a = if time <= tf3 then 0 else if time >= tf4 then 0 else SFGlcd2
SFGlcd3a = if time <= tf4 then 0 else if time >= tf5 then 0 else SFGlcd3
SFGlcd4a = if time <= tf5 then 0 else if time >= tf6 then 0 else SFGlcd4
SFGlcd5a = if time <= tf6 then 0 else if time >= tf7 then 0 else SFGlcd5
SFGlcd6a = if time <= tf7 then 0 else SFGlcd6

SFGlnd1 = 4.5 {mM}
SFGlnd2 = 4.5 {mM}
SFGlnd3 = 4.5 {mM}
SFGlnd4 = 5 {mM}
SFGlnd5 = 5 {mM}
SFGlnd6 = 5 {mM}

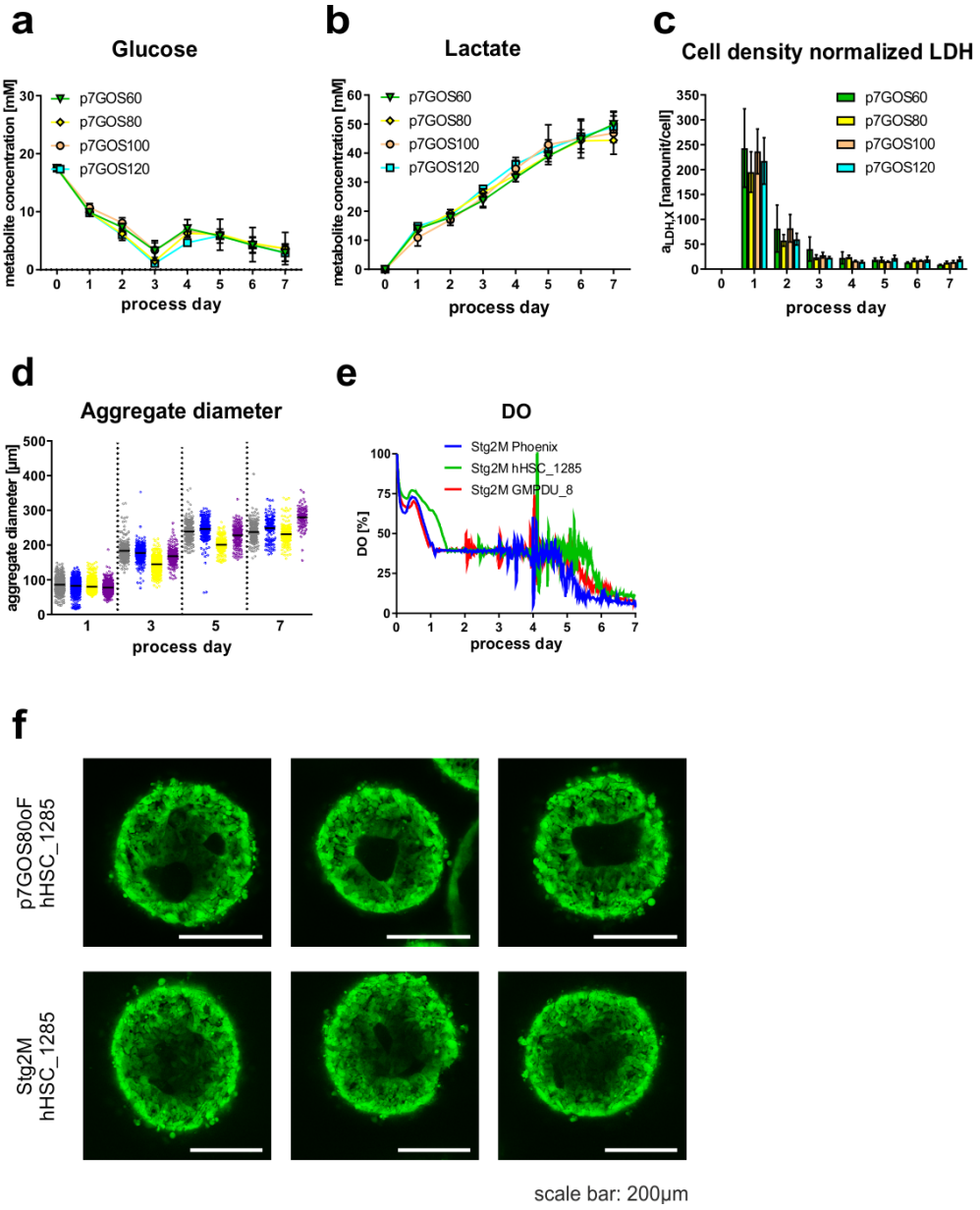
SFGlnd1a = if time <= tf2 then 0 else if time >= tf3 then 0 else SFGlnd1
SFGlnd2a = if time <= tf3 then 0 else if time >= tf4 then 0 else SFGlnd2
SFGlnd3a = if time <= tf4 then 0 else if time >= tf5 then 0 else SFGlnd3
SFGlnd4a = if time <= tf5 then 0 else if time >= tf6 then 0 else SFGlnd4
SFGlnd5a = if time <= tf6 then 0 else if time >= tf7 then 0 else SFGlnd5
SFGlnd6a = if time <= tf7 then 0 else SFGlnd6

