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Utilizing Advanced Flow Cytometry and Live-Cell Imaging to Evaluate iPSC Pluripotency During Cell Line Selection and Differentiation Procedures

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Introduction

Since the initial discovery of induced pluripotent stem cells (iPSCs) in 2006, there has been significant advancement in the field of stem cell biology.[1] Their capacity to differentiate into any cell type combined with infinite self-renewal potential means iPSCs have been used extensively in drug discovery, regenerative medicine, and disease modeling.[2] Moreover, reprogramming of a patient's own body cells to produce iPSCs offers an invaluable resource for personalized medicine.[3] iPSC-derived cells have enhanced research in a spectrum of therapeutic areas, from modeling neuro-degenerative diseases such as Alzheimer's, to evaluating the efficacy of novel drugs for cardiovascular disease, to liver-based screening of drug toxicity.[4]

When generating iPSCs for use in downstream applications, it is commonplace to perform initial checks for key attributes such as viability and pluripotency.[5] This allows for assessment of the quality of the produced iPSC culture, which may differ due to numerous factors such as the reprogramming technology used. For example, lentiviral miRNA infection is known to produce a much higher yield of pluripotent cells compared to adenoviral methods.[6] Some studies suggest that the somatic cell source from which an iPSC was derived can impact its ability to differentiate into certain lineages, potentially due to epigenetic variances.[7] It is therefore important that we have simple, robust, and standardized techniques for evaluating and comparing individual iPSC lines to ensure we choose the best lines for lengthy and expensive studies.

Conventional techniques for evaluating iPSC pluripotency and differentiation, such as traditional flow cytometry, can:

- Require lengthy workflows for staining, including fixation, and multiple wash steps
- Necessitate large sample volumes, reducing the amount of sample remaining for downstream expansion, characterization, and differentiation
- Involve low throughput instrumentation, increasing workflow time and reducing capacity for intra- and inter- experiment replication
- Demand manual data analysis and complicated compensation optimization

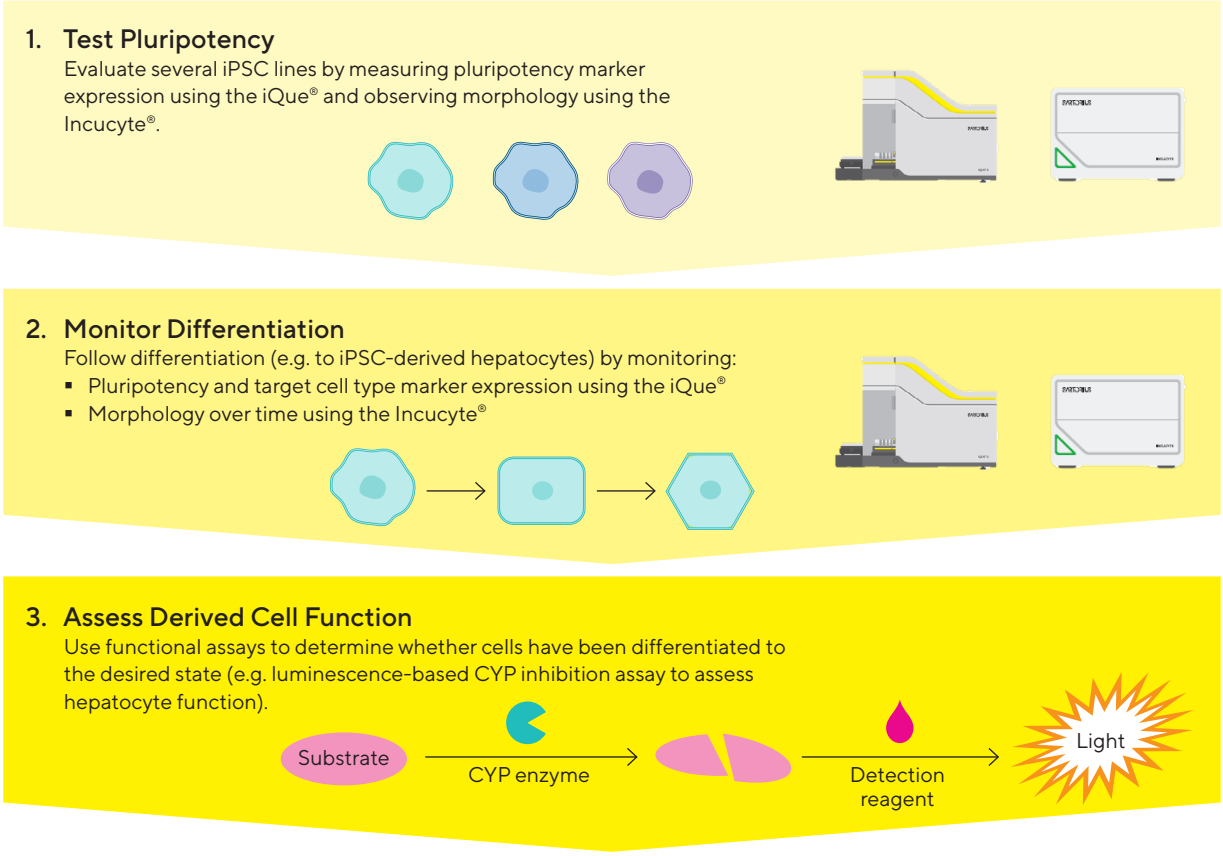
Here we describe a combined iQue® Advanced Flow Cytometry and Incucyte® Live-Cell Analysis approach for stem cell evaluation during cell line selection and differentiation. Fast sample acquisition, low volume requirements (10 µL), and plate-based data analytics for the iQue® conserve precious time and sample during iPSC phenotyping. Meanwhile, Incucyte® images provide continuous morphological assessment for easy monitoring of iPSC colonies throughout differentiation.

