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Supplemental Information

Robotic high-throughput biomanufacturing and functional differentiation of human pluripotent stem cells

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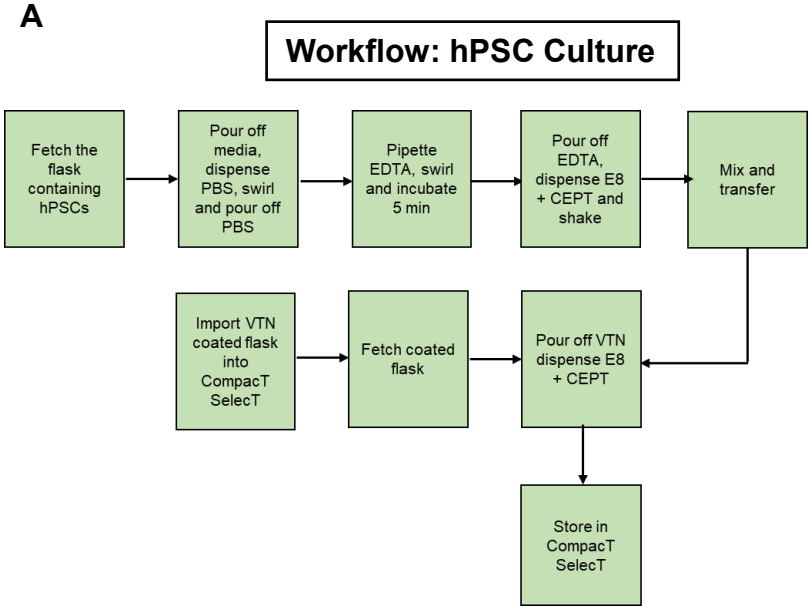
Figure S1

A



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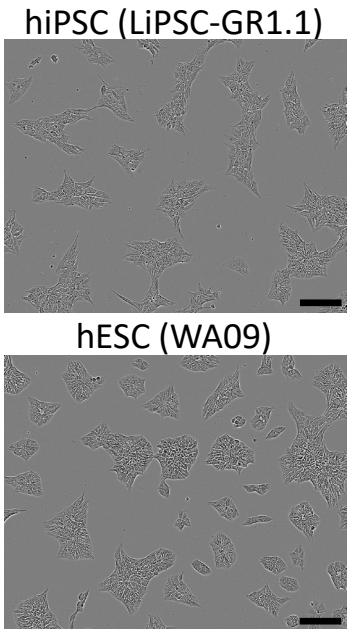

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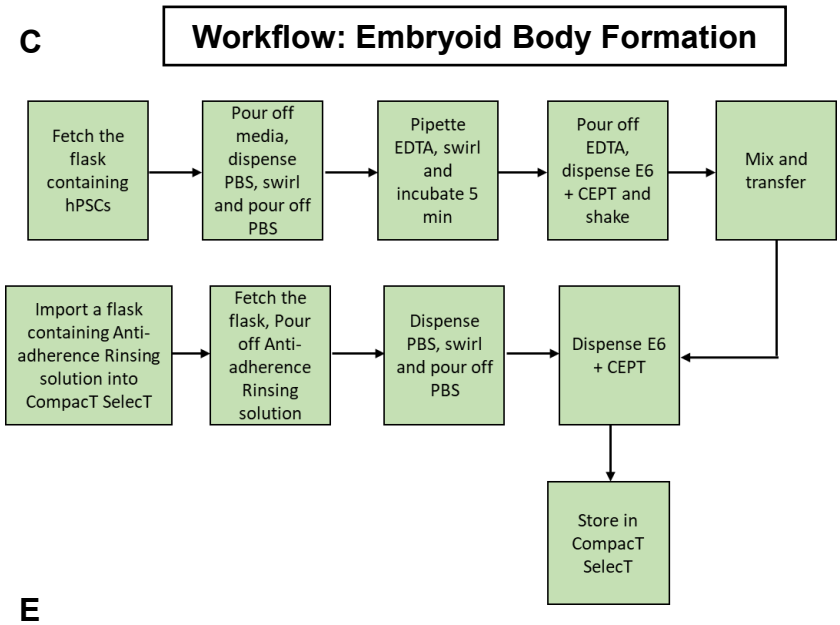

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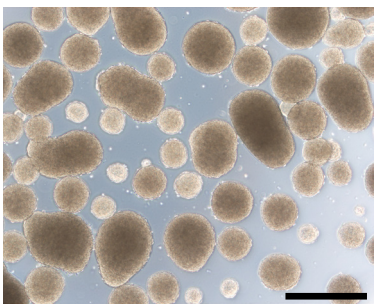


E

Sample Name	Self-renewal	Ectoderm	Mesoderm	Endoderm
hESC EB Manual	-5.05	1.30	3.07	0.56
hESC EB Automated	-4.74	1.24	2.58	0.16
hiPSC EB Manual	-0.80	0.29	0.13	-0.69
hiPSC EB Automated	0.18	0.63	0.23	-0.51

Gene expression relative to the reference standard

Upregulated						Downregulated
x > 1.5	1.0 < x ≤ 1.5	0.5 < x ≤ 1.0	-0.5 ≤ x ≤ 0.5	-1.0 ≤ x < -0.5	-1.5 ≤ x < -1.0	x < -1.5

Figure S1: Robotic workflow for culturing hPSCs and embryoid body (EB) formation
(A) Standardized protocol developed for routine culture of hPSCs using CTST under chemically defined conditions.
(B) Phase-contrast images of robotically cultured hESCs (WA09) and hPSCs (LiPSC-GR1.1) after passaging with the CEPT cocktail. Note the high quality of cultures and absence of cellular debris at 24 h post-passage.
(C) Protocol established for scalable production of EBs by using the CTST system under chemically defined conditions.
(D) Representative phase-contrast image of robotically generated EBs (day 7), which can be cultured and scaled up in large T175 flasks. Scale bar, 500 µm.
(E) ScoreCard analysis of EBs generated manually or robotically from hESCs (WA09) and hiPSCs (LiPSC-GR1.1) show differentiation potential into the three germ layers at day 7.
Data are from n > 3 biological replicates using two independent cell lines (B-C).
Representative images shown for two different cell lines. Optimal cell viability and culture was also observed using other cell lines as summarized in Table S1. Scale bar, 200 µm.

