Long-term single cell passaging of human iPSC fully supports pluripotency and high-efficient trilineage differentiation capacity

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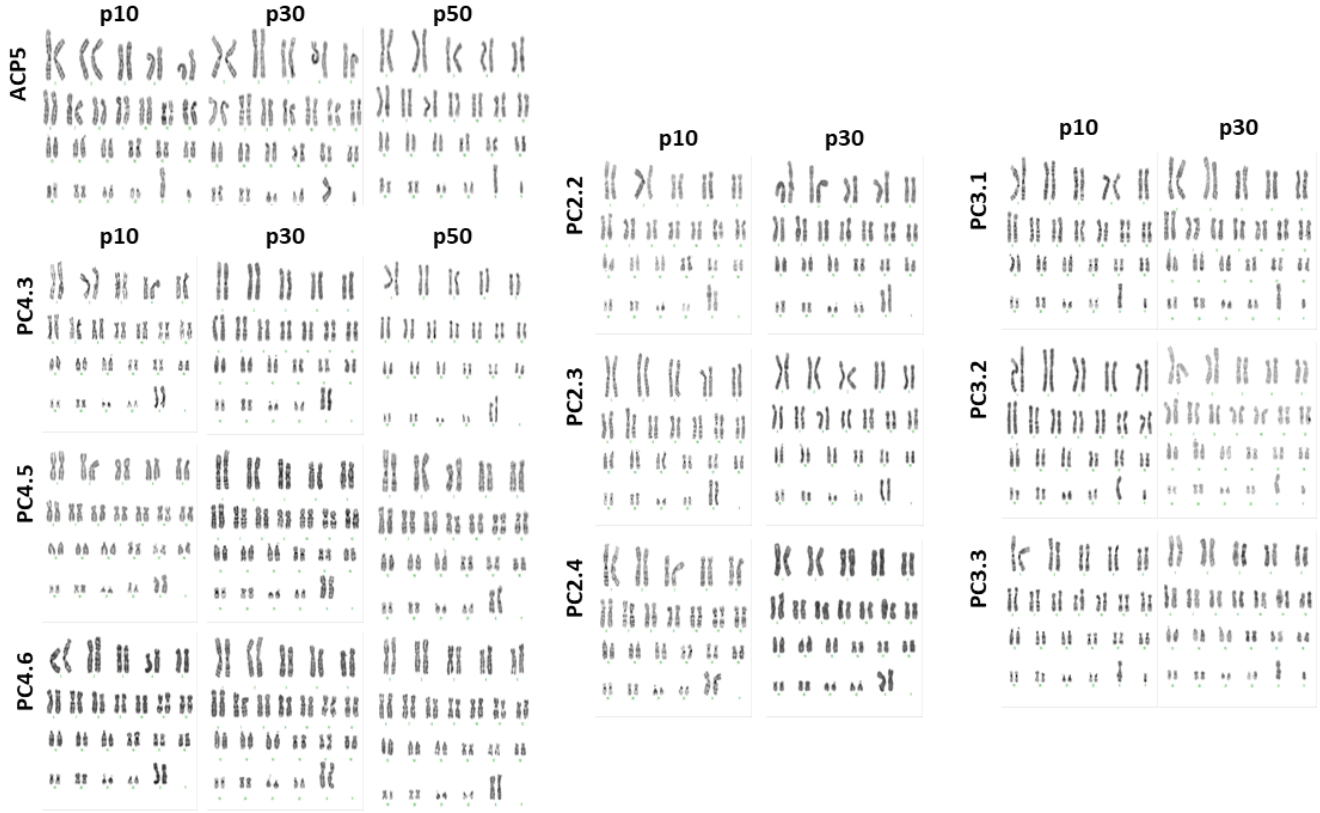
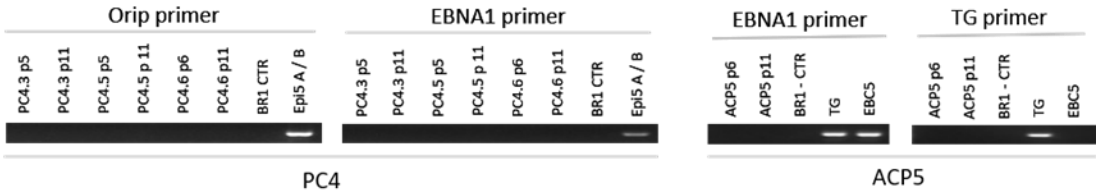
Karyotype