Methods

In this application note, we exemplify the combined use of the iQue® and Incucyte® systems in an integrated workflow for iPSC line assessment and monitoring during differentiation (Figure 1). Measurement of iPSC pluripotency was performed by a simple, one-wash labeling protocol. This analysis aided cell line selection before directed differentiation stimuli was applied to generate iPSC-derived hepatocytes.

Figure 1

Schematic Highlighting the Combined iQue® and Incucyte® Workflow for iPSC Line Selection and Differentiation Monitoring



Note. To ensure the best iPSC line was used for the differentiation protocol, three lines were first evaluated for their pluripotency via assessment of marker expression and colony morphology. One cell line was then differentiated to hepatocyte-like cells, a process which was monitored over time by measuring pluripotency and lineage-specific markers using the iQue® and observing changes in morphology using the Incucyte®. At the end point of the differentiation protocol, functional analysis of the derived cells was performed using a CYP inhibition assay as an indicator of hepatocyte activity.

iPSC Thaw and Expansion

Three iPSC lines: Gibco™ Human Episomal iPSC Line (iPSC line 1), ATCC-DYS0100 (iPSC line 2), and ATCC-HYS0103 (iPSC line 3) were thawed into Vitronectin XF™ (1:25 dilution) coated plates in mTeSR™ Plus media (Stem Cell Technologies) containing ROCK inhibitor Y-27632 (10 µM) at a density of 1×10^6 cells/well in a 6-well dish. Cells were passaged every 3–4 days using Gentle Cell Dissociation Reagent (Stem Cell Technologies) to a density of 1×10^5 cells/well. Plates were placed in the Incucyte® and phase images (10X magnification 9–16 images per well) were gathered every 4 hours to monitor colony formation and confluency. The confluence of colonies was analyzed using the Incucyte® software integrated AI confluence algorithm and passaged when confluency reached 60–70%.

iPSC Pluripotency Assessment

During cell passage, or at desired timepoints, cells were lifted from culture-ware and dissociated to a single-cell suspension using Gentle Cell Dissociation Reagent. The dissociated cells (2×10^6/mL, 10 µL /well, 3–5 wells) were plated in a V-bottom plate (Corning® 3363) and 10 µL of 2X antibody solution was added for 1 hour (RT, dark) to label differentiation marker (SSEA-1) and two cell surface pluripotency markers (SSEA-4 and TRA-1-60). Viability assessment was performed using iQue® Cell Membrane Integrity (B/Red) Dye. Following labeling, wash buffer (PBS + 2% FBS, 100 µL/well) was added prior to centrifugation (300 g, 5 min) and aspiration of supernatant. The iQue® plate shaker was used to resuspend cells (2000 RPM, 60 secs) prior to addition of 20 µL wash buffer.

Samples were acquired using the iQue® Advanced Flow Cytometer with an 8-second sip time. Data analysis was performed using the integrated iQue Forecyt® software with plate-based data visualization features.

Directed Differentiation to Hepatocyte-Like Cells

Cells were differentiated using Cellartis® iPS Cell to Hepatocyte Differentiation System (Takara Bio) (Figure 2). This is a forty-day culture protocol involving expansion and differentiation of iPSCs in a series of cell culture mediums and extracellular matrices to generate iPSC-derived hepatocyte cells (see manufacturer's instructions).

iQue® Assessment of Differentiating Cells

Cell surface marker expression during differentiation was monitored as described for pluripotency assessment with an extended panel to include markers from different stages in hepatocyte development: CD184 (CXCR4) and CD99.