{limits}

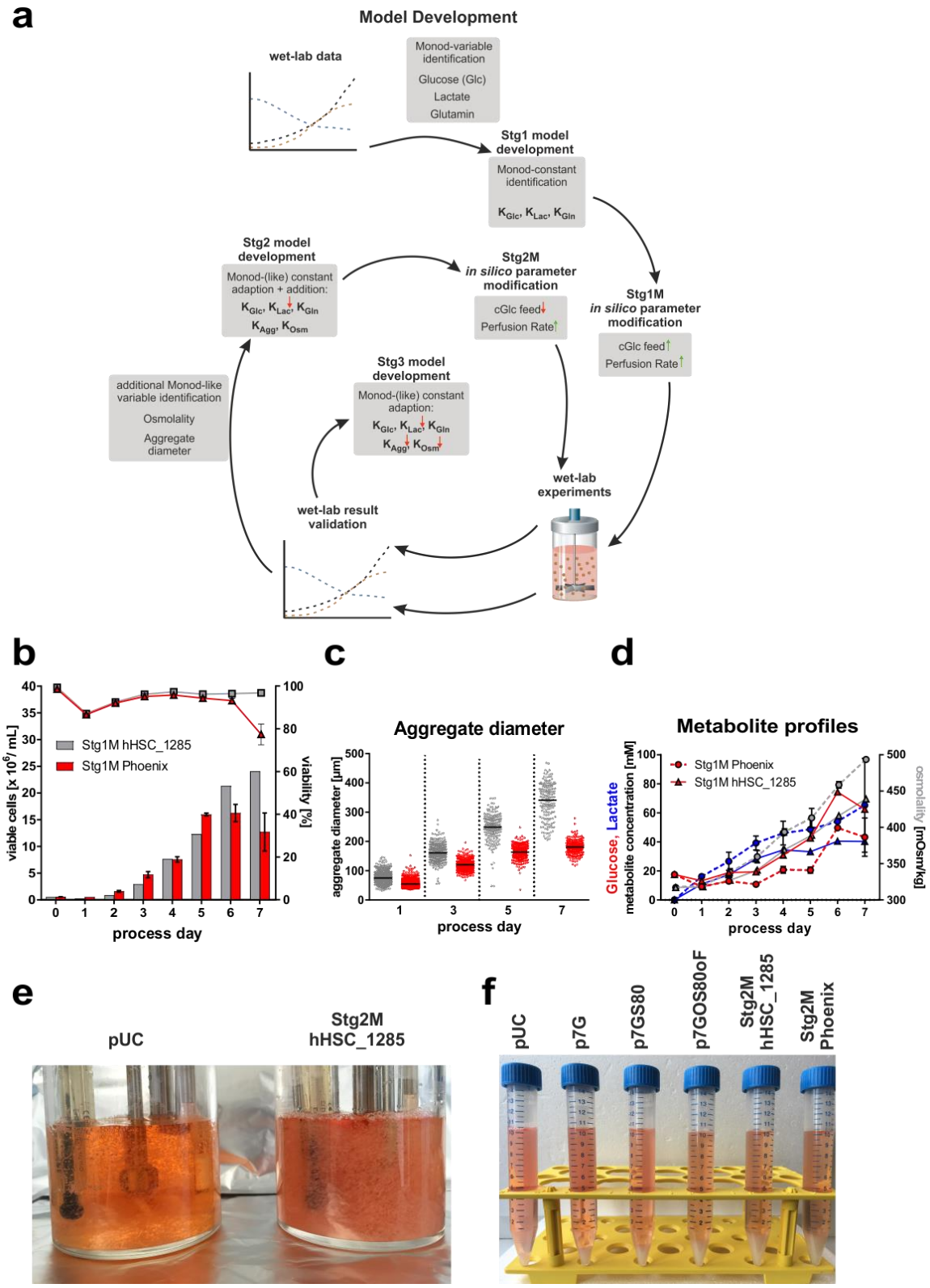
limit Glc >= 0
limit Gln >= 0
limit Lac >= 0
limit Base >= 0



Sup. Figure 1: Impact of process conditions on hiPSC aggregate formation and size. Representative light microscopy pictures of process-derived aggregate samples on days 1, 3, 5 and 7 (scale bars = 200 µm). Abbreviation: d, day. All images displayed in this figure were generated with the cell line hHSC_1285_iPS2 unless stated otherwise.