S1 Figure. Karyotype confirmed absence of karyotypic abnormalities for all hiPSC clones. G-Banding karyotype of hiPSC clones ACP5, PC2.2-2.4, PC3.1-3.3, PC4.3-4.6, after 10, 30 or 50 single-cell passages. No chromosomal abnormalities were observed.

Integration PCR

ACP5 and PC4 clones were obtained by transfection with plasmids pEB-C5, pEB-TG (both used in ACP reprogramming) and Epi 5 (used in PC4 reprogramming). To check for any episomal integration into host DNA, we performed an integration PCR (S2 Figure) using three sets of primers (S1 Table) targeting specific sites of the plasmids in gDNA as described by Chou and colleagues[1]. As positive control we used each iPSC line respective plasmids at dilution ratios of 1:200 (pEB-C5), 1:20,000 (pEB-TG) and 1:500,000 (Epi5).



S2 Figure. RT-PCR confirmed no vector integration into iPSC clones. Integration PCR analysis confirmed no episomal integration into host DNA. As positive control for PC4 clones, we used plasmid Epi5 at a dilution of 1:500,000. For ACP5, plasmids pEB-C5 (1:200) and pEB-TG (1:20,000) were used. BR1 (hESC) was used as negative control.

Flow cytometry and Immunofluorescence

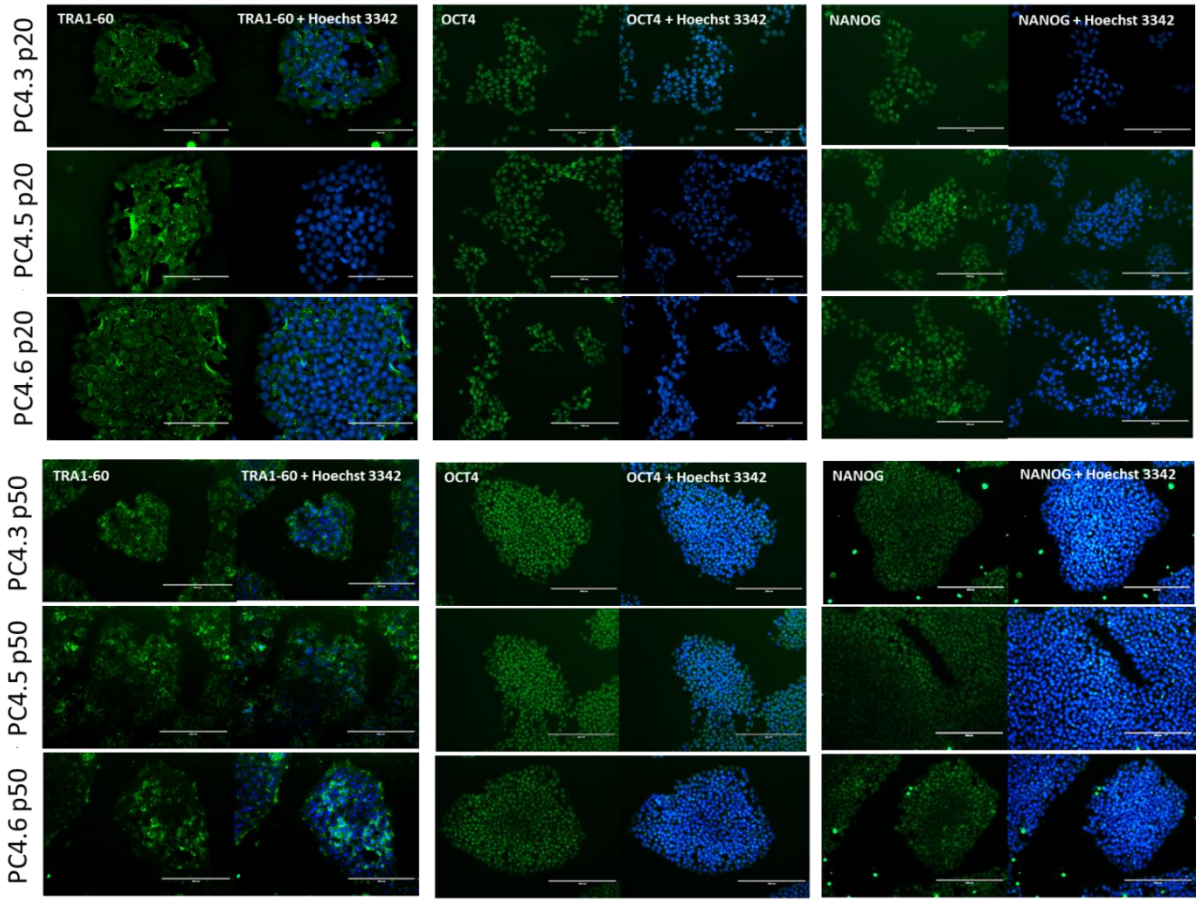
Protein expression was analyzed by Flow Cytometry (FC) and Immunofluorescence (IF). hiPSCs were plated in 96-well plates coated with GELTREX and cultivated for 2 days for IF. Cells were fixed with 4% PFA, permeabilized with Triton 0.1% and Saponin 0.1% (both from Sigma Aldrich, EUA). Cells were stained with antibodies anti-OCT4, NANOG and TRA-1-60. Nuclei were stained using 1 µg/mL of Hoechst 33342 (Sigma Aldrich, EUA) (Figure S3). As for FC, (Figure S4) iPSC were grown until they reached 70-80% confluence and harvested with Versene. Next, cells were fixed and stained using Fluorescent Human ES/iPS Cell Characterization Kit (Merck Millipore Sigma, USA). Both procedures followed manufacturer's instructions.