Incucyte® Monitoring of Differentiation

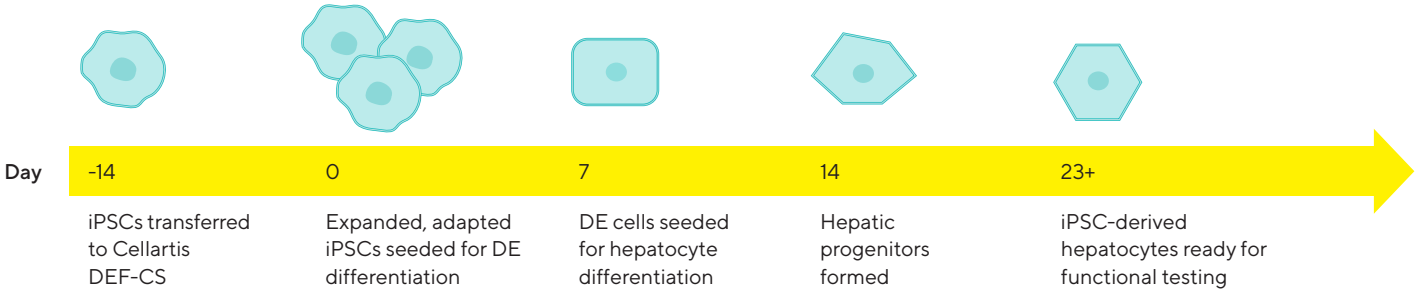
Phase images (10X) were taken using the Incucyte® on a 3–4 hour repeating scan schedule, so that characteristics such as cell morphology and confluency could be observed throughout the differentiation process. The Incucyte® can acquire phase images from an extensive range of culture vessels, meaning the cells could be monitored throughout the expansion process, from 24-well dishes to T75 flasks.

Functional Testing of Differentiated Cells

A P450-Glo™ CYP1A2 Assay (Promega) was used to assess the hepatocyte-like function of the derived cells. Substrate was added directly to wells (1:1000 dilution in PBS) alongside CYP inhibitor (α-naphthoflavone, 1 µM) for 1 hour. Control wells contained no inhibitor. 25 µL/well of the reaction volume (4 replicates per well) was transferred to a white plate (Thermo Scientific™ 236108) alongside detection reagent for 20 min (37 °C). Release of luciferin substrate indicated CYP1A2 activity and was detected as an increased luminescent signal as quantified using a CLARIOstar® microplate reader. Control wells contained cells and media alone. Wells containing substrate and detection reagent alone were included for background subtraction.

Figure 2

Summary of the iPSC-Hepatocyte Differentiation Protocol



Note. The chosen iPSC line was transferred into the Cellartis DEF-CS culture system for adaptation and expansion for 14 days (Day -14 to Day 0). On Day 0, cells were seeded for differentiation to definitive endoderm (DE). On Day 7, DE cells were re-seeded to begin hepatocyte differentiation, first to hepatic progenitor cells and then to the more mature hepatocyte-like phenotype. Cells were ready for functional testing by Day 23 (we tested on Day 25).

Results

Cell Line Selection and Pluripotency Assessment

Evidence suggests that factors such as the reprogramming method or somatic cell type used to generate iPSCs can affect the pluripotency and differentiation capacity of the cell line produced.[6,7] It is important to make an informed decision on which iPSC line to use for lengthy differentiation protocols, such as the forty-day hepatocyte differentiation exemplified in this application note.

Here we evaluated three iPSC lines through measurement of viability and cell surface markers: SSEA-1 (marker of differentiated cells), SSEA-4 and TRA-1-60 (pluripotency markers) using the iQue® Advanced Flow Cytometer (Figure 3). Each cell line was reprogrammed from a different source cell type and donor. iPSC line 1 was reprogrammed from cord blood cells using an Epstein–Barr virus nuclear antigen-based episomal system. iPSC line 2 was derived from human foreskin fibroblasts and reprogrammed through Sendai viral transduction. iPSC line 3 was derived from primary hepatic fibroblasts and was also reprogrammed using Sendai virus. THP-1 cells were included as a non-pluripotent cell control. NCCIT cells were included as a positive control for expression of pluripotency markers.