Figure S2

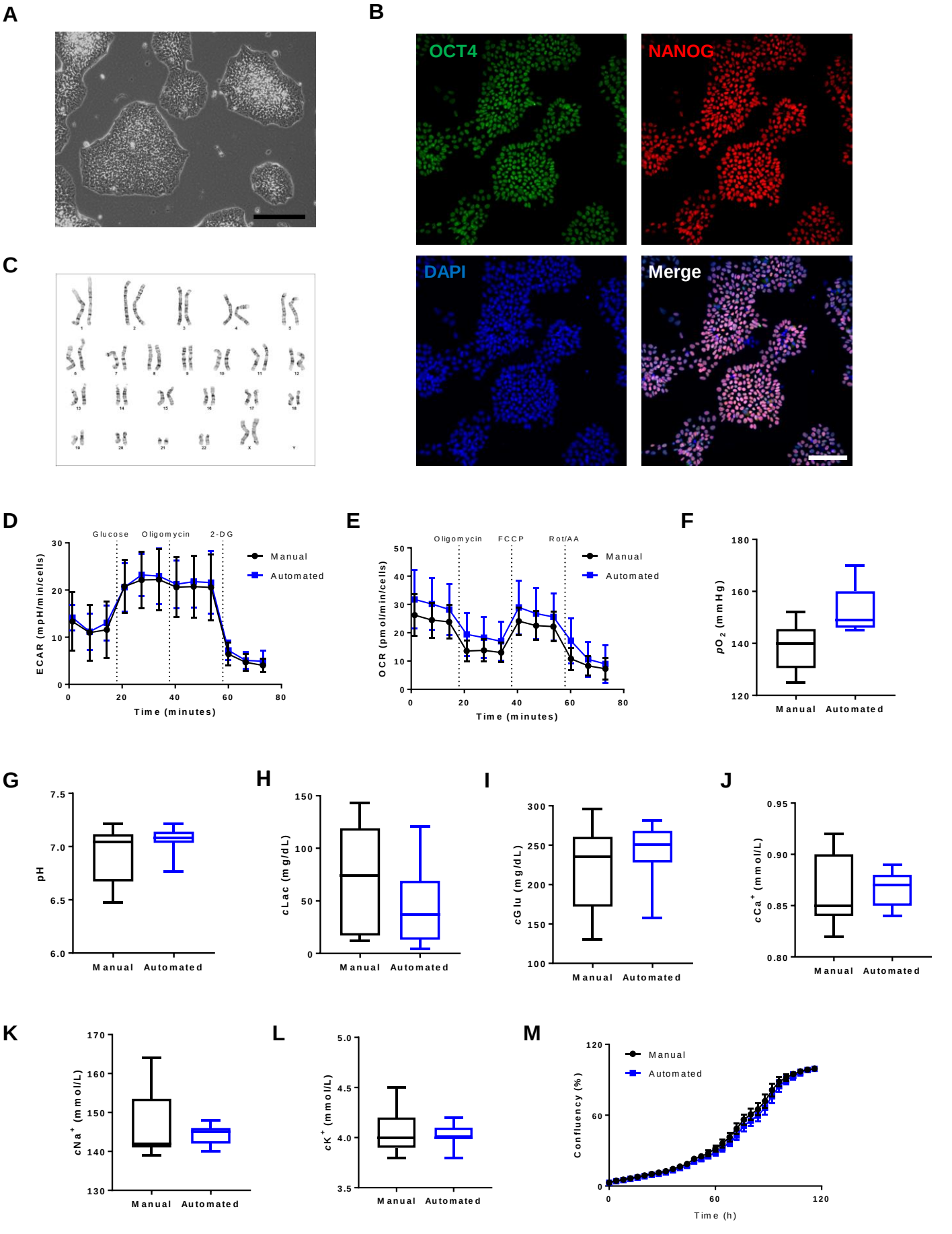

A
B
OCT4
NANOG
DAPI
Merge
C
D
Glucose
Oligomycin
2-DG
Manual
Automated
ECAR (mpH/min/cells)
Time (minutes)
E
Oligomycin
FCCP
Rot/AA
Manual
Automated
OCR (pmol/min/cells)
Time (minutes)
F
pO2 (mmHg)
Manual
Automated
G
pH
Manual
Automated
H
cLac (mg/dL)
Manual
Automated
I
cGlu (mg/dL)
Manual
Automated
J
cCa+ (mmol/L)
Manual
Automated
K
cNa+ (mmol/L)
Manual
Automated
L
cK+ (mmol/L)
Manual
Automated
M
Manual
Automated
Confluency (%)
Time (h)

Figure S2: Characterization of hESCs (WA09) cultured by CTST

(A) Representative overview of pluripotent stem cell colonies. Scale bar, 200 µm.

(B) Immunocytochemical analysis showing expression of pluripotency-associated markers OCT4 and NANOG in hESCs. Scale bar, 100 µm.

(C) Robotically cultured hESCs maintain a normal karyotype (passage 43).

(D-J) Supernatants of cultures maintained either manually or by automation were analyzed daily by using the Vi-Cell MetaFLEX Bioanalyte Analyzer (Beckman). Box plots show the variation of spent media from hESC cultures.

(K) Seahorse XF Glycolysis Stress Test profile comparison of glycolytic function in hESCs maintained by automated or manual cell culture. Cells were treated with serial injections of metabolic modulators (glucose, oligomycin, 2-deoxyglucose (2-DG)).

(L) Seahorse XF Mitochondrial Stress Test profile comparison of mitochondrial function in hESCs maintained by automated or manual cell culture. Cells were treated with serial injections of metabolic modulators (oligomycin, FCCP and Rotenone/Antimycin A (Ret/AA)).

(M) Image-based analysis comparing cell growth in hESC cultures expanded manually and robotically.

Data are expressed as mean ± SD, n > 3 biological replicates using two independent cell lines (D-M). $P > 0.5$, unpaired *t*-test.

Figure S3

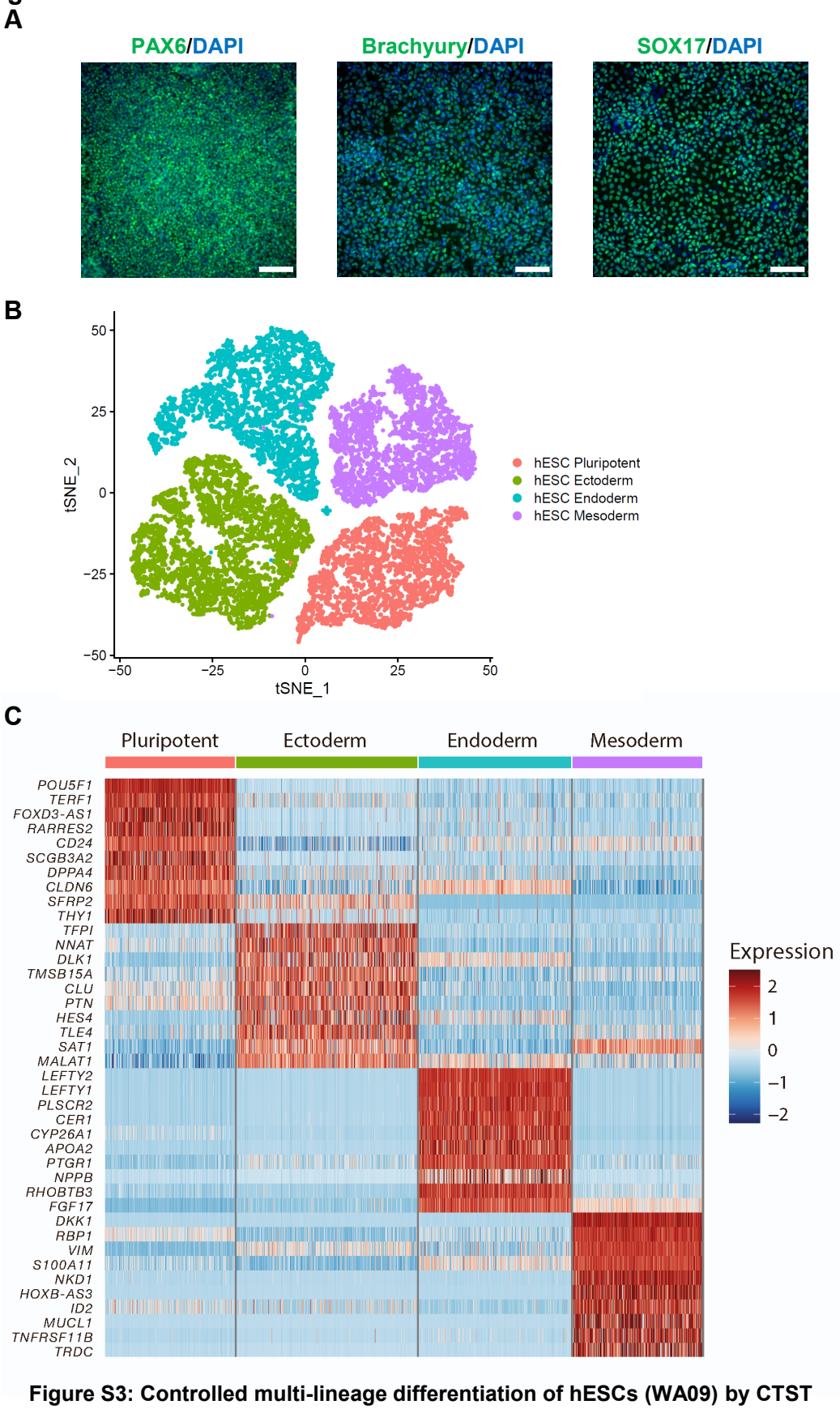


Figure S3: Controlled multi-lineage differentiation of hESCs (WA09) by CTST

(A) Immunocytochemical analysis of hESC-derived ectoderm (PAX6) at day 7, endoderm (SOX17) at day 5 and mesoderm (Brachyury) at day 5 cultures differentiated by CTST. Scale bar, 200 µm.

(B) Single-cell RNA-seq analysis of pluripotent and differentiated hESCs.

(C) Heatmap showing the highly expressed genes for pluripotent cells (WA09) and differentiated cultures representing ectoderm (day 7), endoderm (day 5) and mesoderm (day 5).

Data are from n = 4 biological replicates (A). Data are from n = 19,759 or 16,582 single cells obtained from n = 4 independent experiments using two independent cell lines (B). Single-cell RNA-seq data were analyzed in the Seurat R package. See also legend of Figure 5 for cell counts analyzed per sample.