Cardiomyocytes at the 30th day of differentiation were plated in 96-well plates coated with GELTREX and cultivated for 5 days for IF. Cells were also fixed as previously described. Next, cells were stained with antibodies anti-TNNI1, TNNI3, TNNT2, ACTN2, MYH7, and NKX2.5. For FC, cardiomyocytes were plated in 6-well plates coated with GELTREX and cultivated for 7 days. After cell dissociation, they were fixed with 1% PFA, permeabilized with Triton 0.1% and Saponin 0.1% (both from Sigma Aldrich, EUA) and stained with antibodies anti-TNNI1, TNNI3, TNNT2, ACTN2, and NKX2.5. Titration of antibodies was done until no staining was found in iPSC.

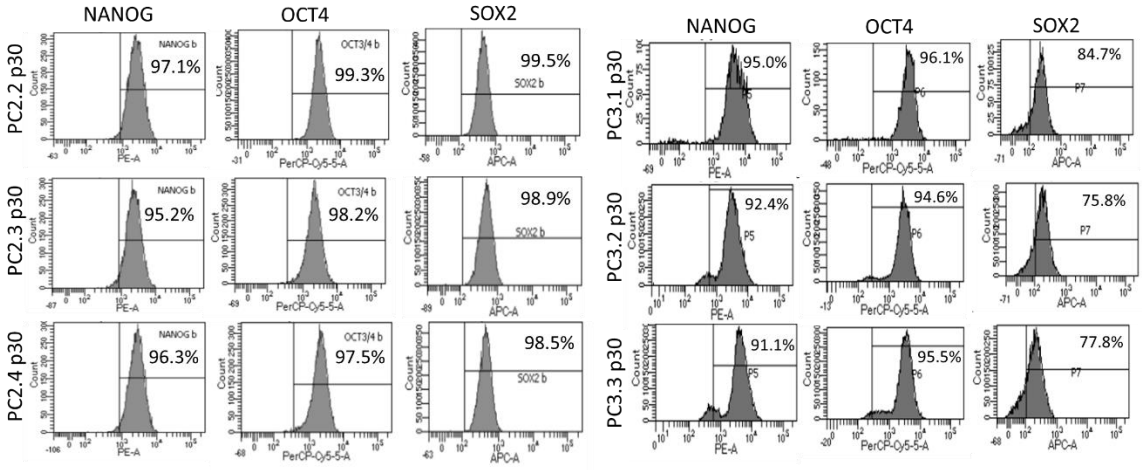
For IF, keratinocytes were plated in a 96-well plate, fixed with 4% PFA, permeabilized and blocked with 0.1% Triton X-100 and 10% fetal bovine serum (Thermo Fisher, USA). Then cells were stained with antibodies anti-K10, K14, Ki67, p63, and CD104. Nuclei were stained using 1 µg/mL of Hoechst 33342 (Sigma Aldrich, EUA). Image was generated in EVOS FL (Thermo Fisher, USA). As for FC, keratinocytes were fixed with 90% methanol ice cold solution and stained with Alexa488 conjugated anti-keratin 14 antibody for FC.