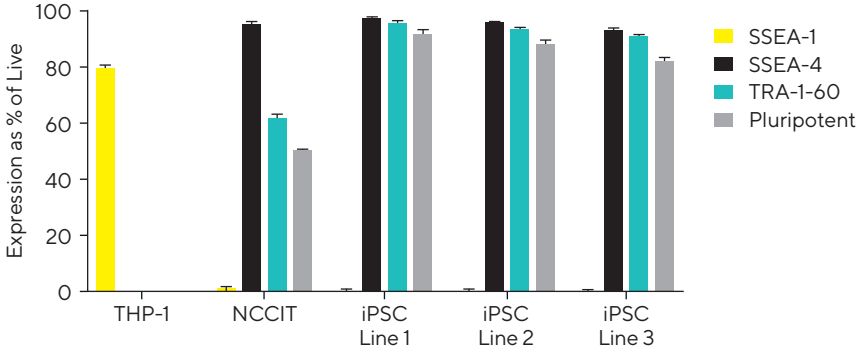
Figure 3

Measuring Pluripotency and Viability to Evaluate Which iPSC Line to Differentiate

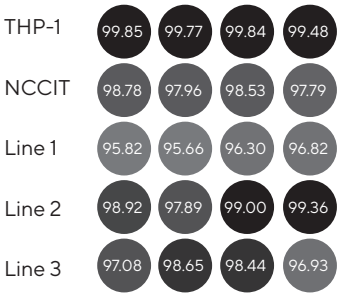
A.

Cell Type	Expected Pluripotency?	Reprogramming Method	Somatic Cell Source
THP-1	N	N/A	Monocytic leukemia
NCCIT	Y	N/A	Mediastinum
iPSC line 1	Y	Episomal	Cord blood
iPSC line 2	Y	Sendai virus	Foreskin fibroblasts
iPSC line 3	Y	Sendai virus	Hepatic fibroblasts

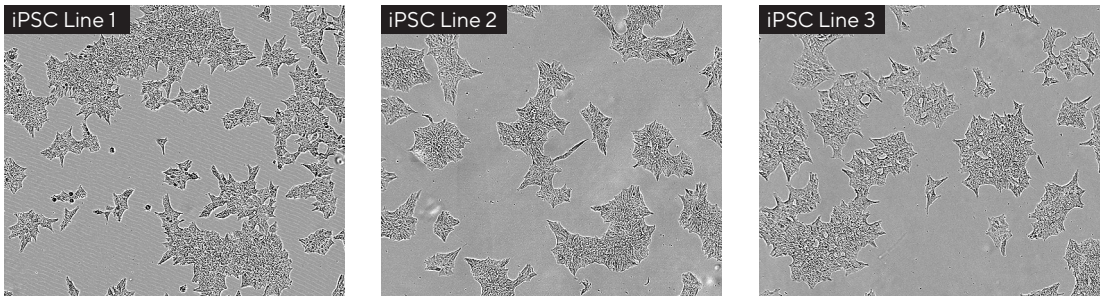
B. Marker Expression



C. Viability (%)



D.



Note. Three iPSC lines were labeled using fluorophore conjugated anti-SSEA-1 (marker of differentiated cells), anti-SSEA-4 and anti-TRA-1-60 (two pluripotency markers) antibodies, and a viability dye (iQue® Cell Membrane Integrity (B/Red) Dye). THP-1 and NCCIT cells were included as positive and negative controls for pluripotency marker expression, respectively. Analysis was performed using the iQue®. (A) Table listing cell lines and characteristics; (B) Marker expression (as % of live, ± SEM) for each cell type (n = 4), 'Pluripotent' refers to the phenotype SSEA-1-/SSEA-4+/TRA-1-60+; (C) Heat map showing % viability for each cell type (n = 4); (D) Incucyte® phase images (10X) of colonies of each iPSC line taken 48 hours after seeding.

As anticipated, the three iPSC lines did not express SSEA-1 (< 0.3%), but SSEA-1 was expressed on the non-pluripotent control THP-1 cells (Figure 3B). Expression of SSEA-4 and TRA-1-60 was relatively high in all three iPSC lines, with highest expression on line 1 and lowest on line 3. While differences in expression of each marker were minor, gating through to the fully 'pluripotent' phenotype (SSEA-1-/SSEA-4+/TRA-1-60+) revealed a 10% drop in the number of pluripotent cells from line 3 compared to line 1. Final values of 94, 90, and 83% pluripotency were measured for the three cell lines, respectively. Viability was high across all cell lines (>96%) (Figure 3C).