Figure S4

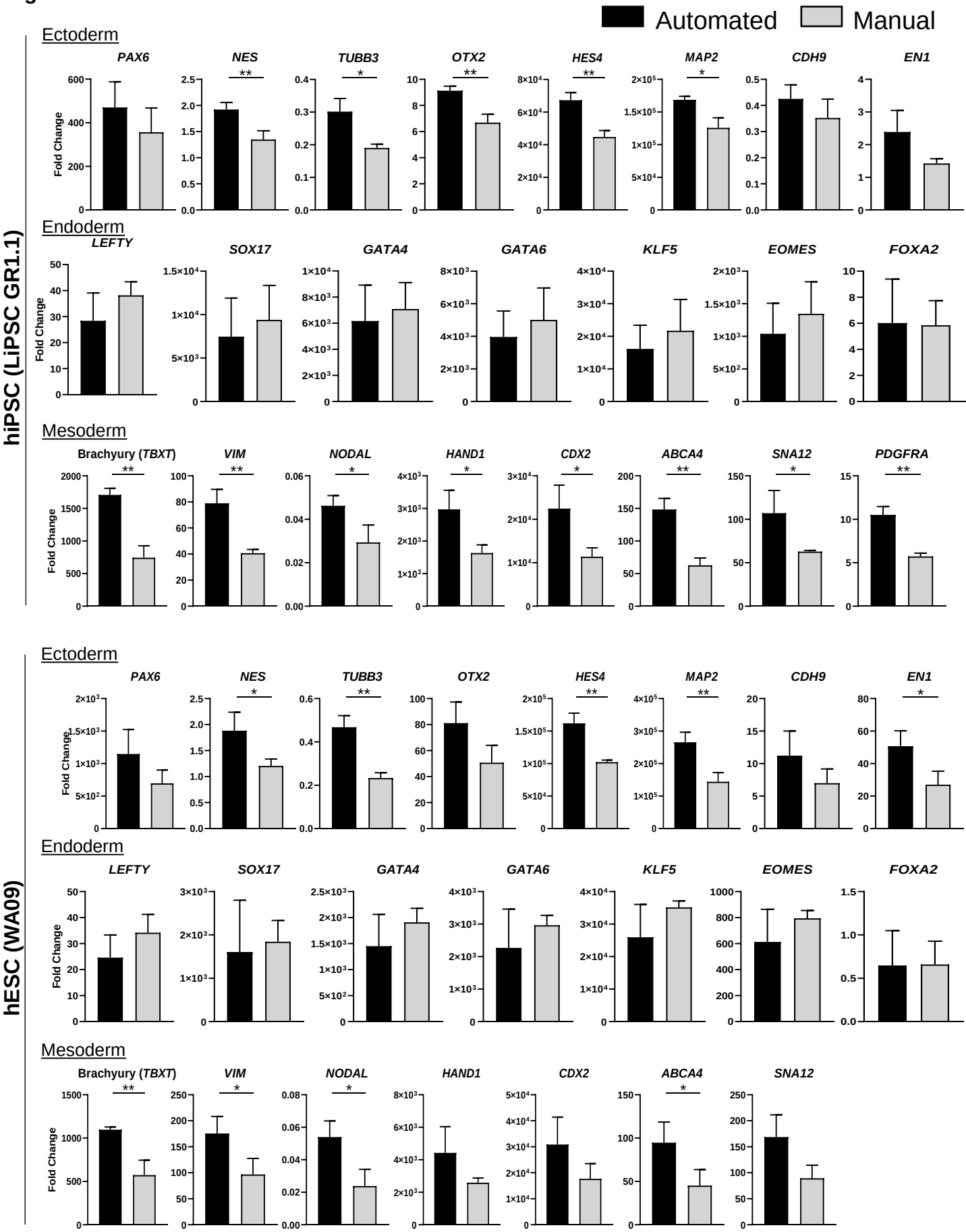


Figure S4: RT-PCR analysis and comparison of multilineage cells differentiated manually and robotically
Expression of typical gene expression of ectoderm at day 7, endoderm and mesoderm at day 5 post differentiation. Data are expressed as mean ± SD, n ≥ 3 biological replicates using hiPSCs (LiPSC-GR1.1) or hESCs (WA09). Note that *NES*, *TUBB3*, *HES4, MAP2,* Brachyury (*TBXT*), *VIM*, *NODAL* and *ABCA4* are expressed at significantly higher levels in automated versus manual differentiation. $P < 0.05$ (*) and $P < 0.01$ (**), unpaired *t*-test.

Figure S5

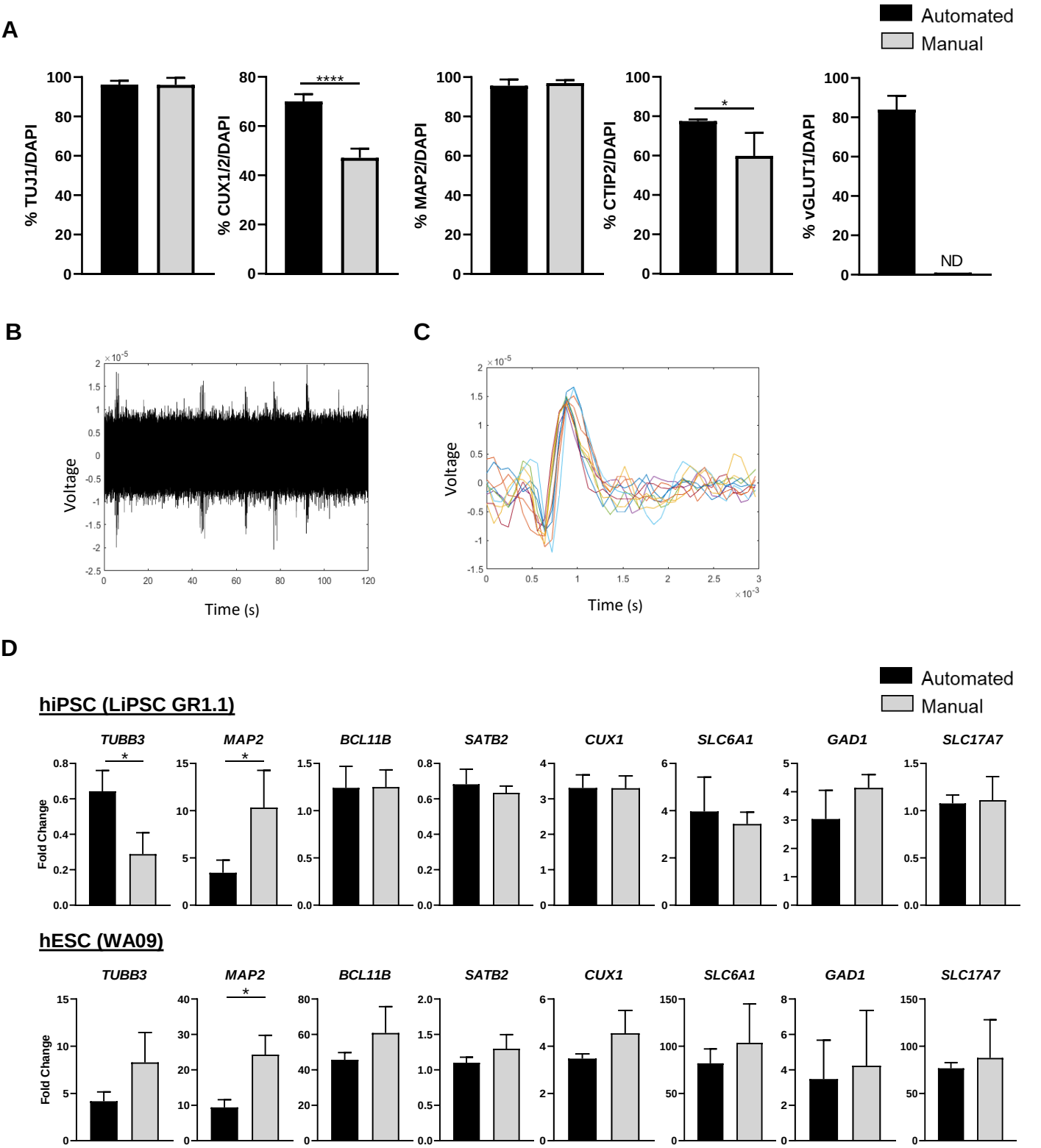


Figure S5: Quantification and characterization of neuronal cultures differentiated manually and robotically

(A) Quantification of cells expressing TUJ1, CUX1, MAP2, CTIP2, GABA and vGLUT1. Cells were differentiated for 40 days (LiPSC-GR1.1) either manually or by CTST. See also Figure 6 for representative immunostain.