For definitive endoderm, at the end of day 3, cells were fixed and permeabilized as described previously for cardiomyocytes. Cells were stained with antibodies anti-FOXA2 and anti-CXCR4. For fluorescence visualization, cells were stained with Alexa 488 and Alexa647 Nuclei staining and image acquisition and performed as previously described. For FC, after cell dissociation, they were fixed with 1% PFA, permeabilized with Triton 0.1% and Saponin 0.1% and stained with antibodies anti-FOXA2. hiPSC-CM and hiPSC were used as negative control. To design the gates for positive events we used samples incubated exclusively with secondary antibodies. All FC data was acquired using Canto BD equipment and analyzed by FlowJo Software considering 1% of false positive events.

hiPSC were used as negative control for the reaction for definitive endoderm, keratinocytes and cardiomyocytes. As for the iPSC staining, fibroblasts were used as negative control. Further information about the antibodies used can be found in S3 Table.



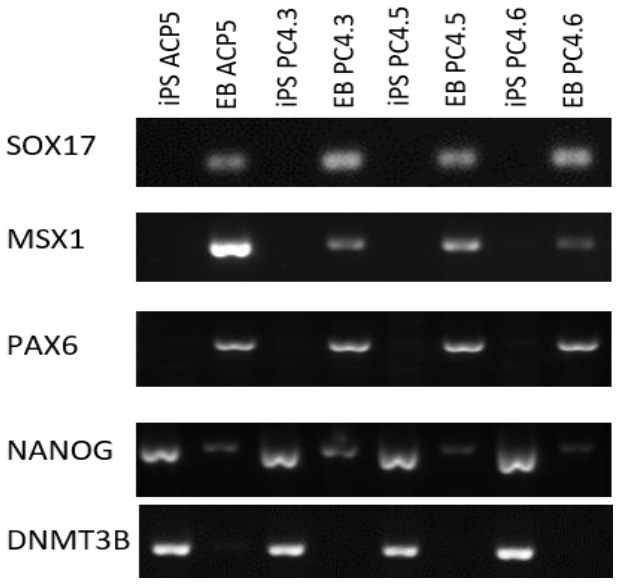
S3 Figure. Immunocytochemistry confirmed expression of pluripotency markers at passages 20 and 50. Immunostaining of clones PC4.3, PC4.5 and PC4.6 at passages 20 and 50 showing expression of pluripotency markers OCT4, NANOG (both nuclear) and TRA-1-60 (membranous).



S4 Figure. PC2 and PC3 clones preserved their pluripotency at high passages. Flow cytometry results for pluripotency markers NANOG, OCT4 and SOX2 in clones PC2 and PC3 at passage 30.

