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Characterization and Optimization of Induced Pluripotent Stem Cell Culture Using Advanced Flow Cytometry and Live-Cell Analysis

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Introduction

In 2006, Japanese researchers published an important discovery outlining a method for creating induced pluripotent stem cells (iPSCs) from primary mouse fibroblasts by activating the expression of key transcription factors.[1] Since this discovery, the field of stem cell biology has rapidly expanded and iPSCs form the basis of many new areas of research. They are intrinsically valuable due to their unique characteristics and the control they afford to researchers and clinicians over the building blocks of the body. The major benefits of the use of iPSCs are the number of different cell types that can be differentiated from them and their capacity for infinite expansion.[2] This flexibility provides many opportunities for the development of specific cell and tissue models both in 2D and 3D for pharmacological testing,[3] cancer research,[4] organoid modeling of tissues,[5] and neurodevelopmental biology.[6] In addition, iPSCs are increasingly used in translational applications, targeting eventual use in the clinic via autologous cell therapies and for individualized medicine approaches.[7] Increasingly, there is a need to improve the culture and expansion methods of iPSCs away from 2D plate-based methods and towards more physiologically relevant methods such as 3D culture. In particular, 3D suspension cultures, such as cell aggregates and spheroids, can maintain greater cell-to-cell contact, produce endogenous extracellular matrix to promote growth conditions similar to *in vivo*, and are readily available for downstream applications.[8]

Some limitations are inherent in any system, however, and iPSCs are high maintenance, expensive, and require constant monitoring to ensure they maintain pluripotency, viability, and homogeneity.[9] Long-term culture of iPSCs can result in genotypic and phenotypic heterogeneity, even in a cell line derived from a single source cell; therefore, it is vital that methods for monitoring, detecting, and reducing heterogeneity in iPSC lines are developed.[10]

Increasing use of stem cells in both clinical and research settings necessitates fast, reliable, and relatively inexpensive solutions for the growth, characterization, and maintenance of this valuable biological resource.

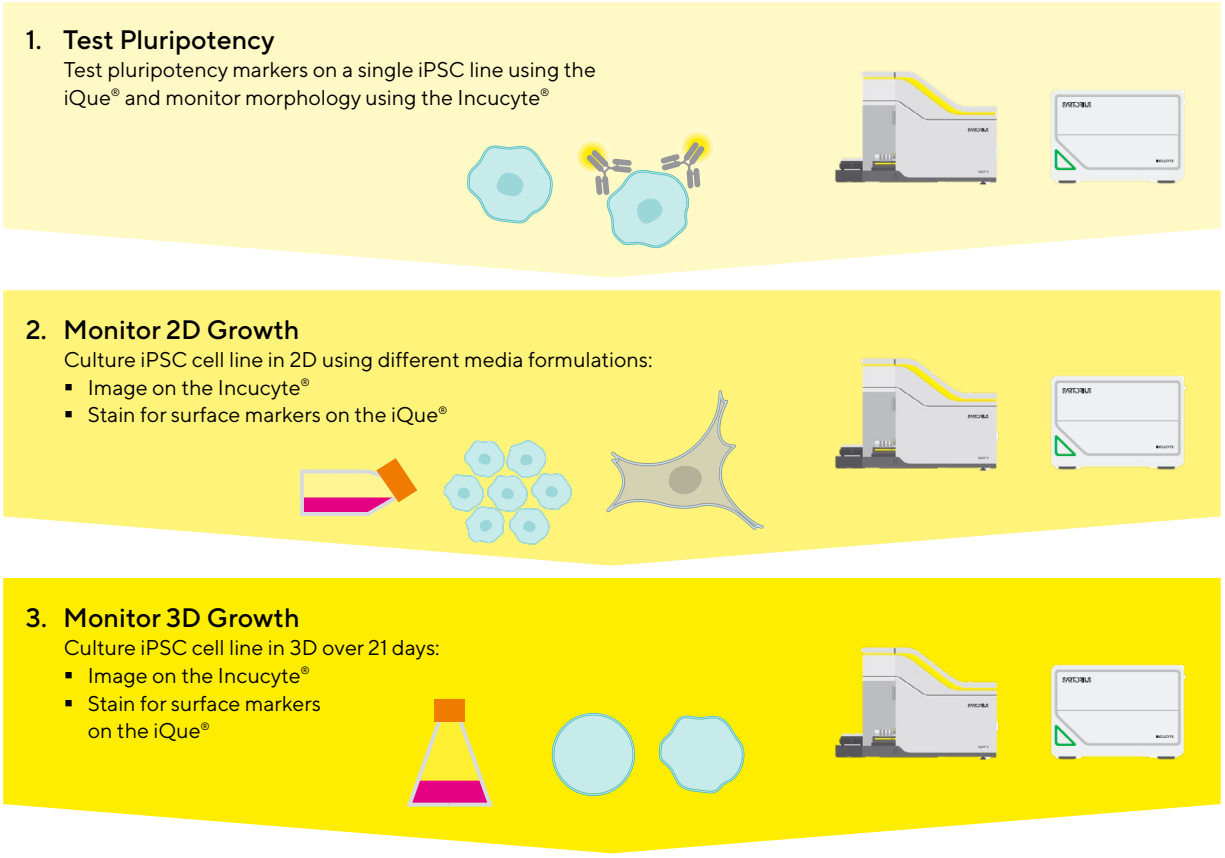
Conventional methods for monitoring iPSC characteristics during culture, such as traditional flow cytometry, can:

- Be labor-intensive and time-consuming, requiring multiple steps including fixation, staining, and washing
- Require large sample volumes, necessitating the use of more precious cells and expensive consumables
- Demand in-depth manual manipulation and analysis of raw data and require compensation optimization
- Often use low-throughput solutions, increasing the time to results

This application note describes methods for the characterization, successful maintenance, scale up, and selection of ideal iPSCs in a combined workflow approach using real-time imaging on the Incucyte® Live-Cell Analysis System for easy morphological analysis and rapid expression profiling using minimal sample volumes (10 µL) on the iQue® Advanced Flow Cytometry platform.

Figure 1

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



Note. Using a combination of two Sartorius instruments, researchers can test iPSC pluripotency and monitor iPSC growth both in 2D and 3D culture, as illustrated above.

Methods

The following methods describe the processes used to characterize and monitor healthy, pluripotent iPSCs grown in 2D and 3D using a combined workflow for the Incucyte® Live-Cell Analysis System and the iQue® Advanced Flow Cytometry platform. Figure 1 outlines the key steps during this workflow.

Cell Culture and Maintenance

Control Cell Line Culture

THP-1 cells (human monocyte derived from an acute monocytic leukemia patient) were grown in suspension at a density of 1×10^5/mL. NCCIT cells (human developmentally pluripotent cell line derived from a mediastinal mixed germ cell tumor) were grown in 2D monolayers until 70–80% confluent before passaging and use in experiments. Both lines were grown in RPMI 1640 medium supplemented with 10% FBS, L-glutamine 2 mM, Penicillin/Streptomycin 100 µg/mL.

Thawing and Culturing iPSC Lines (2D)

A single iPSC line (ATCC-DYS0100 cells derived from human foreskin fibroblasts) was thawed and plated onto Vitronectin XF™ (1:25 dilution in CellAdhere™ Dilution Buffer) precoated 6-well plates at a seeding density of 1×10^6/well in 1 mL growth medium (mTESR™ Plus) supplemented with Y-27632 (ROCK inhibitor, 10 µM) and incubated at 37 °C. iPSCs were monitored using the Incucyte® system to assess confluency, colony formation, and general cell morphology and health. The confluence of colonies was analyzed using the integrated Incucyte® AI confluence software algorithm. Passages were performed every 3–4 days at approximately 60–70% confluence using Gentle Cell Dissociation Reagent and replated at 1×10^5/well. Medium changes were performed daily, with a two-day duration over weekends. For the non-optimized iPSC culture, cells were grown as above except for using RPMI 1640 medium supplemented with 10% FBS, L-glutamine 2 mM, Penicillin/Streptomycin 100 µg/mL.

Culturing iPSC Lines (3D)

Once healthy colonies had been established, iPSCs were transferred from 2D culture into 125 mL Erlenmeyer flasks at a density of 1.5×10^5/mL in 20 mL StemScale™ PSC Suspension Medium supplemented with Y-27632 (10 µM) and incubated at 37 °C on a shaker at 70 rpm. 3D spheroid size and shape were monitored at regular intervals by taking 1 mL samples from the flask and plating into 24-well plates for analysis using the Incucyte®. Passaging was performed at least once a week using Accutase® once spheroid sizes reached a limit of 400 µm. Medium changes were performed every other day, with a two-day duration over the weekend.