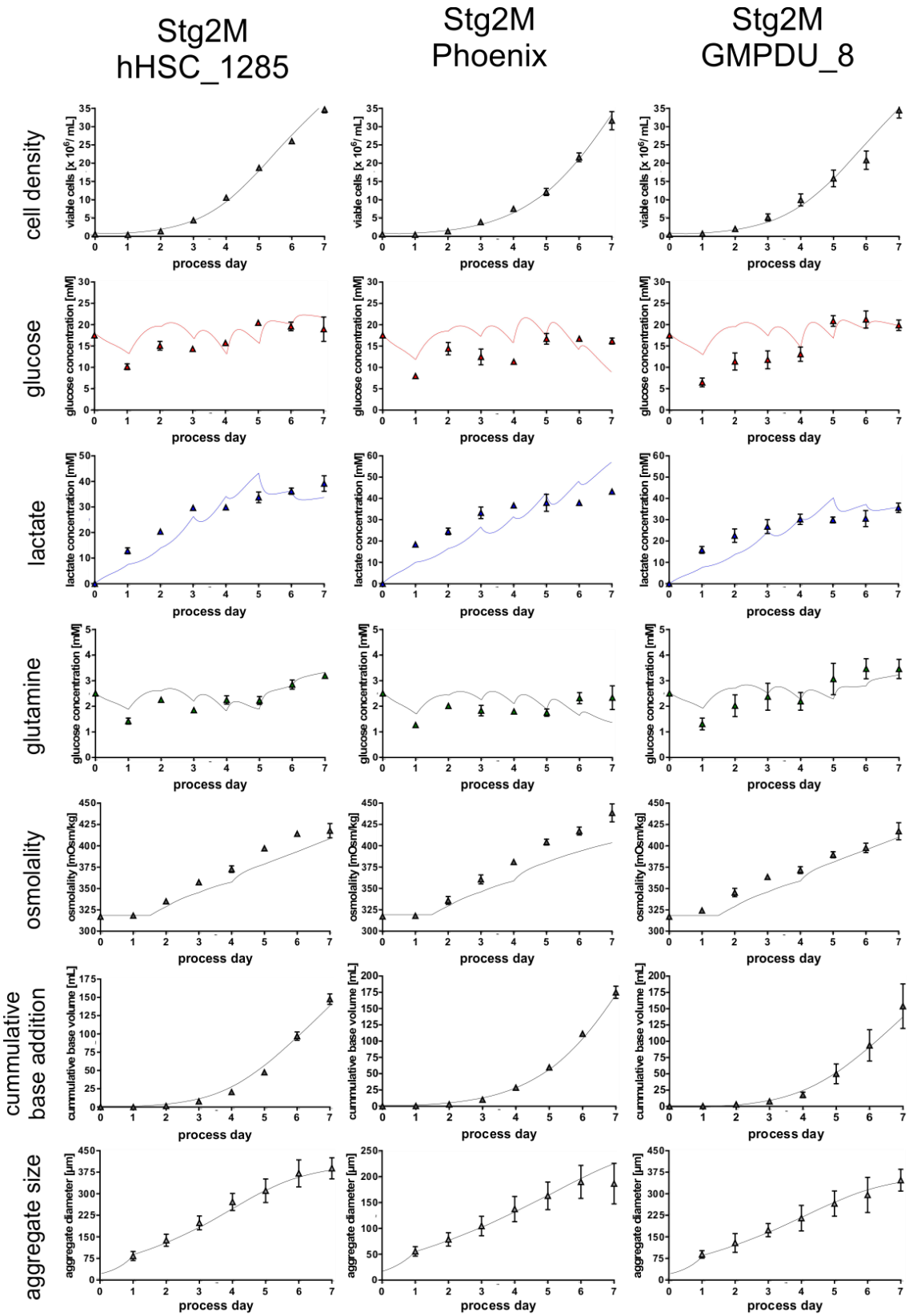


Sup. Figure 2: Metabolic activity in stirring speed altered processes. Respective process-dependent patterns of Glc **(a)** and Lac **(b)** concentrations in cell culture supernatants over the cultivation process. **(c):** Cell density normalized concentration of Lactate dehydrogenase (LDH) as an indicator for cell rupture. **(d):** Impact of optimized feeding strategy on the distribution of aggregate diameters over the cultivation time. **(e):** Model-based *in silico* process optimization. Online process parameter measurement of DO. To ensure clarity of illustration, representative single DO are shown only. **(f):** 2-photon microscopy images with 50 µm depth of Calcein-stained aggregates at the end stage of cultures of hHSC_1285_iPS2 under p7GOS80oF and Stg2M conditions. Images taken at 50 µm virtual aggregate depth. Scale bars = 200 µm. All Data displayed in this figure was generated with the cell line hHSC_1285_iPS2 unless stated otherwise.