As a further quality control check for the iPSC lines, their growth was monitored using the Incucyte® Live-Cell Analysis Platform. Images of each cell line taken 48 hours after seeding are shown in Figure 3D. These showed that all cell lines had formed distinct, tightly packed colonies, as is characteristic of iPSC growth. Good quality iPSC colonies tend to have rounded morphology with 'glowing' edges, which we concluded was most evident in images of cell line 2. Combining the Incucyte® images with iQue® pluripotency data, we decided that iPSC line 2 had the most desirable characteristics, and this cell line was utilized for the differentiation experiment.

Monitoring Loss of Pluripotency During Differentiation

Using iPSCs in research conveys several advantages over primary cells, as they provide an unlimited supply of cells and can be differentiated to produce any cell type. This makes them a particularly useful tool for projects like large-scale screening, where the effects of gene editing or large drug libraries may be explored.[8] iPSCs are routinely differentiated into a wide range of cell types for use in translational research, such as cardiomyocytes, neurons, and hepatocytes. iPSC-derived hepatocytes are useful for assessing liver functions such as drug metabolism and cytotoxicity as well as modeling hepatological diseases such as α1-antitrypsin deficiency or familial hypercholesterolemia.[9]

When differentiating iPSCs, it is crucial that we have simple and robust methods for monitoring the loss in their pluripotency over time. In the experiments displayed in Figure 4, we combined the Incucyte® and iQue® platforms to monitor the loss in pluripotency of iPSCs during differentiation to hepatocyte-like cells. THP-1, AU565, and HepG2 cells were included as non-pluripotent cell controls. HepG2s are a liver cell line, expected to display similarities in phenotype and function to the derived hepatocytes.

The three control cell types expressed the marker of differentiated cells SSEA-1 and did not express the pluripotency markers SSEA-4 and TRA-1-60 (Figure 4B). Conversely, the undifferentiated iPSCs did not express SSEA-1, but were highly pluripotent, with 99% of cells expressing the fully pluripotent phenotype (SSEA-1-/SSEA-4+/TRA-1-60+). The pluripotency of iPSC line 2 had improved since earlier testing (Figure 3B) suggesting that optimized cell culture protocols favor the pluripotent phenotype and lead to elimination of non-specifically differentiated cells.

From Day -14 to Day 0, cells were adapting to and expanding in the Cellartis DEF-CS culture system. This is a system designed for high density iPSC culture in a tightly packed monolayer, rather than in the standard iPSC colony morphology. SSEA-4 and TRA-1-60 expression remained high throughout this adjustment period (Figure 4C); however, compared to the undifferentiated iPSCs grown in mTESR, there was an increase in SSEA-1 expression in the Cellartis culture system, implying there may have been some spontaneous differentiation.

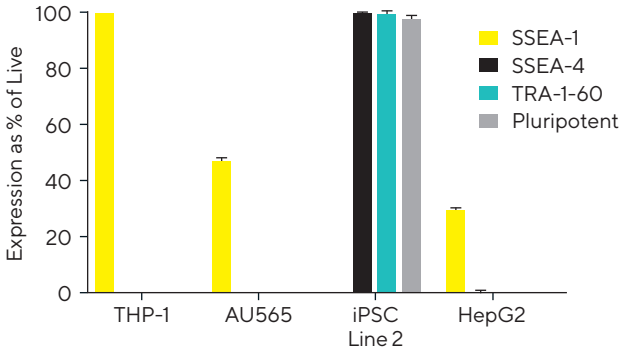
Figure 4

Monitoring Loss in Pluripotency Markers and Morphology Over Time

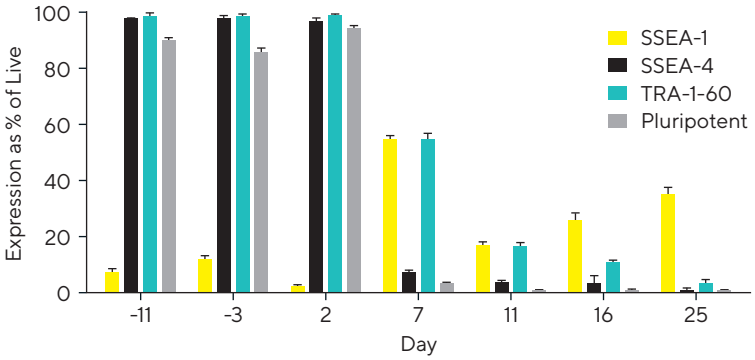
A.