Characterization and Monitoring of Pluripotency

Pluripotency Characterization: iQue®

iPSCs were dissociated to single cells during passage and at specified timepoints using Gentle Cell Dissociation Reagent for 2D culture and Accutase® for 3D culture. Single-cell suspensions were stained with cell surface marker antibodies (in PBS + 2% FBS) for one non-pluripotent marker, SSEA-1, and two pluripotency markers, SSEA-4 and TRA-1-60, in addition to the iQue® Membrane Integrity (B/Red) Dye, for viability analysis. Cells were seeded at 2×10^4/well in a V-bottom 96-well plate; they were stained with the cocktail of antibodies described, centrifuged (300 x g, 5 sec), briefly shaken using the iQue® plate station (2000 rpm, 20 sec), and incubated at RT in the dark for 30 min. PBS + 2% FBS (100 µl) was added to each well and centrifuged (300 x g, 5 min), supernatant was aspirated, plate shaken (3000 rpm, 60 sec), and the samples resuspended in PBS + 2% FBS (20 µL), prior to being analyzed on the iQue®. Analysis of data was performed using the iQue Forecyt® software after compensation had been optimized for each of the antibodies.

Monitoring Pluripotency and Cell Health: Incucyte®

During the experiments, iPSCs were monitored for changes in morphology and confluency associated with pluripotency using the Incucyte® Live-Cell Analysis System. 2D-cultured iPSC lines were monitored by high definition (HD) phase contrast at 4-hour intervals using a repeating scan schedule at 10X. Nuclear to cytoplasmic ratios were calculated by staining iPSC nuclei using the Incucyte Nuclight® Rapid Red Reagent (1:1000) and measuring the cytoplasmic area (confluence mask) and the nuclear area (fluorescence mask) using basic masking on the Incucyte® Live-Cell Analysis System to quantify pluripotency/normal iPSC morphology.

3D-cultured iPSC spheroids were sampled every day and plated into 24-well plates for analysis on the Incucyte® system using a one-off scan at 10X. Spheroid diameter was measured, and the circularity of each spheroid was analyzed as a morphological marker of pluripotency.

Results

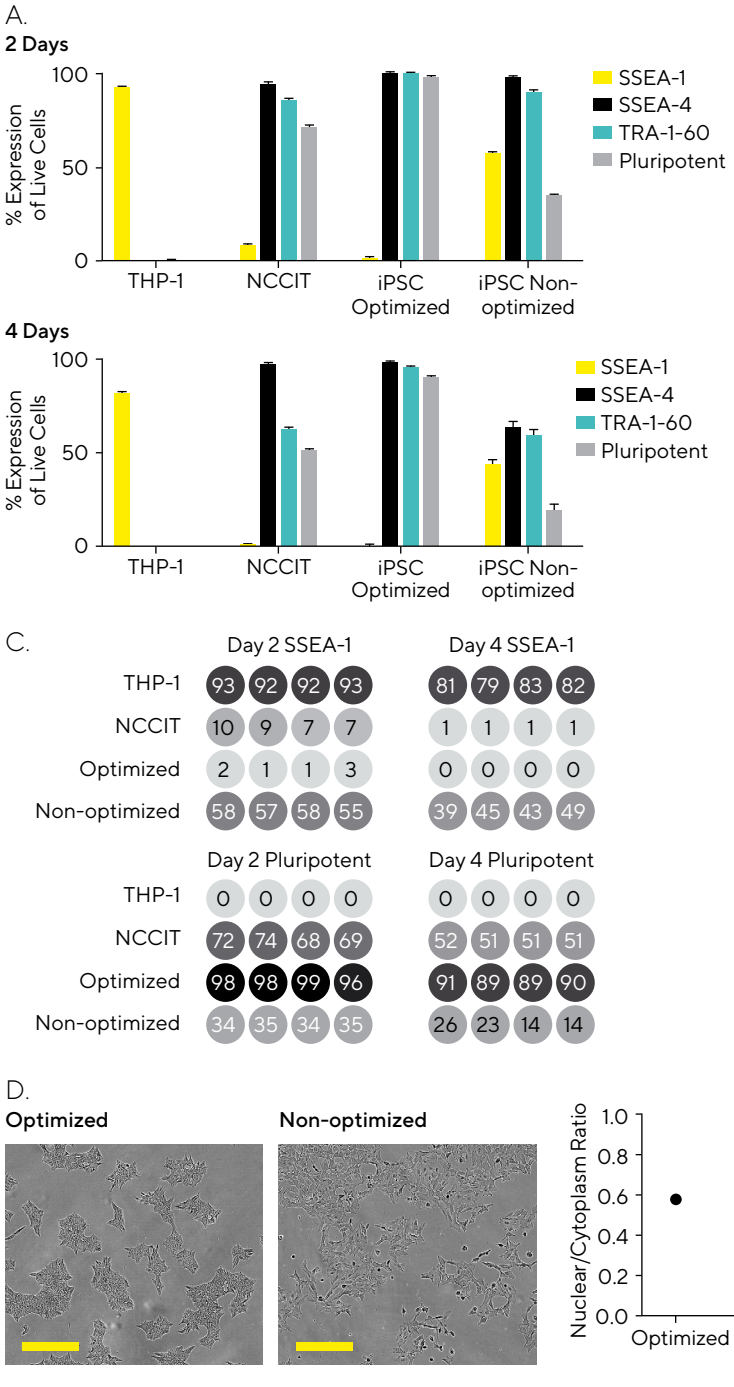
iPSCs require highly specific growth conditions to maintain pluripotency and viability. Determining optimal conditions for their growth and expansion in 2D and 3D during the development of new reagents, techniques, and experimental research can be time-consuming and cost-prohibitive. The data presented here showcase the benefits of using the Incucyte® Live-Cell Analysis platform in conjunction with the iQue® Advanced Flow Cytometry system to monitor the culture of iPSCs during optimization of growth conditions for high levels of pluripotency.