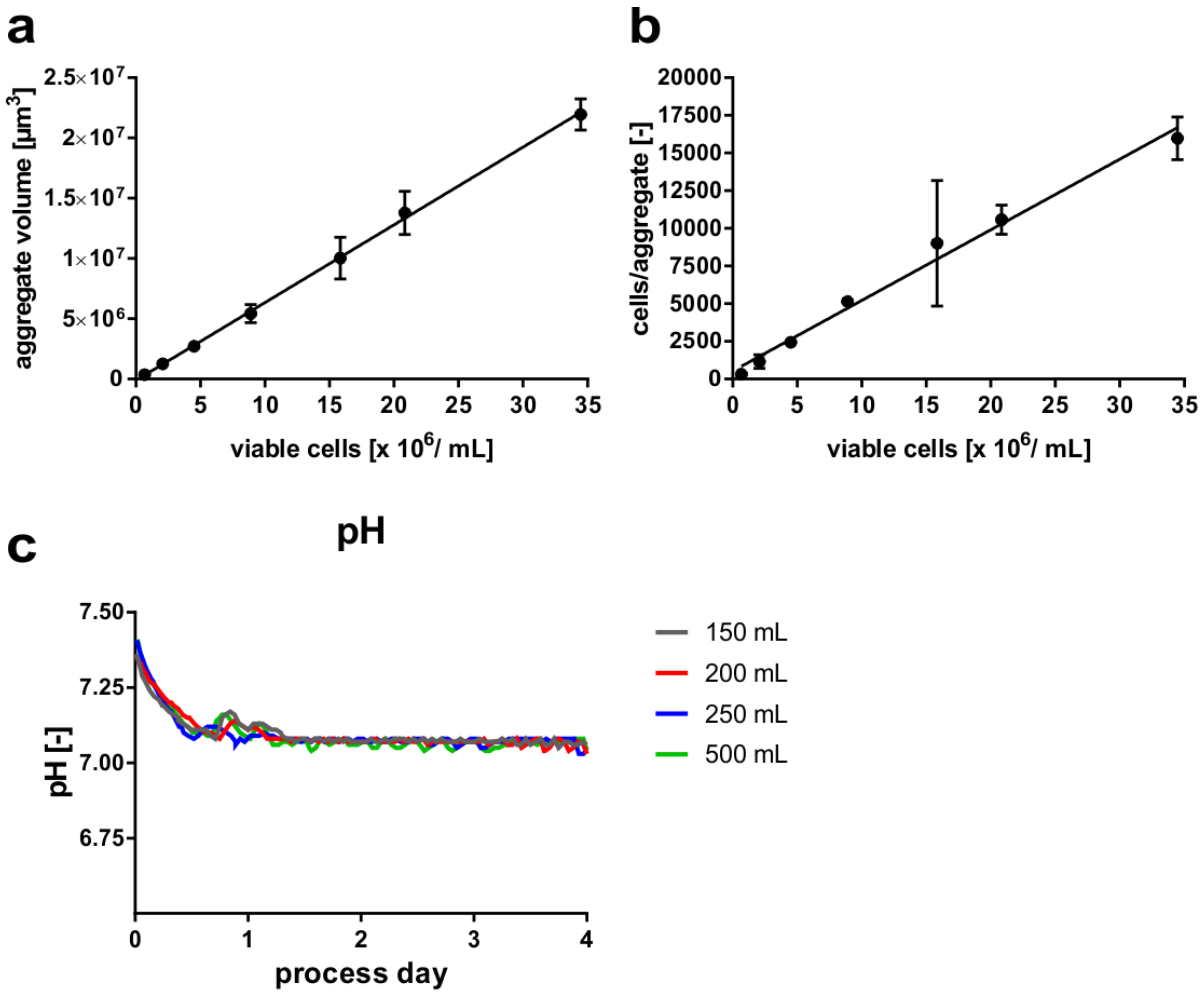


Sup. Figure 3: (a): Schematic outline of the iterative model building process. **(b):** Viable cell densities (bar chart) and viability (line graphs) for processes using the cell lines Phoenix (red; n=2) and hHSC_F1285T_iPS2 (gray, n=2). Process strategy followed the results of the stage 1 model. **(c):**

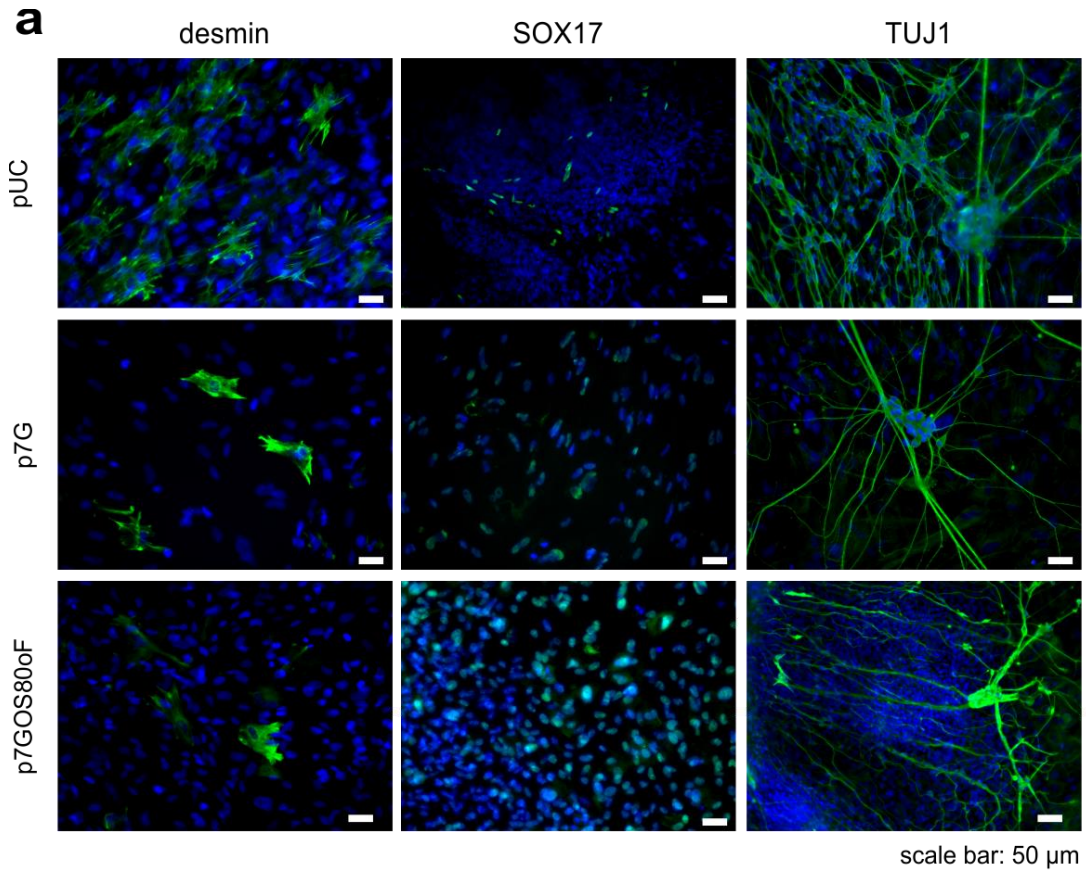
Distribution of aggregate diameters over the cultivation time. **(d):** Respective process-dependent patterns of Glc (red) and Lac (blue) concentrations as well as osmolality (gray) in cell culture supernatants using the cell lines Phoenix (dashed lines; n=2) and hHSC_F1285T_iPS2 (solid lines, n=1). **(e):** Visual comparison of day 7 cultures of hHSC-1285T_iPS2 cultured under pUC and Stg2M conditions. **(f):** 10 mL of day 7 culture suspension of each of the respective process were centrifuged to visualize the cell mass produced under each of these conditions. All Data displayed in this figure was generated with the cell lines hHSC_1285_iPS2 and Phoenix.