Cell Type	Expected Pluripotency?	Cell Line Description
THP-1	N	Monocytic leukemia
AU565	N	Breast cancer
iPSC line 2	Y	iPSC (for more information, see Figure 3A)
HepG2	N	Liver cancer

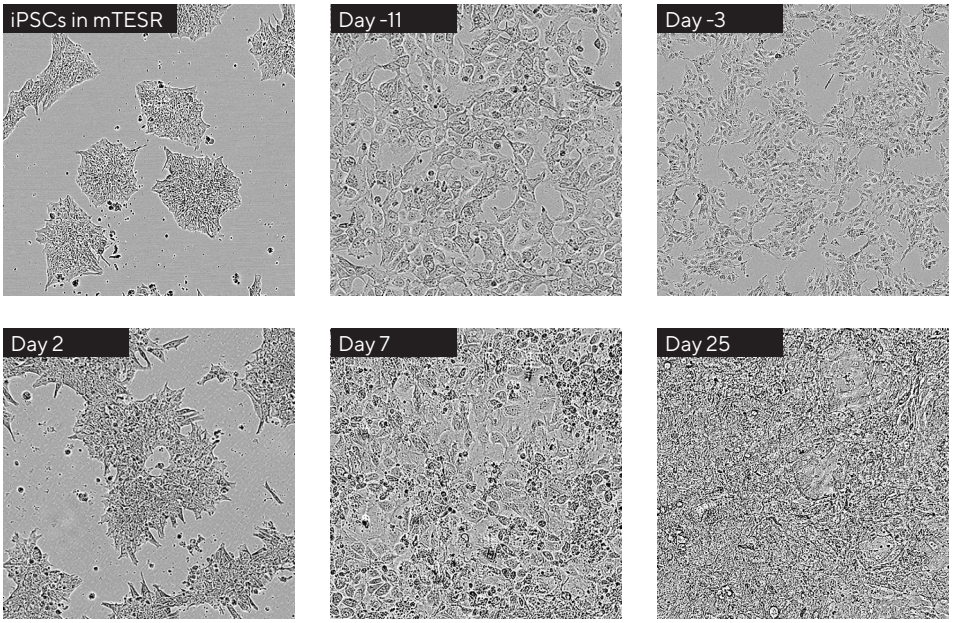
B. Control Cells



C. iPSC Differentiation



D.



Note. iPSCs (line 2) were differentiated to hepatocyte-like cells over forty days using the Cellartis differentiation system. THP-1, AU565, and HepG2 cells were included as controls. Pluripotency marker expression (SSEA-1, SSEA-4, and TRA-1-60) was measured at defined timepoints using the iQue®. (A) Table listing cell types used; (B) Control cell expression (± SEM) of pluripotency markers (n = 4); (C) Marker expression (± SEM) on iPSCs during differentiation (n = 4); (D) Incucyte® phase images (10X) of differentiating iPSCs in comparison to iPSCs grown conventionally in mTESR.

For the remainder of the time course, SSEA-1 expression was largely varied, peaking at 54.8 ± 0.6% on Day 7. This may be unsurprising considering how varied the SSEA-1 expression is between the control cells (THP-1, AU565, and HepG2). Expression of both SSEA-4 and TRA-1-60 declined throughout the differentiation process, with SSEA-4 decreasing more rapidly, leaving only 7.6 ± 0.1% expression remaining by Day 7 compared to 54.8 ± 0.9% expression of TRA-1-60.

Incucyte® images facilitated visual monitoring of changes in phenotype and loss of pluripotency over time (Figure 4D). After 3 days in the Cellartis DEF-CS culture system (Day -11), the morphology of the cells had changed compared to the iPSCs grown in mTESR, becoming more spread out and losing the colony-based formation. On Day 2, the cells regained the 'colony-like' spatial distribution, but this was lost once they differentiated to definitive endoderm by Day 7. From Day 7, the cells became increasingly tightly packed in a monolayer, with the ability to distinguish individual cell features lost, as is evidenced in the image from Day 25.