EB formation

EBs were generated using a hybrid protocol based on previous publication.[2,3] Briefly, hiPSCs were dissociated, centrifuged and resuspended at 80,000 cells/mL concentration. 25uL drops (with 2,000 cells each) were done at a petri dish lid; then the lid was carefully inverted and placed on the top of the dish containing 10mL of HBSS1x. After 48h, cell aggregates were collected and transferred to a low attachment 12-well plate (Sarstedt) with Essential 6 medium. Half of the medium was replaced every three days until day 13, when RNA was collected using Trizol (Thermo Fisher, USA). We performed an end-point RT-PCR using differentiation markers *SOX17*, *MSX1* and *PAX6* (endo, meso, and ectoderm, respectively) and pluripotency marker *DNMT3B* and *NANOG*. As negative control, we used samples extracted from the respective iPSC clones at passage 20. PCR analysis showed that all the hiPSC clones generated EBs containing differentiated progeny of all three germ layers (Figure S5).

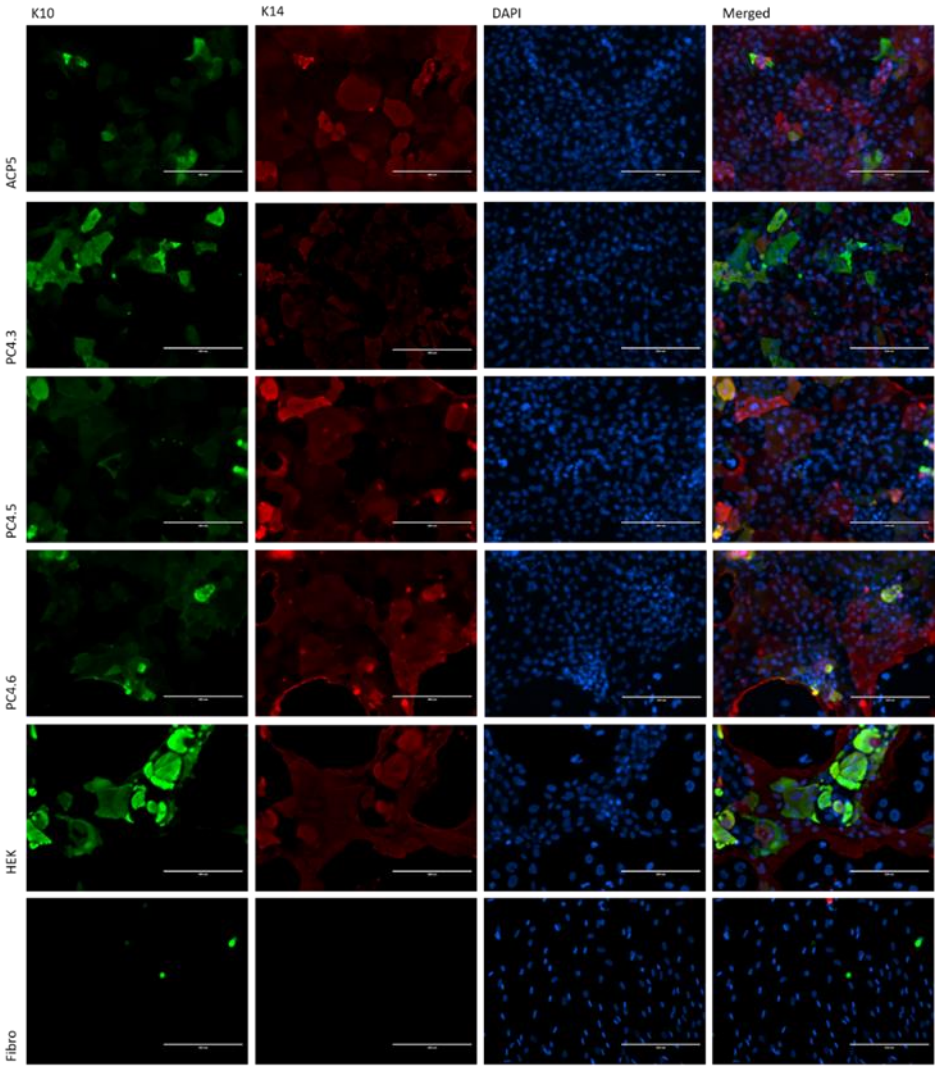


S5 Figure. iPSC clones generated EBs containing differentiated progeny of all three germ layers. Endpoint RT-PCR of EB showing expression of markers of derivatives lines from endoderm (*SOX17*), mesoderm (*MSX*1) and ectoderm (*PAX6*); and showing no expression of pluripotent markers *DNMT3B* and *NANOG*. *GAPDH* was used as housekeeping control.