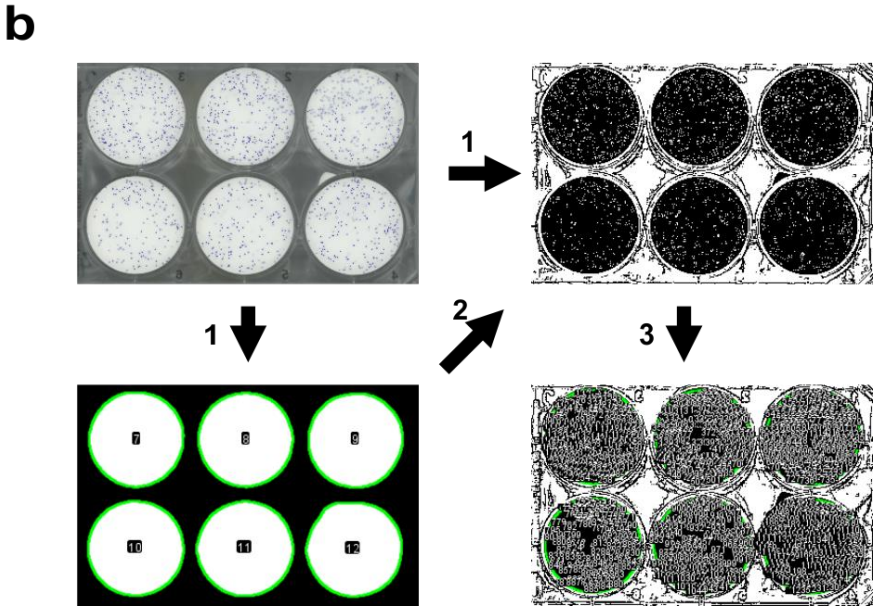


Sup. Figure 4: Overlay of wet-lab data of hHSC_1285_iPS2, Phoenix and GMPDU_8 under Stg2M conditions (points) with in silico modelled curves (under Stg3 model conditions; lines).



Sup. Figure 5: Linear correlation between the (spherical) aggregate volume and the cell density **(a)** as well as the calculated cell number per aggregate and the cell density **(b)** at any process stage. **(c)** Online process parameter measurement of pH for scalable processes. To ensure clarity of illustration, representative single pH is shown only. All Data displayed in this figure was generated with the cell line hHSC_1285_iPS2.





Sup. Figure 6: (a): Process-derived hPS cell aggregates maintained their potential to differentiate into all three germ layers. Representative pictures of immunofluorescence staining of day 7-derived aggregates after induction of undirected differentiation revealed the expression of marker proteins representative of the three germ layers as shown for Desmin (mesoderm), Sox17 (endoderm) and TUJ1 (ectoderm). Positive staining is shown in green. Isotype controls confirmed staining specificity (not shown). DAPI

stained nuclei in blue. Scale bars = 50 µm. **(b):** Exemplary outline of the strategy to analyze aggregate density. Images of Coomassie brilliant blue-stained aggregates were analyzed using ImageJ where the software first recognized all white wells of an image (bottom left). Afterwards, the original image was altered in a way to better visualize stained aggregates as white spots (top right), which are finally counted if they were in the areas originally recognized as wells (bottom right). All Data displayed in this figure was generated with the cell line hHSC_1285_iPS2.

Supplemental Materials and Methods:

Calculation of specific growth rate

Specific growth rate μ [1/d] was calculated as

1) $$\mu = \left(\frac{X_{t_{n+1}} - X_{t_n}}{t_{n+1} - t_n}\right) \times \frac{1}{\bar{X}_{t_{n+1}}}$$

where *X* is the cell concentration [cells/L] at the given time point *t* and $\bar{X}$ the mean cell concentration [cells/ L] calculated as

2) $$\bar{X}_{t_{n+1}} = \frac{X_{t_{n+1}} - X_{t_n}}{\ln(X_{t_{n+1}}) - \ln(X_{t_n})}$$

Glucose and Lactate analysis

Glc and Lac concentrations from cell-free supernatant were analyzed using an YSI 2300 Stat Plus Glucose & Lactate Analyzer (YSI Incorporated Life Sciences) and the Biosen C-line (EKF Diagnostics).

Specific Glc consumption rates *qGlc* [pmol/ (cell x d)] for the process days without medium change were calculated as

3) $$qGlc_{t_{n+1}} = -\left(\frac{Glc_{t_{n+1}} - Glc_{t_n}}{t_{n+1} - t_n}\right) \times \frac{1}{\bar{X}_{t_{n+1}}}$$

where *Glc* is the Glucose concentration [pmol/ L], *t* the process time point [d]

In the perfused cultures the specific Glucose consumption rate is calculated as

4) $$qGlc_{t_{n+1}} = -\left[\left(\frac{Glc_{t_{n+1}} - Glc_{t_n}}{t_{n+1} - t_n}\right) + \frac{F}{V}(\overline{Glc}_{t_{n+1}} - Glc_f)\right] \times \frac{1}{\bar{X}_{t_{n+1}}}$$

where *F* is the flow rate of feed and waste stream [L/ d], *V* the culture volume [L], Glc_f the Glucose concentration in the feed stream [pmol/ L]

and $\overline{Glc}$ is the mean Glucose concentration in the culture calculated as

5) $$\overline{Glc}_{t_{n+1}} = \frac{Glc_{t_{n+1}} - Glc_{t_n}}{\ln(Glc_{t_{n+1}}) - \ln(Glc_{t_n})}$$

Specific Lactate production rates *qLac* [pmol/ (cell x d)] for the process days without medium change are calculated accordingly as

6) $$qLac_{t_{n+1}} = \left(\frac{Lac_{t_{n+1}} - Lac_{t_n}}{t_{n+1} - t_n}\right) \times \frac{1}{\bar{X}_{t_{n+1}}}$$

where *Lac* is the Lactate concentration [pmol/ L].