Optimization of Growth Conditions for Successful 2D iPSC Propagation

Using a single iPSC line grown in 2D, we tested the effects of a non-optimized growth condition on the phenotypic presentation of cell surface markers and morphology over the course of two timepoints, 2- and 4-days post-treatment. iPSCs were grown in their usual optimized conditions until colonies formed, at which point they were challenged by changing their medium from mTESR Plus (optimized) to RPMI 1640 with supplements (non-optimized). Control cells, THP-1 and NCCIT, represented a non-pluripotent control line and a line expressing pluripotent surface

Figure 2

Evaluate Optimal Media Formulation to Retain Pluripotency in 2D-Cultured iPSCs



Note. iPSCs were grown in optimized (mTESR Plus) and non-optimized (RPMI) media to induce 'differentiation' for 2 and 4 days (± SEM, n = 4) (A). Prior to treatment with experimental growth conditions, iPSCs were grown under optimized conditions until medium-sized colonies formed at approximately 3–4 days from initial seeding at 100K cells per well of a 6-well plate. 'Pluripotent' are a population of SSEA-1 negative cells that are positive for both SSEA-4 and TRA-1-60, representing pluripotent cells. (B) Dot plots showing the raw data collected by the iQue® system at 2 days comparing the optimized and non-optimized iPSCs, note the shift in SSEA-1 expression in the non-optimized iPSCs and the subsequent losses in pluripotency marker expression (n = 4). (C) Percentage expression 'heatmaps' produced in the iQue Forecyt® software showing the changes in marker expression over 2 and 4 days of 'differentiation' of iPSCs (n = 4). (D) iPSCs grown in optimized and non-optimized growth conditions display distinct morphological differences linked to differentiation which can be imaged and analyzed effectively using the Incucyte® (representative images taken at 10X magnification). Graphed quantification of the Nuclear/Cytoplasm ratio analyzed in the Incucyte® from the representative images, right. Scale bar indicates 400 µm.

markers, respectively. Cells were imaged using the Incucyte® Live-Cell Analysis System and surface markers were analyzed using the iQue® Advanced Flow Cytometry platform.

Analysis shown in Figure 2 highlights the rapid loss of pluripotency in iPSCs when they are grown in non-optimized conditions. This is illustrated by the increase in non-pluripotent marker SSEA-1 expression (57.5 ± 0.7%) as early as 2 days post-treatment (Figure 2A). Further, we observe decreases in the expression of pluripotency markers SSEA-4 (97.3 ± 0.8%), TRA-1-60 (89.8 ± 0.9%), and pluripotent population (34.6 ± 0.3%), with this decrease amplified after 4 days of treatment (SSEA-4 63.4 ± 2.9%, TRA-1-60 58.9 ± 2.9%, pluripotent population 19.3 ± 3.0%). When we compare this data to the optimized iPSCs, where we see no marked differences in expression profile over the time course of these studies (95 ± 0.4% for pluripotent markers and less than 1.8 ± 0.5% for SSEA-1, with one exception being the pluripotent marker at 4 days 89.6 ± 0.6%), there is a clear deviation from the pluripotent profile, i.e. increased SSEA-1 and reduced SSEA-4, TRA-1-60, and combined pluripotency marker expression.