iPSC-derived keratinocytes calcium treatment

iPSCs at different passages (passage 25 to 50) were plated with mitomicyn C-inactived 3T3 cells (donation from Monica Mathor's laboratory) at a 20% confluence. After 2 days, defined-KSFM medium (Thermo Fisher, USA) was supplemented with 10 ng/mL of BMP4 (R&D Systems, USA) and 1 µM retinoic acid (Sigma Aldrich, USA) as described by Itoh and colleagues.[4] At day 4, cells were cultivated with fresh defined-KSFM medium

and, after 28 days of differentiation, iPSC-derived keratinocytes (iPSC-KC) were supplemented with 1 µM CaCl for 7 days. Medium was exchanged every 2 to 3 days. After treatment with calcium, iPSC-KC were stained using epithelial specific markers K10 and K14. Immunofluorescence showed that after calcium treatment some K10-positive cells were found in all cell clones (S6 Figure).



S6 Figure. Immunostaining showed up-regulation of K-10 after calcium treatment for Keratinocytes derived from all iPSC clones. Cell line HEK and original PC4 fibroblasts were used as positive and negative controls for the reaction, respectively.

iPSC-derived cardiomyocytes beating culture

[Insert Video 1 - https://youtu.be/T9JADZXKfck (40x magnification)]

[Insert Video 2 - https://youtu.be/XLOut0VpXTo (100x magnification)]

Supplementary Videos. Cardiomyocytes generated by long-term cultivating iPSC spontaneously beating. Videos recorded at day 11 of differentiation, showing cardiomyocytes beating on monolayer. The software wondershare filmora was used to add legend and chronometer to the video and the codec (compression-decompression program) used was H.246.

CellCountAnalyser

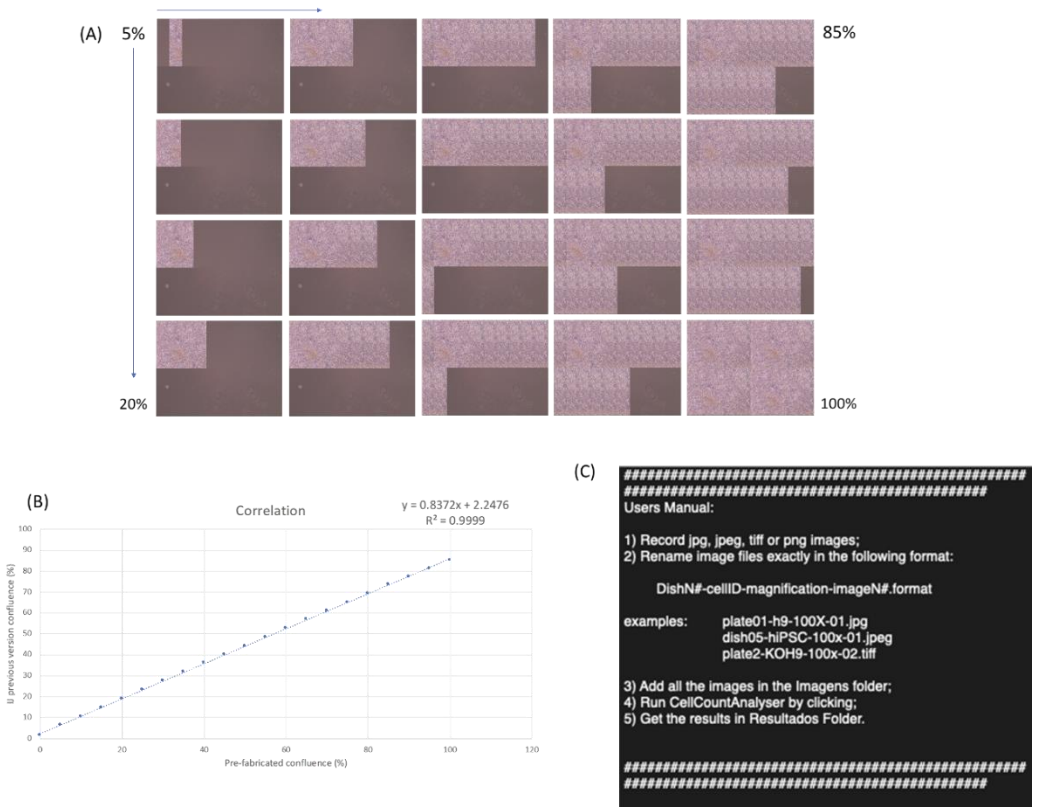
Cells confluence monitoring is a regular practice in a cell culturing laboratory. Nevertheless, visual monitoring can cause significant misinterpretations, especially considering different operators. Since the high-efficient protocols of differentiation using hiPSCs, in general, are dependent on the proliferative state of these cells, to keep them growing in log-phase seems to be a mandatory step for success.