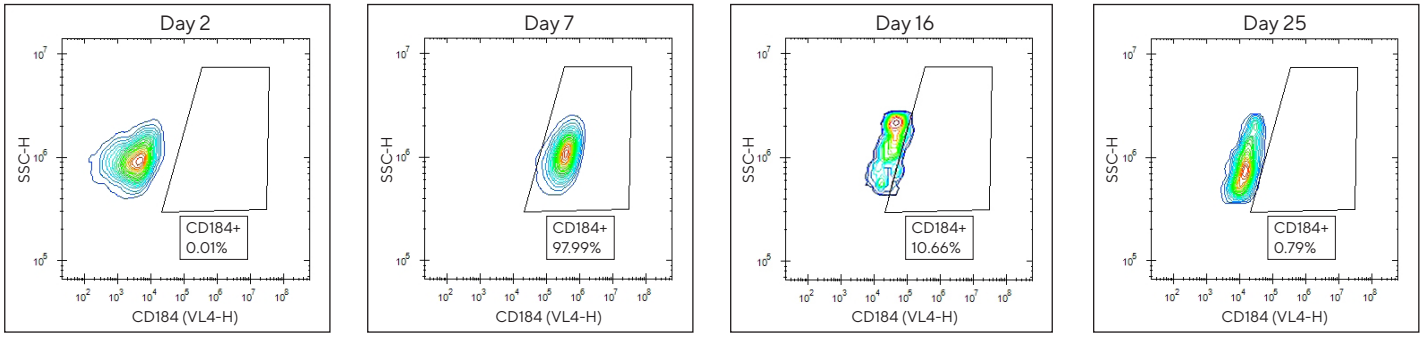
Phenotypic and Functional Assessment of Differentiating Cells

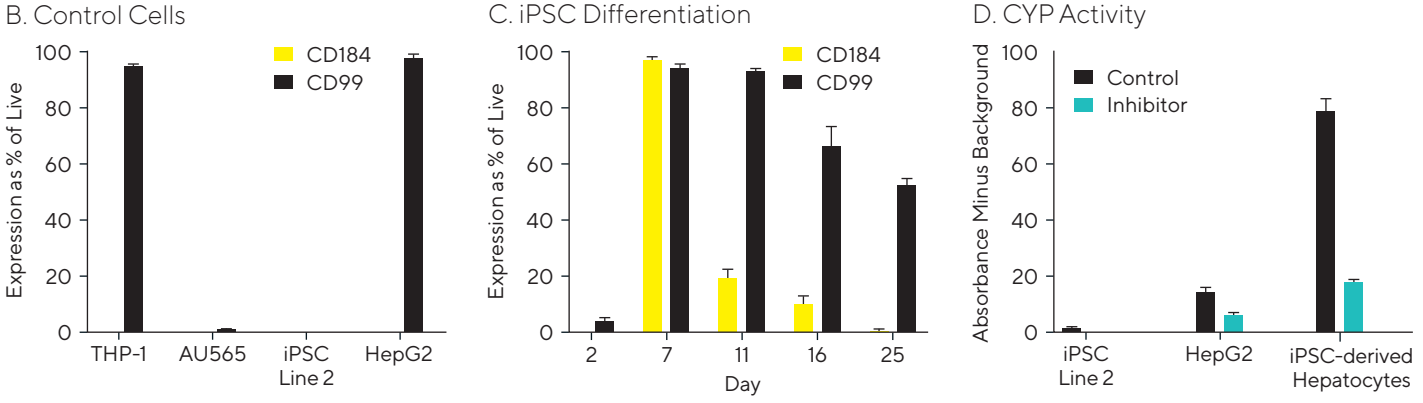
To ensure that the differentiation process is successfully progressing towards the desired lineage, it is important to monitor the gain in specific characteristics over time, concurrently with the loss in pluripotency. In the same panel used to assess the pluripotency markers in Figure 4, we included two additional markers with the aim of measuring the gain of hepatocyte-like phenotype over time (Figure 5). The markers were chosen to represent progressive stages in the hepatocyte differentiation pathway, from definitive endoderm (CD184) to the more mature hepatocyte (CD99).[10]

Figure 5

Phenotype and Function Analysis to Evaluate Gain in Hepatocyte-Like Cell Features During iPSC Differentiation

A.





Note. Within the same panel as used in Figure 4, markers were included to monitor progression of the hepatocyte differentiation pathway (CD184 and CD99). (A) Contour plots show CD184 expression over time; (B) Marker expression (± SEM) on control cells (n = 4); (C) Expression of markers (± SEM) at defined timepoints in iPSC differentiation (n = 4); (D) CYP450-Glo™ luminescence assay comparing CYP1A2 activity (± SEM) across cell types in the presence and absence of inhibitor α-napthoflavone (1 µM) (n = 4). Background subtraction was determined using wells containing substrate and detection reagent alone.

CD184, the marker for definitive endoderm cells peaked on Day 7 (Figure 5A and C) at 98 ± 0.1%, indicating highly successful differentiation to the DE phenotype. Expression of CD99 was high on the HepG2 cells as expected, but also on the THP-1 cells, suggesting it is not exclusively a hepatocyte marker (Figure 5B). CD99 was expressed from Day 7 onwards on the differentiating cells, declining towards Day 25, with 52 ± 1.3% expression on the fully differentiated cells.