In Figure 2B, (dot plots taken directly from iQue Forecyt® software) there is a clear shift of the population from the Optimized 1.63 % SSEA-1 positive to the Non-optimized 57.5 % SSEA-1 positive (top two dot plots). The lower plots further demonstrate the shift away from pluripotency marker expression in the Non-optimized conditions, where the Optimized iPSCs present a compact population in the upper right quadrant of the plot (SSEA-4+, TRA-1-60+), while the Non-optimized iPSCs present a much more spread population shifting into the TRA-1-60 negative portion of the plot. Heatmaps produced in iQue Forecyt® software present the same data for SSEA-1 and pluripotent expression for the two timepoints, highlighting the flexibility of this platform (Figure 2C).

Incucyte® images of the iPSCs at Day 2, show a marked difference in morphology between the Optimized and Non-optimized conditions. iPSCs grown in Optimized conditions form tightly packed colonies with clearly defined edges, that 'glow' under phase images. By contrast, Non-optimized iPSCs are much more spread out and no longer form colonies, they are beginning to resemble fibroblast cells (Figure 2D). Quantification of these morphological differences was performed using the adherent cell by cell scan at 10X magnification, and nuclear and cytoplasm area measurements were made using the Basic Analyzer and AI Confluence analysis using the following equation to provide a Nuclear/Cytoplasm ratio, a standard measurement used when studying iPSCs.

$$\frac{total\ nuclei\ area}{total\ cytoplasmic\ area} = nuclear\ cytoplasm\ ratio$$

The more iPSC-like, and thus pluripotent, a cell is, the higher the Nuclear/Cytoplasm ratio. The graph in Figure 2D illustrates the reduction in this ratio in the Non-optimized conditions, from 0.6 to 0.4.

Optimization of Growth Conditions for Successful 3D iPSC Propagation

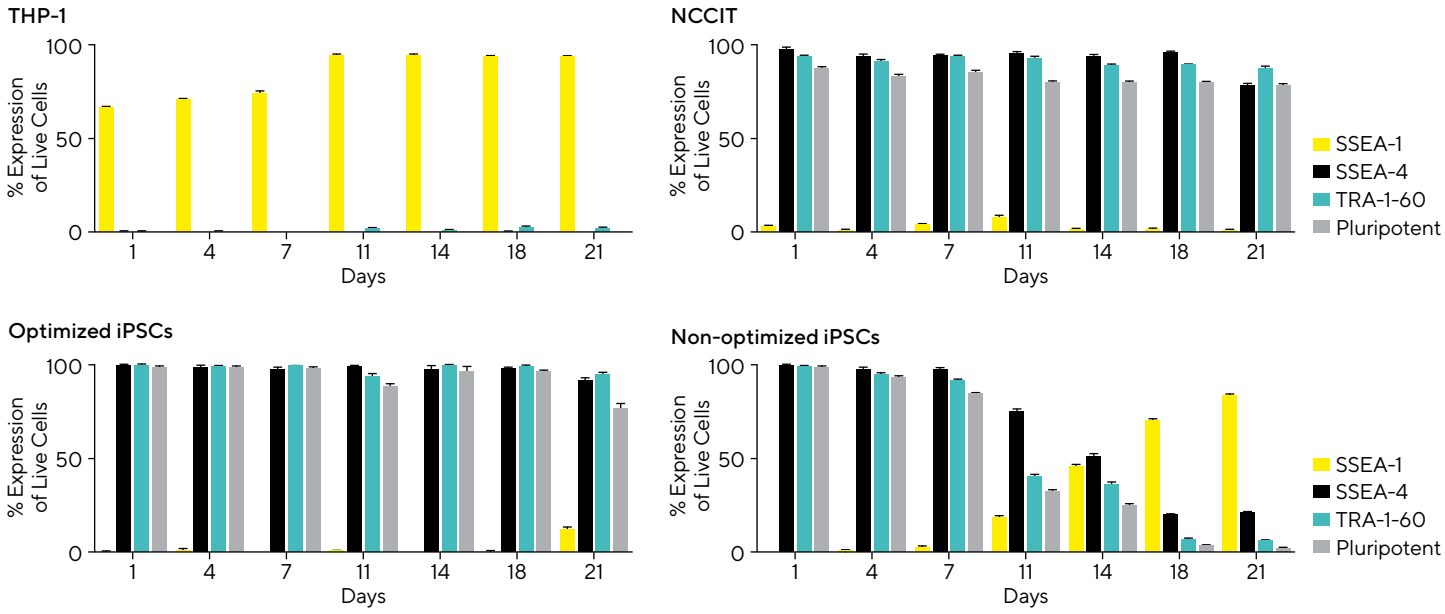
Scaling up iPSC culture requires changes to the conditions under which the cells are grown. Due to the relative flexibility of iPSCs, they can be grown in both 2D and 3D, facilitating scale-up in bioreactors of good quality iPSC lines for a range of downstream processes.