In the perfused cultures the specific Lactate production rate is calculated as

7) $$qLac_{t_{n+1}} = -\left[\left(\frac{Lac_{t_{n+1}} - Lac_{t_n}}{t_{n+1} - t_n}\right) + \frac{F}{V}\overline{Lac}_{t_{n+1}}\right] \times \frac{1}{\bar{X}_{t_{n+1}}}$$

where $\overline{Lac}$ is the mean Lactate concentration in the culture calculated as

8) $$\overline{Lac}_{t_{n+1}} = \frac{Lac_{t_{n+1}} - Lac_{t_n}}{\ln(Lac_{t_{n+1}}) - \ln(Lac_{t_n})}$$

The yield coefficient of Lactate from Glucose *Y(qLac/qGlc)* [-] is calculated as follows

9) $$Y(qLac/qGlc) = \left|\frac{qLac}{qGlc}\right|$$

Amino Acid analysis

Amino acid levels were determined via high-performance liquid chromatography using a precolumn derivatization method with ortho-phthaldialdehyd (OPA) utilizing a Shimadzu RF-10AxL fluorescence detector (excitation 330 nm, emission 420 nm), an Agilent 1200 pump (Agilent), a triathlon autosampler (Spark), a 3-channel degasser (Sykam), a T-1 column stove (Techlab) and a Waters Resolve C18 column (5 µm, 3.9 x 150 mm). Protein precipitation was performed at -20 °C overnight via addition of 100 µL methanol to 25 µL cell-free supernatant. After centrifugation, 30 µL protein-free samples were incubated with 50 µL OPA-reagent (27 mg OPA, 5 mL ethanol, 0.2 mL mercaptoethanol, 45 mL 0.4 M potassium borate buffer, pH 9.5) for 1.5 min and subsequently 10 µL were loaded onto the column. Chromatography was performed with a flow rate of 1 mL/min at 30°C utilizing a gradient of 0% to 100% with elution buffer A (0.05 M sodium acetate, 0.05 M sodium dihydrogen phosphate, pH 7, 2% tetrahydrofuran and 2% methanol) and elution buffer B (54% methanol).
Specific amino acid consumption rates *qAc* [pg/ (cell x d)] for the process days without medium change were calculated as

10) $$qAc_{t_{n+1}} = -\left(\frac{Ac_{t_{n+1}} - Ac_{t_n}}{t_{n+1} - t_n}\right) \times \frac{1}{\bar{X}_{t_{n+1}}}$$

where *Ac* is the amino acid concentration [pg/ L],

In the perfused cultures the specific amino acid consumption rate is calculated as

11) $$qAc_{t_{n+1}} = -\left[\left(\frac{Ac_{t_{n+1}} - Ac_{t_n}}{t_{n+1} - t_n}\right) + \frac{F}{V}(\overline{Ac}_{t_{n+1}} - Ac_f)\right] \times \frac{1}{\bar{X}_{t_{n+1}}}$$

where Ac_f the amino acid concentration in the feed stream [pmol/ L] and $\overline{Ac}$ is the mean Glucose concentration in the culture calculated as

12) $$\overline{Ac}_{t_{n+1}} = \frac{Ac_{t_{n+1}} - Ac_{t_n}}{\ln(Ac_{t_{n+1}}) - \ln(Ac_{t_n})}$$

Osmolality

Culture osmolality was analyzed via freezing point osmometer OSMOMAT 3000 basic (Gonotec).

Aggregate concentration

To determine the aggregate concentration, 1 mL of culture broth was harvested and the aggregates were stained for 5 min at room temperature with 150 µL of Comassie brilliant blue, subsequently aggregates where harvested for 3 min at 1000 x g and resuspended in 1 mL PBS. Afterwards stained aggregate suspension was distributed a 100 µL into 3 wells of a 6-well plate and covered with 2 mL of condensed milk per well. The plate was then imaged on a normal scanner, while aggregates were automated counted with a macro an ImageJ. A exemplarily analysis is shown in Fig. S6b.

Flow cytometry

Assessment of pluripotency-associated surface markers SSEA-3, SSEA-4 and TRA-1-60 was performed by incubation of cells with antibodies against SSEA-3, SSEA-4 and TRA-1-60 in a triple staining for 12 min at RT. Antibodies were diluted in PBS w/o.

For intracellular pluripotency- and growth-associated markers OCT-3/4, NANOG and KI-67, single cell suspensions were fixed with 4% paraformaldehyde at room temperature for 15 min and subsequently incubated with antibodies against OCT-3/4 + NANOG in a double staining and KI-67 in a single staining for 12 min at RT. Antibodies were diluted in a 1:1 ratio of PBS w/o and FIX&PERM® Solution B (Nordic-MUbio). Assessment of definitive endoderm surface markers CXCR4 and C-KIT was performed by incubation of cells with respective antibodies for 30 min on ice. Antibodies were diluted in PBS w/o

Supplemental Table 5: Directly labeled antibodies used for flow cytometric analysis.

Name	Class	Species recognition	Conjugate	Supplier	Dilution
Anti-SSEA-3	IgM	Human	DyLight 650	Thermo Fisher	1:250
Anti-SSEA-4	IgG1	Human	VioBlue	Miltenyi Biotec	1:25
Anti-TRA 1-60	IgG1	Human	PE	Miltenyi Biotec	1:25
Anti-OCT-3/4 Isoform A	IgG1	Human/ mouse	PE	Miltenyi Biotec	1:25
Anti-NANOG	IgG1	Human	APC	Miltenyi Biotec	1:25
Anti-KI-67	IgG1	Human/ mouse	PerCP-Vio700	Miltenyi Biotec	1:25
Anti-CXCR4	IgG2a	Human	APC	eBioscience	1:20
Anti-C-KIT	IgG1	Human	PE	eBioscience	1:20

Afterwards, cells were washed twice with PBS w/o and analyzed using a MACSQuant® Analyzer 10 Flow Cytometer (Miltenyi Biotec). Subsequently, data were further processed using FlowJo v10 software.