At the endpoint of the differentiation (Day 25), a CYP1A2 inhibition assay was performed to determine whether the derived cells were functionally similar to hepatocytes (Figure 5D). CYP1A2 is an enzyme in the CYP450 family in the liver and plays a major role in drug metabolism.[11] In this assay, a Luciferin-1A2 substrate was added to cells, which the CYP1A2 enzyme converted to Luciferin causing an increase in the luminescence of the sample. CYP activity was measured in the presence and absence of a CYP1A2 specific inhibitor, α-napthoflavone (1 µM). Results showed high CYP1A2 activity in the iPSC-derived hepatocyte cells, which was reduced by 4.4-fold in the presence of the inhibitor. There was no CYP activity in the undifferentiated iPSCs and low-level activity in the HepG2 cells, which was reduced by the addition of inhibitor.

Conclusions

Combining the pluripotency and hepatocyte marker expression, Incucyte® images, and endpoint functional analysis provided strong evidence to suggest that we successfully differentiated iPSCs to produce hepatocyte-like cells. Overall, these data exemplify the ease with which the iQue® and Incucyte® platforms can be used to monitor the temporal progression of differentiation. The development of simple, robust methods such as these for monitoring iPSC differentiation has promise to enhance the scalability and throughput of applications utilizing iPSCs, such as liver toxicity screening.

Measuring the pluripotency and viability of iPSC lines to aid selection of the most appropriate cell line to use for differentiation, we gave ourselves the best chance of success, reducing the risk of yield-limiting factors such as excessive cell death or non-specific differentiation to other lineages. The experiments in this note have highlighted some of the advantages of this workflow:

- Pluripotency marker expression and viability of iPSCs are easily measured using the iQue® with simple, one-wash labeling protocols
- The miniaturized assay format due to the low volume requirements of the iQue® reduces sample wastage, meaning more cells are available for downstream analysis, expansion, and differentiation
- High-throughput sample acquisition using the iQue® (15 minutes for a full 96-well plate) reduces workflow time and facilitates enhanced replication, meaning more robust data
- Plate-level, real-time data visualization tools via advanced flow cytometry enable rapid comparison between cell lines, resulting in the fastest path to actionable results
- Monitoring cultures using the Incucyte® provides an easy way to check for pluripotency, confluency, and differentiation over time
- Measuring differentiation marker expression over time using the iQue® complements endpoint functional analysis to provide full confidence in the progression of your differentiation workflow.

Together, this creates a streamlined workflow for iPSC characterization with potential applications in research and drug discovery.

References

1. Omole A, Fakoya A. Ten years of progress and promise of induced pluripotent stem cells: historical origins, characteristics, mechanisms, limitations, and potential applications. *Peer J*. 2018;6: p.e4370.
2. Singh V, Kalsan M, Kumar N, Saini A, Chandra R. Induced pluripotent stem cells: applications in regenerative medicine, disease modeling, and drug discovery. *Frontiers in Cell and Developmental Biology*. 2015;3.
3. Liu G, David B, Trawczynski M, Fessler R. Advances in pluripotent stem cells: History, mechanisms, technologies, and applications. *Stem Cell Reviews and Reports*. 2019;16(1) pp.3–32.
4. Ko H, Gelb B. Concise review: Drug discovery in the age of the induced pluripotent stem cell. *Stem Cells Translational Medicine*. 2014;3(4), pp.500–509.
5. Sullivan S, et al. Quality control guidelines for clinical-grade human induced pluripotent stem cell lines. *Regenerative Medicine,* 2018;13(7), pp.859–866.
6. Rao M, Malik N. Assessing iPSC reprogramming methods for their suitability in translational medicine. *Journal of Cellular Biochemistry*. 2012;113(10), pp.3061–3068.
7. Sanchez-Freire V, et al. Effect of human donor cell source on differentiation and function of cardiac induced pluripotent stem cells. *Journal of the American College of Cardiology*. 2014;64(5), pp.436–448.
8. Corbett J, Duncan S. iPSC-Derived hepatocytes as a platform for disease modeling and drug discovery. *Frontiers in Medicine*. 2019;6, p.265.
9. Karagiannis P, et al. Induced pluripotent stem cells and their use in human models of disease and development. *Physiological Reviews*. 2019;99(1), pp.79–114.
10. Teng Ang L, et al. A roadmap for human liver differentiation from pluripotent stem cells. *Cell Reports*. 2018;22(8), pp.2190–2205.
11. Boon R, et al. Amino acid levels determine metabolism and CYP450 function of hepatocytes and hepatoma cell lines. *Nature Communications*. 2020;11(1), 1393.

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