(B) MEA experiment showing spontaneous neuronal activity (spike profile) at 6 weeks of robotic differentiation of hiPSCs (LiPSC-GR1.1).

(C) Representative overlay of 10 spikes detected from one channel of a MEA recording to demonstrate similarity between the spikes detected.

(D) RT-PCR analysis of typical neuronal gene expression after 6 weeks of differentiation.

Data are expressed as mean ± SD, n ≥ 3 independent fields of view (A), MEA recordings (B-C) or RT-PCR (D). $P < 0.05$ (*) and $P < 0.0001$ (****), unpaired *t*-test. N.D. indicates data not collected.

Figure S6

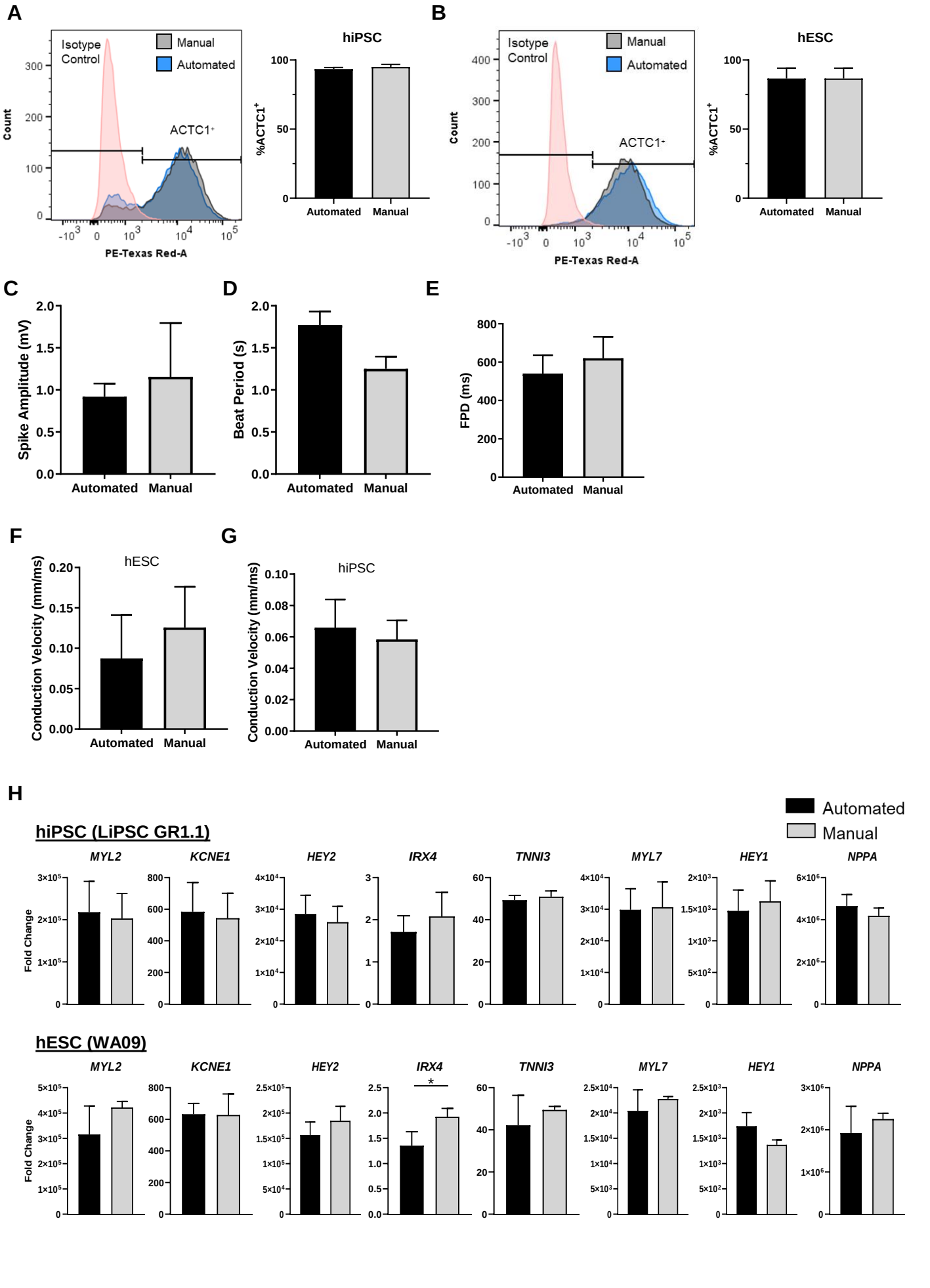

A
Isotype Control
Manual
Automated
ACTC1+
Count
PE-Texas Red-A
hiPSC
%ACTC1+
Automated
Manual
B
hESC
C
Spike Amplitude (mV)
D
Beat Period (s)
E
FPD (ms)
F
hESC
Conduction Velocity (mm/ms)
G
hiPSC
H
Automated
Manual
hiPSC (LiPSC GR1.1)
hESC (WA09)
MYL2
KCNE1
HEY2
IRX4
TNNI3
MYL7
HEY1
NPPA
Fold Change
*

Figure S6: Functional analysis and characterization of cardiomyocytes generated manually and robotically

(A, B) Flow cytometry analysis and quantification of alpha-cardiac actin (ACTC1)-expressing cells at day 24 derived from hiPSCs (LiPSC-GR1.1) and hESCs (WA09).

(C) Comparison of spontaneous spike amplitudes in cardiomyocyte cultures differentiated manually and robotically from hESCs (WA09). MEA experiment was performed at day 24.

(D) Comparison of beat periods in hESC-derived cardiomyocyte cultures (WA09) generated by manual versus automated cell differentiation. MEA experiment was performed at day 24.

(E) Comparison of field potential duration in cardiomyocyte cultures after manual and automated differentiation of hESCs (WA09) as measured by MEA on day 24.

(F, G) MEA analysis of conduction velocity in cardiomyocyte cultures derived from hESCs (WA09) and hiPSCs (LiPSC-GR1.1) after manual and robotic cell differentiation (day 24).

(H) RT-PCR analysis and comparison of typical cardiomyocyte-specific genes expressed after 24 days of manual or robotic differentiation. Note that virtually all genes tested are expressed at similar levels irrespective of manual or automated differentiation.

Data are expressed as mean ± SD, n ≥ 3 biological replicates using two independent cell lines. $P < 0.05$, unpaired *t*-test.