In this regard, several groups have been proposing, from the simple to very complex, semi-automatic or automatic systems to measure cell confluence based on microscope imaging techniques.[5–8] Despite the advantages to monitor cell confluence by automated methods, significant issues associated to the quality of images obtained by optical microscopy from cell cultures can also cause some misinterpretation for impairing daily cell maintenance and differentiation protocols.

We have been monitoring cell confluence based on a modified version of the software previously proposed by Busschots et. al..[8] Despite the easy platform designed to be used in ImageJ software, we have found at least two important issues in this software, 1) there are too many steps to evaluate the confluence spending long time to reach final results and 2) there are significant misinterpretation of results, as higher as the confluence (S4 Table).

Considering these problems, we have created a "One-click Python-based platform" (named CellCountAnalyser) to automate the calculation of confluence in batches of images and also, we created a correction factor based on pre-fabricated images with known confluences from 0 to 100% (5% intervals, S7-A Figure). Based on the quantification of prefabricated images, a linear equation to correlates measured (by original software) vs. real expected confluences was generated. The implementation of the linear equation step generated a new corrected value for measured confluence closer to that visualized in the culture dishes (S7-B Figure and S5 Table). We also created a command line to generate an excel report with quantified confluences and mean ± STD for all the class/name images. Additionally, an easy step-by-step user manual was written (S7-C Figure). The package with CellCountAnalyser is available to download in this article's supplementary material.

Furthermore, five images with 100X magnification from predefined (and marked to be used in following days) areas of the center and borders of each plate were recorded and quantified daily to monitor cell confluence.



S7 Figure. CellCountAnalyser yielded more accurate cell confluence predictions. A) Set of pre-fabricated images with known confluences from 0-100%. **B)** Correlation of the confluences measured by the original software and the real values. **C)** CellCountAnalyser users manual.

S1 Table. Set of primers used for Integration PCR

Primer	Sequence			Amplicon
EBNA1 *forward*	5'-ATCGTCAAAGCTGCACACAG-3'	pEB-C5, pEB-Tg	Epi5,	666 bp
EBNA1 *reverse*	5'-CCCAGGAGTCCCAGTAGTCA-3'			
Orip *forward*	5'-TTCCACGAGGGTAGTGAACC-3'	Epi5		544 bp
Orip *reverse*	5'-TCGGGGGTGTTAGAGACAAC-3'			
TG *forward*	5'-GCCAGGTGGGTTAAAGGAGC-3'	pEB-Tg		127 bp
TG *reverse*	5'-GGTACTTATAGTGGCTGGGCTGT-3'			