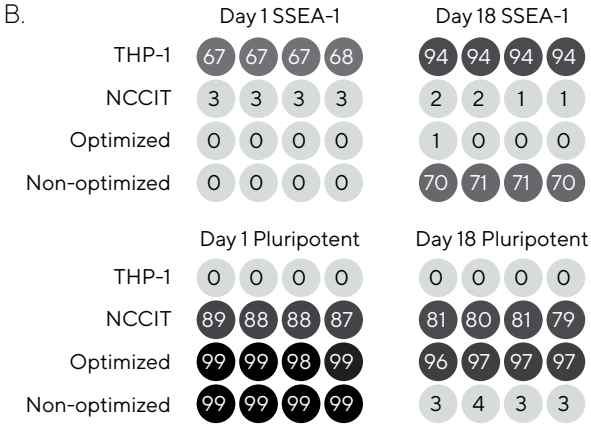
Transferring the culture of the iPSC line used in the 2D experiments to a 3D culture, we investigated the effects of Non-optimized growth conditions on the expression of surface markers for pluripotency and the formation of 3D spheroids over 7 timepoints covering a total of 21 days. iPSCs were grown in 2D culture for at least 2 passages before transfer into 3D culture for at least 1 passage before they were seeded and subjected to Optimized (passaging at each timepoint) and Non-optimized (no passaging) conditions over the time course. Control cells, THP-1 and NCCIT represented a non-pluripotent control line and a line expressing pluripotent surface markers, respectively. Cells were imaged using the Incucyte® Live-Cell Analysis System and surface markers were analyzed using the iQue® Advanced Flow Cytometry platform.

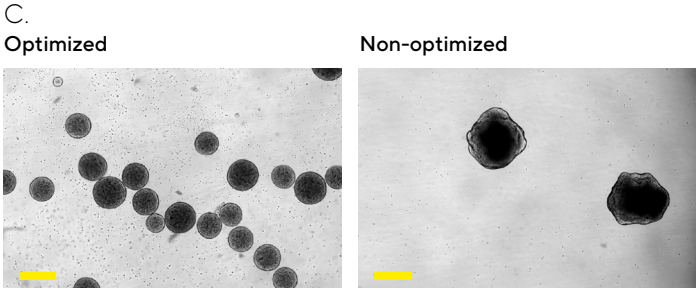
When iPSCs are grown as 3D spheroids, marker expression analysis, Figure 3A, shows that dramatic changes in phenotype only began to present on Day 11 in Non-optimized conditions, where we saw an increase in SSEA-1 expression (18.5 ± 0.39%) and a reduction in pluripotent marker expression, SSEA-4 (75.2 ± 1.2%), TRA-1-60 (40.5 ± 0.5%), and pluripotent (32.9 ± 0.5%). This trend continued until the endpoint at Day 21 where we observed a final SSEA-1 expression of 83.5 ± 0.7%, SSEA-4 at 20.7 ± 0.3%, TRA-1-60 at 5.9 ± 0.1%, and the pluripotent population at 2.2 ± 0.1%. This data indicates a loss of pluripotency throughout the time course when we contrast the results with the expression profile of our Optimized iPSCs, which stay consistently pluripotent until Day 21. At this timepoint, we start to see an increase in SSEA-1 expression (12.4 ± 0.6%) and a decrease in the pluripotency markers, SSEA-4 (91.1 ± 1.8%), and the pluripotent population (76.5 ± 2.6%). Although there are fluctuations across the time course in the expression profile, Day 21 is increased over the observed variation, suggesting a 21-day cut-off point for successful culture of 3D iPSC spheroids in the system we used.