Figure S7

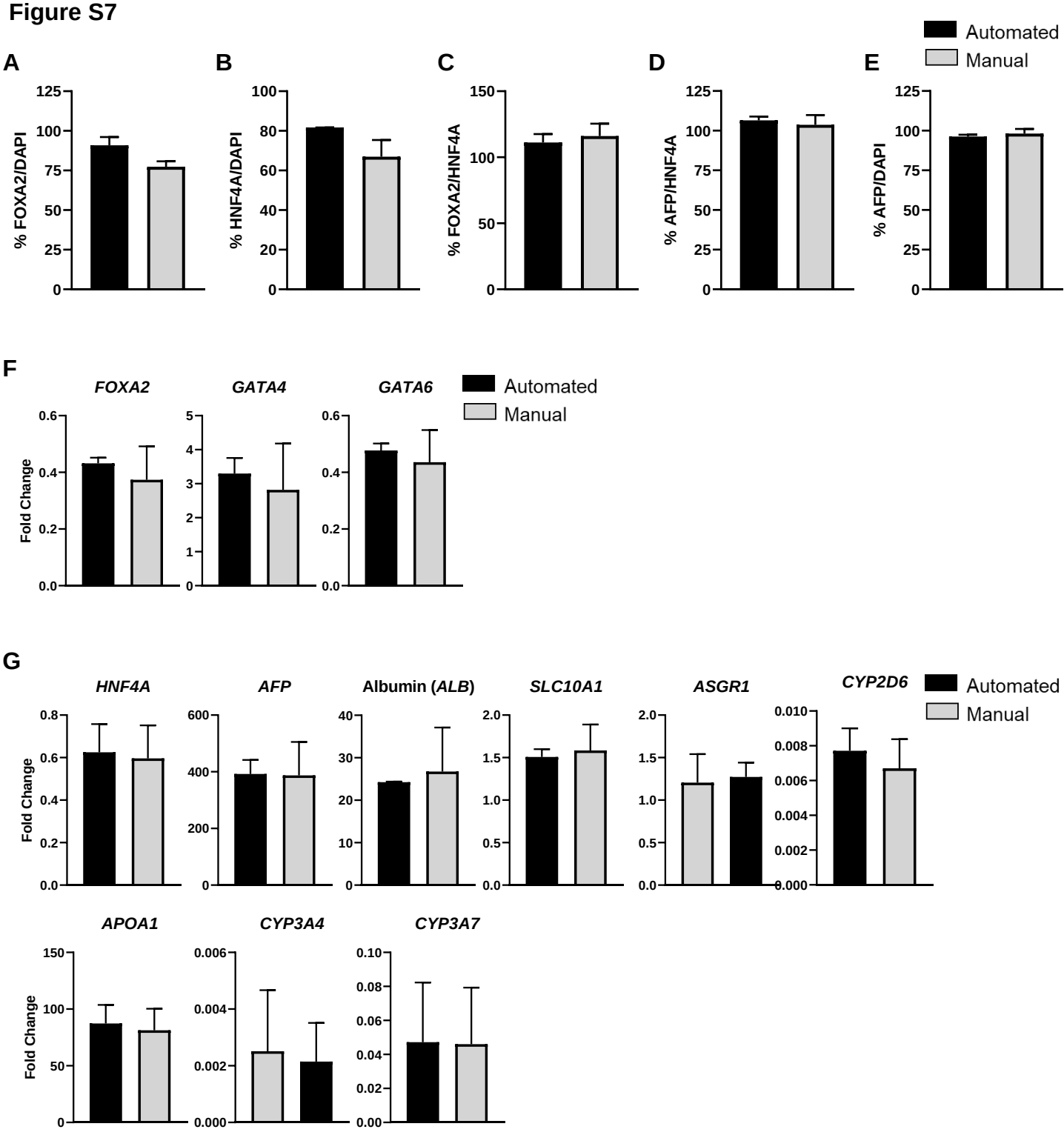


Figure S7: Quantification of endodermal and hepatic markers after manually and robotic cell differentiation

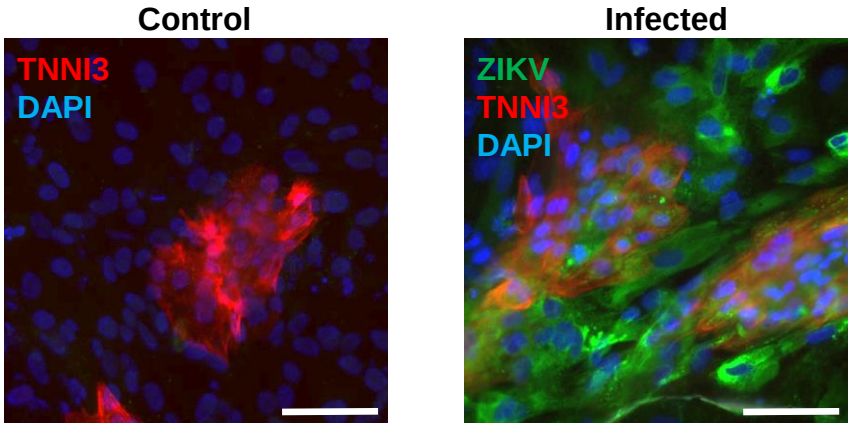
(A-E) Quantification and comparison of immunolabeled cells expressing FOXA2, HNF4A and AFP in hiPSCs cultures (LiPSC-GR1.1) differentiated into hepatocytes manually and robotically. See also Figures 7I-J for representative immunostain. Data are expressed as mean ± SD, n≥ 3 independent fields of view. $P > 0.5$, unpaired *t*-test.

(F-G) RT-PCR analysis and comparison of manual and robotic hepatocyte differentiation at day 10 and 20, respectively. Note that virtually all genes tested are expressed at similar levels irrespective of manual or automated differentiation.

Data are expressed as mean ± SD, n ≥ 3 biological replicates using the LiPSC-GR1.1 cell line. $P > 0.5$, unpaired *t*-test.

Figure S8

A



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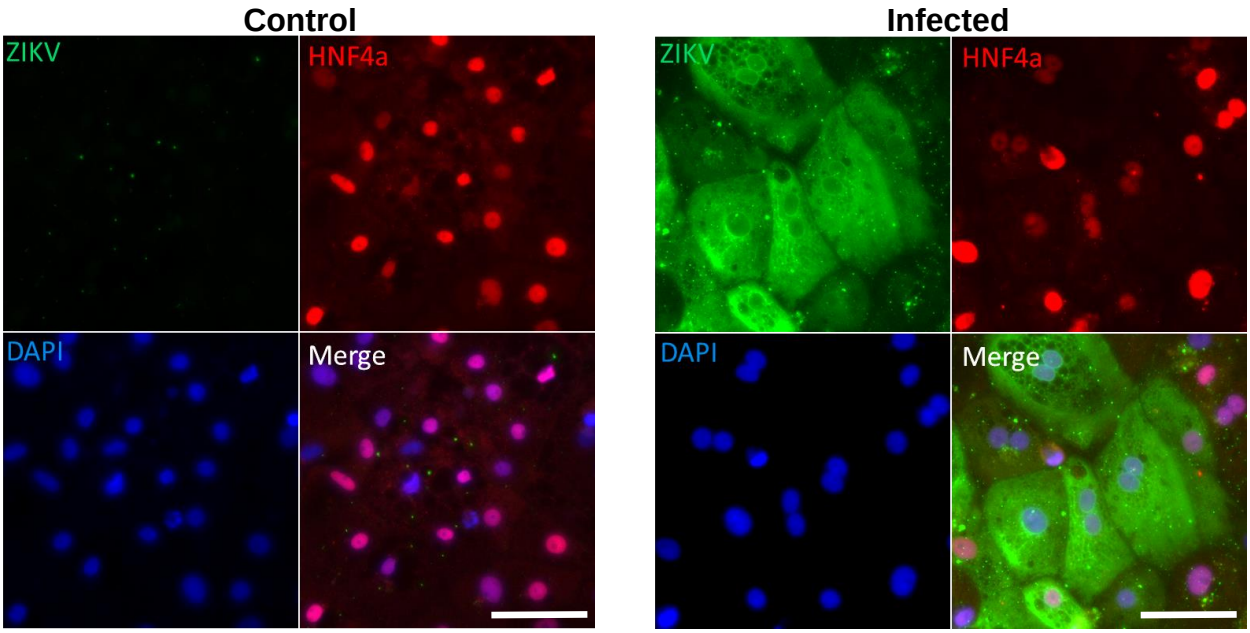

Figure S8: Infection of robotically generated cardiomyocytes and hepatocytes by ZIKV
(A) Cardiomyocytes were derived from hiPSCs (LiPSC-GR1.1) and exposed to ZIKV for 24 h. A specific antibody against flavivirus antigen shows that cells expressing cardiac troponin (TNNI3) can be infected by ZIKV. Scale bar, 75 µm.
(B) Hepatocytes were derived from hiPSCs (LiPSC-GR1.1) and exposed to ZIKV for 24 h. A specific antibody against flavivirus antigen shows that cells expressing HNF4A can be infected by ZIKV. Scale bar, 75 µm. Data are from n ≥ 3 biological replicates.

Cell Line	Source
BU NKX2.1-GFP	Boston University
CDI IPS 8621	Cellular Dynamics
GM23225	Coriell
GM23279	Coriell
GM23476	Coriell
GM23720	Coriell
GM25256	Coriell
GM26107	Coriell
ESI-035	ESI BIO
HUES 8	Harvard Stem Cell Institute
HUES 9	Harvard Stem Cell Institute
HUES 53	Harvard Stem Cell Institute
HUES 64	Harvard Stem Cell Institute
NCRM4	NIH
NCRM5	NIH
ND1-4	NIH
CMT2A-1.1	WiCell
CMT2A-1.2	WiCell
CMT2A-2.1	WiCell
CMT2A-2.2	WiCell
CMT2A-3.1	WiCell
CMT2A-3.2	WiCell
JHU078i	WiCell
JHU198i	WiCell
MCW027i	WiCell
MCW032i	WiCell
WA01	WiCell
WA01 Oct4-GFP	WiCell
WA09	WiCell
WA09 Syn-GFP	WiCell
WA13	WiCell
WA14	WiCell
WA17	WiCell
WA26	WiCell

Table S1. Overview of cell lines cultured with CTST and experiments represented in this study
List of hESC and hiPSC lines that were robotically cultured over the last 5 years by the stem cell team at NCATS and used for various projects. To date, the listed cell lines have undergone robotic cell expansion to generate more than 3720 cryovials with ten million hESCs or hiPSCs per vial. The cell lines WA09 and LiPSC-GR1.1 were systematically characterized in this study as indicated.