S2 Table. Set of primers used for RT-PCR and RT-qPCR

Primer		Sequence	Amplicon
DNMT3B	Forward	5’- ATAAGTCGAAGGTGCGTCGT -3’	203 bp
	Reverse	5’- GGCAACATCTGAAGCCATTT -3’	
NANOG	Forward	5’- CAGCCCTGATTCTTCCACCAGTCCC-3’	391 bp
	Reverse	5’- TGGAAGGTTCCCAGTCGGGTTCACC-3’	
MSX1	Forward	5’-CGAGAGGACCCCGTGGATGCAGAG-3’	307 bp
	Reverse	5’-GGCGGCCATCTTCAGCTTCTCCAG-3’	
PAX6	Forward	5’-GCCAGCAACACACCTAGTCA-3’	138 bp
	Reverse	5’-TGTGAGGGCTGTGTCTGTTC-3’	
SOX17	Forward	5’CAAGGGCGAGTCCCGTATC-3’	58bp
	Reverse	5’GTCCTTAGCCCACACCATGA-3’	
K5	Forward	5’ATCTCTGAGATGAACCGGATGATC-3’	75 bp
	Reverse	5’-CAGATTGGCGCACTGTTTCTT -3’	
K10	Forward	5’- GGGCTCTGGAAGAATCAAAC -3’	185 bp
	Reverse	5’- ATTGTCGATCTGAAGCAGGA -3’	
IVL	Forward	5’- TCCTCCTCCAGTCAATACCC -3’	181 bp
	Reverse	5’- CTGTGGCTCCTTCTGCTGT -3’	
GAPDH	Forward	5’- GTGGACCTGACCTGCCGTCT -3’	152 bp
	Reverse	5’- GGAGGAGTGGGTGTCGCTGT -3’	

S3 Table. Set of antibodies used for iPSC, IPSC-derived -cardiomyocytes and -keratinocytes.

Protein target	Dilution used		Manufacturer, Catalog number
	IF	FC	
OCT4	Kit	Kit	Merck Millipore Sigma, SCR078
NANOG	Kit	Kit	
SOX2	-	Kit	
TRA-1-60	Kit	-	
Cardiac troponin T (TNNT2)	1:200	1:3500	Fritzgerald, 20R-3024
Myosin (MYH7)	1:500	-	Interprise, DSHB-A4.951
Alfa-actinin (ACTN2)	1:500	1:1000	Abcam, ab9465
Fetal cardiac troponin I (TNNI1)	1:500	1:1000	Thermo Fisher, 701585
Adult cardiac troponin I (TNNI3)	1:30	1:50	Interprise, DSHB-T-I4
NKX2.5	1:100	1:100	Cell Signaling, 8792S
Keratin 10	1:100	-	AbCam, ab9026
Keratin 14	1:1000	1:1000	AbCam, ab192055 (FC) Biolegend, PBR-155P (IF)
Ki67	1:50	-	AbCam, ab15580
p63	1:50	-	Zeta Corporation, Z2003L
integrin alpha-6 (CD49f)	1:100	-	eBioscience/14-0495-82
integrin beta-4 (CD104)	1:100	-	BD Biosystems, BD555721
FOXA2	1:300	1:2000	Abcam, ab60721
CXCR4	1:200	-	Abcam, ab124824

S4 Table. Cell confluence data by previous software

Cell plate	Image	Visual confluence %	Confluence by IJ previous version %
1	640111	100	82.622
	640112	100	79.351
	640113	100	81.033
	640114	100	80.051
	640115	100	82.272
2	640211	100	81.495
	640212	100	80.613
	640213	100	80.46
	640214	100	81.035
	640215	100	81.612
3	640311	100	82.874
	640312	100	84.269
	640313	100	82.688
	640314	100	81.394
	640315	100	83.833

S5 Table. Cell confluence data after modifications in the algorithm

Pre. Frab. Confluence %	Confluence by IJ previous version %	CellCountAnalyser
0	1.503	-0.89
5	6.346	4.90
10	10.457	9.81
15	14.674	14.84
20	18.968	19.97
25	23.234	25.07
30	27.561	30.24
35	31.772	35.27
40	36.11	40.45
45	40.243	45.38
50	44.24	50.16
55	48.481	55.22
60	52.623	60.17
65	56.905	65.29
70	61.082	70.28
75	65.145	75.13
80	69.25	80.03
85	73.476	85.08
90	77.437	89.81
95	81.366	94.50
100	85.348	99.26

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