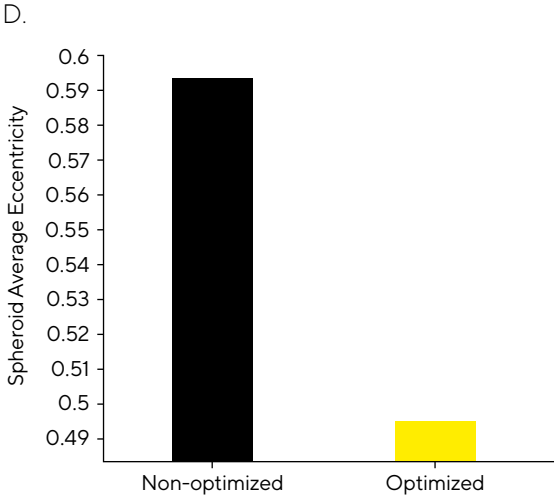
Figure 3

Determine Optimal Cell Culture Conditions for Improved 3D Spheroid Cell Culture









Note. 3D iPSC spheroids were grown in Optimized (passaged) and Non-optimized (non-passaged) conditions to induce 'differentiation' for up to 21 days, and marker expression was measured on the iQue® Advanced Flow cytometer (± SEM, n = 4). (A) 3D iPSC spheroids were grown in 125 mL shaker flasks at a density of 150k cells per mL in 20 mL of StemScale™ PSC Suspension Medium (Gibco). The spheroids were grown until their first passage, at which point they were subjected to the described growth conditions. Heatmaps produced in the iQue Forecyt® software showing the changes in marker expression at 1 and 18 days of Optimized and Non-optimized growth conditions (n = 4). (B) 3D iPSC spheroids grown in Optimized and Non-optimized growth conditions display distinct morphological differences associated with differentiation which can be imaged effectively using the Incucyte®. (C) Spheroid images were taken at 10X magnification at the 14-day timepoint during the 3D 'differentiation' process prior to collection for analysis on the iQue®. Scale bar indicates 400 µm. (D) Spheroid eccentricity was measured by analyzing Incucyte® images using the Incucyte® Organoid Anaylsis Software module.

Heatmaps produced in iQue Forecyt® software present the same data for SSEA-1 and pluripotent expression for two representative timepoints at Day 1 and Day 18, providing a snapshot of the changes in expression during the experiment (Figure 2C).

3D iPSC spheroids were sampled at each timepoint, plated into a 24-well plate, and visualized in the Incucyte® Live-Cell Analysis System (Figure 3C). The images show the morphological differences after 18 days between the Optimized (top) and Non-optimized (bottom) culture conditions. When 3D iPSC spheroids are grown in Optimized conditions, we observe a very distinct, compact spheroid with high levels of circularity, compared to the Non-optimized conditions, in which the spheroids are much larger, far less compact, and lack the circularity of the Optimized spheroids (0.59 vs 0.49 eccentricity, respectively, Figure 3D).

Conclusions

iPSCs are a valuable resource in many areas of research and clinical development; however, they require highly specific conditions for optimal growth to maintain pluripotency, viability, and propagation potential. These requirements are often expensive and culture methods can be time intensive, requiring complicated techniques. We have shown that by using Sartorius platforms we can successfully characterize iPSCs and monitor their pluripotency with ease via surface marker expression and morphological status, both in 2D and 3D, using the iQue® Advanced Flow Cytometer and the Incucyte® Live-Cell Analysis platform. The key advantages of using this combined workflow over conventional methods are:

- Streamlined processes and data analysis save time and reagent costs to reduce attrition due to low-quality cell products
- Miniaturization reduces antibody reagent costs and saves sample for downstream expansion, characterization, and differentiation
- Low sample requirements provide greater resources for multiple replicates easily processed using a plate-based format
- Monitoring iPSC lines throughout the time course provide greater insights into morphology and pluripotency changes over time
- Rapid protocols require no fixation in a single wash workflow for pluripotent surface markers

The data presented here highlight the advantages of using a streamlined workflow combining multiple Sartorius systems for the characterization and optimization of iPSC culture in 2D and 3D for several applications including drug development, disease modeling, and clinical therapy research.

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