Vessel	Surface Area Per Well (cm^2)	Total Vessel Surface Area (cm^2)	CompacT SelecT Capacity	Total Surface Area (cm^2)	Media Change Speed (min)	Media Changes Per Day	Manual Media Changes Per Shift (8h)
T175 Flask	175	175	90	15750	2	720	240
T75 Flask	75	75	90	6750	2	720	240
T175 Triple Flask	525	525	90	47250	2	720	240
6-Well Plate	9.5	57	190	10830	6	240	80
24-Well Plate	1.9	45.6	190	8664	6	240	80
96-Well Plate	0.32	30.72	280	8602	6	240	80
384-Well Plate	0.056	21.504	280	6021	6	240	80

Table S2: Comparing the efficiency of robotic versus manual cell culture
Automated versus manual cell culture features can be compared considering different plate formats, speed of media changes and number of possible media changes based on the scenario that automation allows non-stop 24 h cell culture work, whereas manual cell culture is performed during a typical 8 h workday. In addition, while manual cell culture is typically done in 6-well plates, the CTST system can handle various flask and plate formats listed here.

Table S3. Differentially expressed genes in manually versus robotically cultured cells
List of genes that were up- or downregulated in hiPSCs and hESCs after manual or robotic cell culture. Data are from n = 5573, 4835, 4485 or 3922 single cells obtained from n = 4 independent experiments using two independent cell lines for “hESC Manual”, “hiPSC Manual”, “hESC Auto” and “hiPSC Auto”, respectively. Single-cell RNA-seq data were analyzed in the Seurat R package. See also Figure 3 for more details. **See Supplemental Excel File Table S3.**

Table S4. Helios, TaqMan, Western blot, and Immunofluorescence reagents
A CyTOF antibody panel against 28 targets for pluripotency, DNA damage, apoptosis and stress-signaling pathways as used for the experiment shown in Figure 4. List of TaqMan probes used for RT-PCR analyses. List of antibodies used for western blot and immunofluorescence.
See Supplemental Excel File Table S4.

	Initial (Million)	Final (Million)	Scale-up per Plate or Flask (Million)						
Cell Type	**Cells/cm²**	**Cells/cm²**	**384-well**	**96-well**	**24-well**	**6-well**	**T75**	**T175**	**T175 Triple**
Ectoderm	0.10	0.90	19.4	26.65	41.04	51.30	67.50	157.50	472.50
Mesoderm	0.05	0.45	9.66	13.82	20.52	25.65	33.75	78.75	236.25
Endoderm	0.20	0.40	8.60	12.29	18.24	22.80	30.00	70.00	210.00
Hepatocytes	0.10	0.30	6.45	9.22	13.68	17.10	22.50	52.50	157.00
Cardiomyocytes	0.09	0.10	2.15	3.07	4.56	5.70	7.50	17.50	52.50
Neurons	0.05	0.43	9.30	13.21	19.61	24.51	32.25	75.25	225.75

Table S5. User-friendly scalable production of different cell types by CTST
Depending on experimental needs, various cell types can be derived from hPSCs and produced at large scale in different cell culture vessels.

Reference	Automated System	Culture Medium for hPSCs	Coating Substrate	Passaging Reagent	Differentiation	Automated Scalability	Chemically Defined	Analysis
Thomas et al., 2009	CompacT SelecT	MEF-Conditioned Medium	Matrigel	Trypsin	Manual Embryoid bodies Cardiomyocytes	Partial	No	Pluripotency markers Karyotype MEA
McLaren et al., 2013	CompacT SelecT	N/A	PLO-Laminin	Trypsin	Automated Lt-NES	Partial	N/A	Neural markers
Soares et al., 2014	CompacT SelecT	CDM-PVA	Porcine gelatin-MEF/FBS	Collagenase IV, Dispase	Manual Multi-linage	Partial	No	Pluripotency markers qPCR
Tristan et al., present study	CompacT SelecT	E8 Medium (chemically defined)	Recombinant Vitronectin (chemically defined)	EDTA (enzyme-free)	Automated Monolayer Multi-lineage Embryoid bodies Neurospheres Cortical Neurons Cardiomyocytes Hepatocytes Others (not shown in present study)	Full	Yes	Comparison manual vs. robotic Pluripotency markers Karyotype Scorecard/qPCR Bulk culture RNA-Seq Single-cell RNA-Seq Mass cytometry Metabolic analysis Robotic MEA Disease modeling High-throughput screening

Table S6. Overview and comparison of previous reports and the present study utilizing CTST
Note the various advantages of the present study as compared to previous reports including the use of chemically defined media, enzyme-free passaging and more extensive analysis and characterization of cells generated by automation.

SUPPLEMENTAL MOVIES

Movie S1: Robotic cell culture of hiPSCs using the CTST system

Movie shows a routine step during cell passaging when hiPSCs cultured in large flasks are detached and prepared for plating into new flasks. Full movie showing the various automated functions carried out under sterile conditions and mimicking the manual cell culture process is available here: https://youtu.be/-GSsTSO-WCM

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Automated and manual cell culture

All hESC and hiPSC lines (Table S1) were maintained under feeder-free conditions in E8 medium (ThermoFisher) and VTN-N-coated (ThermoFisher) microplates or T175 flasks. Cells were passaged using 0.5 mM EDTA in phosphate buffered saline (PBS) without calcium or magnesium (ThermoFisher) every 3-4 days. After passage cells were counted using the automated Vi-cell XR counter (Beckman) on the CTST platform and cells were plated at a density of 1.5×10^5 cells/cm^2 in E8 cell culture medium supplemented with the CEPT cocktail for the first 24 h (Chen et al., 2021). All karyotyping analysis were performed by Cell Line Genetics (Madison, WI). Cells were maintained in a humidified 5% CO_2 atmosphere at 37 °C. To optimize cell survival and cytoprotection during cell passaging of pluripotent and differentiated cells, we used the recently developed CEPT cocktail (Chen et al., 2021) consisting of 50 nM Chroman 1 (#HY-15392; MedChem Express), 5 μM Emricasan (#S7775; Selleckchem), Polyamine supplement (#P8483, 1:1000 dilution; Sigma-Aldrich) and 0.7 μM Trans-ISRIB (#5284; Tocris). All manual cell culture experiments were performed in parallel to automated cell culture and matched for cell passage number and density throughout the study.

Live-cell metabolic assays by using Seahorse XF Analyzer

hES/iPS cells were dissociated by using Accutase, counted and seeded at 15,000 cells per well and incubated for 24 hours at 37°C in 5% CO_2 atmosphere. On the day of measurement, media was changed to Seahorse assay media containing 1 mM pyruvate, 2 mM glutamine and 10 mM glucose supplemented with 400 ng/mL of Hoechst and incubated in a CO_2-free incubator for 1 hour. Cell number was obtained by imaging for Hoechst- or DAPI-positive nuclei in a Celigo Image Cytometer (Nexcelom) for data normalization. Mitochondrial metabolism (OCR) and glycolysis (ECAR) were analyzed with Seahorse Mito, Glycolysis and Phenotype Stress Test Kits (Agilent).

Multi-electrode array (MEA)

hES/iPSC-derived neurons were plated at a density of ~5 million neurons/cm^2 in complete media containing laminin (10 μg/mL, ThermoFisher) and Y-27632 (10 μM, Tocris). After confirming the superiority of the CEPT cocktail, we replaced Y-27632 by CEPT. Twenty-four hours post-plating, media was replaced with neuron maintenance media and 50% media exchange was performed every 2-3 day. Recordings were acquired day 7 and 14 post plating. iPSC-derived cardiomyocytes were plated at a density of ~63,000 cardiomyocytes/cm^2 in cardiomyocyte media using CEPT cocktail. Forty-eight hours post-plating media was replaced with cardiomyocyte maintenance media and complete media changes were performed every 2 days. For cardiomyocytes MEA plates were coated with 8 μL of fibronectin (50 μg/mL; Sigma) over the electrode recording area. For plating neuronal cells, MEA plates were coated with poly(ethyleneimine) (0.1%, Sigma) in borate buffer (pH 8.4).