Undirected differentiation

For serum-based undirected differentiation, hiPSC aggregates were transferred to 6 well ultra-low attachment plates (Corning) in differentiation medium consisting of 80% IMDM (Invitrogen) supplemented with 20% fetal calf serum (HyClone), 1 mM L-Glutamine, 0.1 mM β-Mercaptoethanol, and 1% nonessential amino acid stock (all Invitrogen). After 1 week, aggregates were plated on 0.1% gelatin coated 6 well plates and further cultured for 2 weeks in differentiation medium thereby applying media change every 3 – 4 days.

Immunocytological staining

Immunostaining was performed as described previously [1, 2]. In brief, cells were fixed with 4% PFA at room temperature for 10 min and 5 min, respectively. After blocking with 5% donkey serum and 0.25% Triton X-100 (Sigma Aldrich) in Tris-buffered saline for 20 min at room temperature, cells were incubated with respective monoclonal primary antibodies. After washing cells were incubated with secondary antibodies (Alexa Fluor 488 donkey anti-mouse IgG for OCT-3/4, SSEA4, Desmin and TUJ1; Alexa Fluor 488 donkey anti-mouse IgM for TRA-1-60; Cy™3-labeled donkey anti-goat IgG for SOX17; Cy™5-labeled donkey anti-rabbit IgG for SOX2) for 30 min at room temperature. After washing, cells were counterstained with DAPI

(Sigma-Aldrich) for 15 min, and analyzed via Axio Oberserver A1 fluorescence microscope and Axiovision software 4.71 (Zeiss).

Supplemental Table 6: Antibodies used for immunocytologcial staining.

Name	Class	Species	Clonality	Supplier	Dilution
Primary antibodies					
Anti-OCT-3/4	IgG_{2b}	Mouse	Monoclonal	Santa Cruz Biotechnology	1:100
Anti-SSEA4	IgG_3	Mouse	Monoclonal	DSHB	1:100
Anti-TRA-1-60	IgM	Mouse	Monoclonal	Abcam	1:100
Anti-SOX2	IgG	Rabbit	Monoclonal	Cell Signaling	1:200
Anti-Desmin	IgG1	Mouse	Monoclonal	Progen Biotechnik	1:20
Anti-SOX17	IgG	Goat	Monoclonal	R&D Systems	1:200
Anti-TUJ1	IgG_{2a}	Mouse	Monoclonal	Merck	1:400
Secondary antibodies					
Alexa Fluor 488-conjugated Anti-Mouse	IgG	Donkey	Polyclonal	Jackson Immuno Research	1:200
Alexa Fluor 488-conjugated Anti-Mouse IgM, µ chain specific	IgG	Donkey	Polyclonal	Jackson Immuno Research	1:200
Cy™5-conjugated Anti-Rabbit IgG (H+L)	IgG	Donkey	Polyclonal	Jackson Immuno Research	1:200
Cy™3-conjugated Anti-Goat IgG (H+L)	IgG	Donkey	Polyclonal	Jackson Immuno Research	1:200

Cell cycle analysis

Aggregates were dissociated like describe in "Sampling, Aggregate Analysis, and Cell Counting" and afterwards resuspended in 70% (v/v) ethanol in PBS and stored until used at -80°C.

For measurement cells were thawed, washed once with PBS and stained for 5 min at 37°C with DAPI (1:30000 in PBS with 1% Triton X; Sigma-Aldrich). Afterwards washed once with PBS and analyzed using a MACSQuant® Analyzer 10 Flow Cytometer (Miltenyi Biotec) and data were further processed using FlowJo v10 software.

Multiphoton microscopy

Aggregates were stained with Calcein-AM (1 µM in PBS +; Sigma-Aldrich) for 2 h at 37°C and afterwards washed once with PBS +. Multiphoton microscopy images were acquired using a Thorlabs MPM200 multiphoton microscope (Thorlabs GmbH, Germany) with FITC and TRITC emission filters and a

Chameleon Ultra II (Coherent Inc., US) laser source operating at a wavelength of 850 nm. For imaging, aggregates were placed below the objective with NA 1.05 (Olympus XLPLN25WMP2) in PBS +.

***In silico* process modelling**

For the *in silico* modelling an unstructured-unsegregated kinetic model approach was used. As a basis for this approach the following equations were used.

The change in cell concentration over time was calculated as follows:

13) $$\frac{dX}{dt} = \mu X$$

The general calculation of the change of a metabolite *S* over time was calculated as follows, where q_S is the cell specific consumption or production rate of the specific metabolite *S*.

14) $$\frac{dS}{dt} = \frac{\mu}{\mu_{max}} q_S X$$

According to Monod, the specific growth rate μ is dependent on the ratio of a limiting substrate *S* and the sum of this limiting substrate and its Monod constant K_S.

15) $$\mu = \mu_{max} \frac{[S]}{K_S + [S]}$$

The change of the glucose concentration (glutamine works analog to glucose) in perfused cultures over time is calculated as follows:

16) $$\frac{dGlc}{dt} = -\frac{\mu}{\mu_{max}} q_{Glc} \times X - \frac{F}{V} \times (Glc - Glc_f)$$

The change of the lactate concentration in perfused cultures over time is calculated as follows:

17) $$\frac{\mathrm{dLac}}{\mathrm{dt}} = -\frac{\mu}{\mu_{\mathrm{max}}} \mathrm{q_{Lac}} \times \mathrm{X} - \frac{\mathrm{F}}{\mathrm{V}} \times \mathrm{Lac}$$

Applying Monod equation for glucose results into:

18) $$\mu = \mu_{max} \times \frac{[Glc]}{K_{Glc} + [Glc]}$$

With the assumption that the lactate concentration acts as an inhibitor of the glucose metabolism, the following equation results:

19) $$\mu = \mu_{max} \times \frac{[Glc]}{K_{Glc} + \left(1 + \frac{[Lac]}{K_{Lac}}\right) \times [Glc]}$$

After the addition of the potential inhibition of glutamine *Gln* the equation resembles as follows:

20) $$\mu = \mu_{max} \times \frac{[Glc]}{K_{Glc} + \left(1 + \frac{[Lac]}{K_{Lac}}\right) \times [Glc]} \times \frac{[Gln]}{K_{Gln} + [Gln]}$$

Applying the same strategy, terms for the aggregate diameter *Agg* as well as the osmolality *Osm* were added, here the power of 9 was used to establish the term in a more stringent way:

21) $$\mu = \mu_{max} \times \frac{[Glc]}{K_{Glc} + \left(1 + \frac{[Lac]}{K_{Lac}}\right) \times [Glc]} \times \frac{[Gln]}{K_{Gln} + [Gln]} \times \frac{{K_{Agg}}^9}{{K_{Agg}}^9 + Agg^9} \times \frac{{K_{Osm}}^9}{{K_{Osm}}^9 + Osm^9}$$

The total lactate concentration $\sum Lac$ produced over the cultivation time is calculated is the lactate concentration over the cultivation time in equation 17) without the perfusion term:

22) $$\frac{d\sum Lac}{dt} = -\frac{\mu}{\mu_{max}} q_{Lac} \times X$$

This total lactate concentration is used to calculate the cumulative volume of base $NaHCO_3$ V_{NaHCO3} that is added to the culture, as follows:

23) $$V_{NaHCO_3} = 0.1873 \times \sum Lac - 1.576$$

The glucose concentration in the medium without consumption Glc_{wc} is calculated as follows:

24) $$\frac{dGlc_{wc}}{dt} = -\frac{F}{V} \times (Glc_{wc} - Glc_f)$$

The calculation of the osmolality *Osm* over the cultivation time is calculated as follows, it has to be mentioned that the equation is only valid from day 1.5 onwards and therefore no change in osmolality is assumed for the first 1.5 days of culture.

25) $$\frac{dOsm}{dt} = 0.06 \times \frac{\frac{F}{V} \times Glc_f \times 9.25 - \frac{\mu}{\mu_{max}} q_{Lac} \times X \times 3.5 - \frac{\mu}{\mu_{max}} q_{Glc} \times X - \frac{F}{V} \times (Glc - Glc_f)}{\frac{F}{V}} - \frac{F}{V} \times (Glc_{wc} - Glc_f)$$

To described the change in aggregate size, two equations have to be used. The following equation describes the phase of aggregate formation for the first 24 h, where agg_f is the cell specific aggregate forming factor that is also stirring rate depended.

26) $$\frac{dAgg}{dt} = Agg \times 1.7 \times agg_f$$

From day 1 onwards the change in aggregate size *Agg* over time can be described as follows, where agg_g is the cell specific aggregate growing factor.

27) $$\frac{dAgg}{dt} = Agg \times 1.7 \times (agg_g \times \mu - 0.03)$$

The above described equations as well as factors were used to write a code that was modelled with the software Berkeley Madonna. For process optimization the perfusion rate and the glucose feed concentration were *in silico* optimized on a trial and error approach.

To calculate the prediction error of the model approach, first the relative error for each point e_{ri} was calculated like follows, where x_{wl} are wet-lab and x_m are modelled data points, with $x_{wl\,max}$ as the maximal wet-lab data point.

28) $$e_{r_i} = \frac{|x_{wl} - x_m|}{x_{wl\,max}} \times 100$$

The average error $\bar{e}_r$ and the standard deviation for the average error $SD_{\bar{e}_r}$ for each data set were calculated as follows:

29) $$\bar{e}_r = \frac{\sum_{i=1}^{n} e_{r_i}}{n}$$

30) $$SD_{\bar{e}_r} = \sqrt{\frac{1}{n-1} \times \sum_{i=1}^{n} (e_{r_i} - \bar{e}_r)^2}$$

Process upscaling and transition to BioBlock reactor system

The initial settings for preculture handling, process inoculation and control remained unchanged for the DASbox System (conducted equal to Stg2M conditions, see above) used for process volumes of 150, 200 and 250 mL, respectively and was equivalently applied to the DASGIP BioBlock® System used for processes in 500 mL scale. As a key component for the upscaling control of hPSC aggregates, the stirring rate was adjusted to the respective culture volume based on the following equations, putting into relation the: volumetric power input [W/m^3], the reactor system-specific impeller diameter *D* [m], the impeller rotational speed *N* [rps], the liquid density ρ [kg/m^3], the power number N_p [-] and the vessel liquid volume *V* [m^3]

31) $$\frac{P}{V} = \frac{N^3 \times D^5 \times \rho \times N_p}{V}$$

Therefore, the volumetric power input is supposed to constant in the new system:

32) $$\frac{P}{V} = \frac{{N_i}^3 \times {D_i}^5 \times \rho \times N_{p,i}}{V_i} = \frac{{N_o}^3 \times {D_o}^5 \times \rho \times N_{p,o}}{V_o}$$

Since the liquid density is constant between our systems it is neglect able:

33) $$\frac{{N_i}^3 \times {D_i}^5 \times N_{p,i}}{V_i} = \frac{{N_o}^3 \times {D_o}^5 \times N_{p,o}}{V_o}$$

Therefore after conversion of the equation the impeller rotational speed can be calculated:

34) $$N_o = \sqrt[3]{{N_i}^3 \times \frac{V_o}{V_i} \times \frac{{D_i}^5}{{D_o}^5} \times \frac{N_{p,i}}{N_{p,o}}}$$

However, after adjustments it seems that by keeping the impeller geometry as well as the impeller to vessel diameter ratio constant, the power number can be neglected resulting in the following equation:

35) $$N_o = \sqrt[3]{{N_i}^3 \times \frac{V_o}{V_i} \times \frac{{D_i}^5}{{D_o}^5}}$$

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