Mass cytometry time-of-flight (CyTOF)

Human ES/iPS cells were dissociated into single cells with Accutase for 12 min at 37°C. After single cell dissociation, cells were then stained with 2.5 μM Cell-ID Cisplatin (201064, Fluidigm) in MaxPar PBS (201058, Fluidigm) to discern viable (negative) and non-viable cells (positive). Surface antibody staining was performed at RT for 30 min in MaxPar Cell Staining Buffer (201068, Fluidigm). The cells were then fixed with freshly prepared 1.6% formaldehyde (28906, Thermo-Fisher) solution in MaxPar PBS for 20 min. Permeabilization was performed at RT for 15 min using 25% Nuclear Antigen Staining Buffer Concentrate (S00111, Fluidigm) in Nuclear Antigen Staining Buffer Diluent (S00112, Fluidigm). Then the cells were stained with antibodies against intracellular targets in Nuclear Antigen Staining Perm (S00113, Fluidigm) at RT for 45 min.

To identify cellular events, the cells were stained with 250nM Iridium Intercalator (201192B, Fluidigm) in MaxPar Fix and Perm Buffer (201067, Fluidigm). The cells were loaded into the cytometer in Cell Acquisition Solution (201240, Fluidigm) supplemented with 10% EQ™ Four Element Calibration Beads (201078, Fluidigm) for signal normalization. The data acquisition was performed using Helios™, a CyTOF® mass cytometer system (Fluidigm). The acquired data was normalized based on EQ Four Element Calibration Beads signal using R/Shiny package “premessa” (https://github.com/ParkerICI/premessa). Beads were then excluded and dead cells, aggregates and non-cellular events were gated out. The single cellular events were retained and the data were analyzed using a modified CyTOF workflow (Robinson et al., 2017). A total of 200000 events were collected for each sample, including normalization beads. The

numbers of single live cells that passed the gate criteria were used for subsequent analysis were: WA09 auto 32889 cells, WA09 manual 11898 cells, LiPSC-GR1.1 auto 32857 cells, LiPSC-GR1.1 manual 19217 cells. To construct the UMAP plots, 8,000 cells were used from each sample. The panel of antibodies is provided in Table S4.

Flow cytometry analysis of cardiomyocytes

On day 24 of cardiomyocyte differentiation, cells were dissociated using the Cardiomyocyte Dissociation Kit (STEMCELL Technologies). Dissociated cells were resuspended in Intracellular Fixation and Permeabilization Buffer (ThermoFisher) and incubated for 15 min at RT. Cells were stained with primary antibodies against cardiac actin (Sigma, MABT823, 1:50) or IgG control (Millipore, CBL610, 1:100) were added and incubated for 1 h at RT, followed by FITC-conjugated goat anti-mouse secondary antibody (Millipore, cat# 12-506, 1:100) staining for 1 h at RT. Cells were washed twice with staining buffer and analyzed using a BD LSRFortessa Flow Cytometer (BD Biosciences).

Single-cell RNA library preparation and sequencing

Cells were dissociated by 10 min incubation with Accutase (Sigma) at 37 °C to obtain a single cell suspension, washed with PBS, pelleted and resuspended in PBS at a cell concentration of 1,000 cells per µL. GEMs were transferred to PCR 8-tube strips and GEM-reverse transcription was performed in a C1000 Touch Thermal Cycler (BioRad): 53 °C for 45 min, 85 °C for 5 min and held at 4 °C. GEMs were lysed in recovery buffer and single-stranded cDNA was cleaned up using silane DynaBeads (ThermoFisher). cDNA was amplified in a C1000 Touch Thermal Cycler (BioRad): 98°C for 3 min, cycled 12X: 98 °C for 15 sec, 67 °C for 20 sec, 72 °C for 1 min; 72 °C for 1 min and held at 4 °C. Amplified cDNA was cleaned up using the SPRIselect Reagent (Beckman Coulter). Post cDNA amplification QC and quantification was done using a High Sensitivity D5000 ScreenTape Assay (Agilent) on a 4200 TapeStation System (Agilent). Library Construction was done by fragmentation at 32 °C for 5 min, end repair and A-tailing at 65°C for 30 min. Post fragmentation, end repair and A-tailing double-sided size selection was done using the SPRIselect Reagent (Beckman Coulter). Adaptor ligation was done at 20°C for 15 min. Post ligation cleaned up using the SPRIselect Reagent (Beckman Coulter). Sample indexing was done using the i7 Sample Index Plate (Chromium) in a C1000 Touch Thermal Cycler (BioRad): cycled 10-12X: 98 °C for 45 sec, 98 °C for 20 sec, 54 °C for 30 sec, 72 °C for 20 sec; 72 °C for 1 min and held at 4 °C. Post sample index PCR double sided size selection done using the SPRIselect Reagent (Beckman Coulter). Post library construction quantification was done using a High Sensitivity D1000 ScreenTape Assay (Agilent) on a 4200 TapeStation System (Agilent). Sequencing libraries were quantified by qPCR using the KAPA library quantification kit for Illumina platforms (KAPA Biosystems) on a QuantStudio 12K Flex Real-Time PCR System (ThermoFisher). Libraries were loaded on an Illumina HiSeq 3000 using the following: 98bp Read1, 8bp i7 Index and 26bp Read2.

Analysis of single-cell RNA-seq

The Cellranger software package from 10X Genomics, Inc. (version 3.0.1) was used to process raw BCL files from single cell sequencing as follows. Pipeline details can be found at https://github.com/cemalley/Tristan_methods. This work used the computational resources of the NIH HPC Biowulf cluster (http://hpc.nih.gov). Demultiplexing and FASTQ generation were done with the mkfastq command, and the count command created gene expression matrices. Dense matrices were created with the mat2csv command. Embryonic stem cell and iPSC lines were analyzed in the Seurat R package (Seurat 2.3.4; R 3.5.2) (Stuart et al., 2019). The FindMarkers and FindAllMarkers functions were used to perform Wilcoxon rank sum tests of differential expression between samples or clusters. Cluster determination used the FindClusters function with default resolution parameter, which runs a shared nearest neighbor modularity optimization-based clustering algorithm. Expression dynamics due to cell cycle were regressed out using the CellCycleScoring function. See the code at https://github.com/cemalley/Tristan_methods for full details. Further data visualizations were made in R and with the ggplot2 package (3.1.0). Samples were checked for expression of markers of glycolysis, aerobic respiration, pluripotency, the peroxisome, the pentose phosphate shunt and the TCA cycle.

Automated and manual differentiation into embryonic germ layers

For endoderm differentiation, hES/iPS cells were plated at a density of 150,000 cells/cm^2 on VTN-coated T75 flasks in 15 mL of E8 media supplemented with CEPT cocktail and allowed to reach 50-60%

confluency. After reaching 50-60% confluency cell culture media was switched to TeSR-E8 Pre-Differentiation media for 24 h or until cells reached 70% confluency. Cells were single-cell dissociated by 10-15 min incubation with EDTA (0.5 mM, ThermoFisher) in phosphate buffered saline (PBS) without calcium or magnesium (ThermoFisher) at 37 °C and plated at a density of 210,000 cells/cm^2 onto VTN-coated T75 flasks in 15 of TeSR-E8 Pre-Differentiation media supplemented with CEPT cocktail. Twenty-four hours post plating cell culture media, flasks were rinsed with DMEM/F12 and media was replaced with 15 mL of Medium 1 (STEMdiff Definitive Endoderm Basal Medium with STEMdiff Definitive Endoderm Supplement A and STEMdiff Definitive Endoderm Supplement B). The next day cell culture media was exchanged with 15 mL Medium 2 (STEMdiff Definitive Endoderm Basal Medium with STEMdiff Definitive Endoderm Supplement B). On Days 3-5, cell culture media was changed with 15 mL Medium 2 (STEMdiff Definitive Endoderm Basal Medium with STEMdiff Definitive Endoderm Supplement B). Endoderm cells were analyzed on day 5.

For mesoderm differentiation on day 0 cells were plated at a density of 50,000 cells/cm^2 on VTN-coated T75 flasks in 15 mL of E8 media supplemented with CEPT cocktail and incubated for 24 h. On days 1-5 cell culture media was replaced with 22.5 mL of STEMdiff Mesoderm Induction Medium (STEMCELL Technologies). On day 5, mesoderm cells were analyzed.

For ectoderm differentiation cells were plated at a density of 50,000 cells/cm^2 on VTN-coated T75 flasks in 15 mL of E8 media supplemented with CEPT cocktail and allowed to reach 70% confluency. After reaching 70% confluency cell culture media was switched to E6 with 100 nM LDN-193189 and 2 µM A83-01. Media was changed daily for 6 days. On day 7, cells were ready to be assayed. For all differentiation protocols, cells were maintained in 5% CO_2 atmosphere at 37 °C and all media changes were done at 24 h intervals. All automated and manual differentiations were performed in parallel as mentioned above.

Automated neuronal differentiation

For neuronal differentiation, hESCs and hiPSCs by were single-cell dissociated by 10-15 min incubation in EDTA (0.5 mM, ThermoFisher) in phosphate buffered saline (PBS) without calcium or magnesium (ThermoFisher) at 37 °C and plated at a density of 50,000 cells/cm^2 onto VTN-coated T75 flasks in 15 mL of E8 media supplemented with CEPT cocktail. After reaching 70% confluency cell culture media was switched to E6 with 100 nM LDN-193189 and 2 µM A83-01. Cells were then given daily media changes for 6 days. On day 7, cells were single cell dissociated by 5 min incubation with Accutase, rinsed in PBS without calcium or magnesium and resuspended in E6 supplemented with CEPT cocktail and transferred into ultra-low attachment T175 flasks in 30 mL of media. After 24 h, formed neurospheres were allowed to settle and cell culture media was switched to DMEM/12 GlutaMAX (ThermoFisher) supplemented with BDNF (10 ng/mL, R&D Systems), GDNF (10 ng/mL, R&D Systems), N2 (ThermoFisher), B27 without Vitamin A (ThermoFisher), cyclic-AMP (50 µM, Tocris) and Ascorbic Acid (200 µM, Tocris) and maintained in suspension in ULA T-175 flasks. Media was changed every 2 days for two weeks. Cells were then transferred into T175 flasks coated with Geltrex (ThermoFisher) and maintained in DMEM/12 GlutaMAX supplemented with BDNF (10 ng/mL), GDNF (10 ng/mL), N2, B27 without Vitamin A, cyclic-AMP (50 µM, Tocris) and Ascorbic Acid (200 µM, Tocris) for 2 weeks. Thereafter, cells were ready to be assayed. Cells were maintained in a 5% CO_2 atmosphere at 37 °C and all media changes were done at 24 or 48 h intervals. All automated and manual differentiations were performed in parallel as mentioned above.

Automated and manual cardiomyocyte differentiation

hES/iPS cells were single-cell dissociated by 10-15 min incubation with EDTA (0.5 mM, ThermoFisher) in phosphate buffered saline (PBS) without calcium or magnesium (ThermoFisher) at 37°C and plated at a density of 150,000 cells/cm^2 onto matrigel-coated T75 flasks in 15 mL of E8 media supplemented with CEPT cocktail. Daily E8 media changes were done until cells reached 95% confluency. Once cells were 95% confluent (Day 0) cell culture media was exchanged with 15 mL of Cardiomyocyte Differentiation Medium A (STEMdiff Cardiomyocyte Differentiation Basal Media with Supplement A) with Matrigel (1:100, Corning). On day 2, cell culture media was exchanged with 15 ml of Cardiomyocyte Differentiation Medium B (STEMdiff Cardiomyocyte Differentiation Basal Media with Supplement B). On days 4 and 6, cell culture media was exchanged with 15 mL of Cardiomyocyte Differentiation Medium C (STEMdiff Cardiomyocyte Differentiation Basal Media with Supplement C). After day 8, cell culture medium was exchanged every 2 days with 15 mL Cardiomyocyte Maintenance Medium (STEMCELL Technologies). On day 30, cells were ready to be assayed. Cells were maintained in 5% CO_2 atmosphere at 37 °C. All automated and manual differentiations were performed in parallel as mentioned above.

Automated and manual hepatocyte differentiation
hESCs and iPSCs were single-cell dissociated by 5 min incubation in EDTA (0.5 mM, ThermoFisher) in phosphate buffered saline (PBS) without calcium or magnesium (ThermoFisher) at 37 °C and plated at a density of 10,000 cells/cm^2 onto Laminin 521 (62.5 µg/cm^2, Biolamina)-coated 384-well plates in 30 µL of E8 media supplemented with CEPT cocktail or Y-27632 (10 µM, Tocris). After 24 h, differentiation was initiated by daily media changes with 25 µL per well of RPMI 1640/HEPES (ThermoFisher) supplemented with PenStrep (ThermoFisher), NEAA (ThermoFisher), 2% B27 (ThermoFisher), bFGF (20 ng/mL, ThermoFisher), Activin A (50 ng/mL, ThermoFisher) and BMP4 (10 ng/mL, R&D Systems) for 2 days. On days 3-5, media was exchanged daily with 25 µL per well of RPMI 1640/HEPES supplemented with PenStrep, NEAA, 2% B27 and Activin A (50 ng/mL). On days 6-10, media was exchanged daily with 25 µL per well of RPMI 1640/HEPES supplemented with PenStrep, NEAA, 2% B27, bFGF (10 ng/mL) and BMP4 (10 ng/mL, R&D Systems). On days 11-15, media was exchanged daily with 25 µL per well using RPMI 1640/HEPES supplemented with PenStrep, NEAA, 2% B27 and HGF (20 ng/mL, Peprotech). On days 16-20, media was exchanged daily with 25 µL per well of HCM Bullet Kit medium (Lonza). On day 21 cells were ready to be assayed. Throughout all steps, cells were maintained in 5% CO_2 atmosphere at 37 °C and all media changes were done at 24 h intervals. All automated and manual differentiations were performed in parallel as mentioned above.

RT-qPCR
RNA was isolated from automated and manually differentiated hiPSC-derived hepatocyte cell cultures using the RNeasy Plus mini kit (Qiagen, 74136). To increase RNA yields from cardiomyocyte cultures the RNeasy Fibrous Tissue Mini Kit (Qiagen, 74704) was used. RNA was used to synthesize cDNA using the high-capacity RNA to cDNA (Applied Biosystems, 4387406). qPCR was done using the TaqMan Gene Expression Master Mix (Applied Biosystems, 4369016) in a QuantStudio 7 Real Time-PCR system. RPL13A or GAPDH were used as a house-keeping genes and for normalization. List of probes is provided in Table S4.

Immunocytochemistry
Cells were fixed with 4% paraformaldehyde (PFA) in PBS for 15 minutes, followed by permeabilization-blocking with 5% Donkey Serum and 0.1% Triton X-100 in PBS for 1 hour. Cells were then stained with primary antibodies overnight at 4 °C. All antibodies are listed in Table S4. Fluorescence images were taken with the Leica DMi8 microscope using appropriate filters.

Western blotting
Cells were harvested by scraping, pelleted, washed with PBS, flash frozen and stored at -20 °C until processed. Cells were lysed by sonication in RIPA buffer (ThermoFisher) supplemented with halt protease inhibitor cocktail (ThermoFisher). Western blots were performed using a Wes Capillary Western Blot analyzer (ProteinSimple) according to the manufacturer's recommendation. Protein quantification was done using the Compass software. Primary antibodies used are listed in Table S4.

Zika virus experiments
Vero (African green monkey kidney Vero 76) and wild-type Ugandan MR766 Zika Virus (ZIKV) were purchased from the American Type Culture Collection (ATCC; Manassas, VA). Vero cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) plus 10% Fetal Bovine Serum (FBS). ZIKV virus was amplified in Vero cells by inoculation with virus (multiplicity of infection [MOI] =1) for 3 h in a low volume of medium with 4% FBS (3 mL per T175 flask), with rocking every 15 min, before the addition of 37 mL of full growth medium. Virus-infected cells were incubated for 72 h before harvesting the virus-containing supernatant. Before storing virus aliquots at -80^{o}C, virus titer was determined by a viral plaque-forming assay in 4 × 10^5 cells in 6-well plates, as described (Baer and Kehn-Hall, 2014). Cells were seed into culture plates and incubated at 37 °C. For viral infection, the cells were seeded in culture plates and maintained in 5% CO_2 atmosphere at 37 °C overnight to allow cells to attach. The next day, ZIKV was added to the cells with a multiplicity of infection of 1.0. The cells were incubated with ZIKV for 24 h in 5% CO_2 atmosphere at 37 °C. Next, the inoculum was removed, cells were washed twice with PBS, followed by fixation and immunostaining.

Supplemental References

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