SARTORIUS

Application Note

July, 2023

Key words or phrases:
Induced Pluripotent Stem Cells, iPSCs, Pluripotency, Stem Cells, CellCelector Flex, Incucyte, iQue, Cell Culture, iPSC Characterization, iPSC R&D

Solutions for the Culturing, Maintaining and Characterization of Induced Pluripotent Stem Cells

Daryl Cole, PhD — Scientist - Sartorius
Kirsty McBain — Scientist - Sartorius
Nicola Bevan — Manager - Sartorius

Introduction

From drug discovery to organoid modeling of disease, stem cells are increasingly being used in research as a vital tool for scientific investigation. The current trend away from animal models and the push to more relevant systems for simulating the human body require flexible and specific tools to achieve this goal. Induced Pluripotent Stem Cells (iPSCs) are produced from normal tissue, through the forced expression of key transcription factors[1], providing a limitless supply of these precious cells for research and development. Due to the very specialized nature of these cells, their maintenance and culture is more intensive than most cell lines. For this reason, it is important that solutions for the culture and maintenance of these cell types are readily and widely available. Characterization of stem cells can be difficult and unreliable, depending on the methodology used, which is why it is important to develop robust techniques for monitoring stem cells throughout culture and experimental testing. If conditions are not optimal during the maintenance of iPSCs, their pluripotency can be lost.

Reproducibility is highly prized in research and automated solutions can provide high levels of consistency in method and data generation. The CellCelector Flex is an automated platform for targeted cell identification and picking that is not only highly accurate, but also very gentle on cells, providing an ideal solution when working with delicate iPSCs. The Incucyte® Live-Cell Analysis platform automates the imaging processes of iPSC workflows, allowing cells to be monitored over time to analyze changes in morphology and colony formation from within the incubator. This limits the disturbance to precious iPSC culture plates, but also enables real-time tracking of cell growth and health metrics.

Find out more: www.sartorius.com/ipscs

Further characterization of iPSCs can be performed on the iQue® Advanced Flow Cytometer, investigating changes in expression of pluripotency markers integral to maintaining stemness, providing an overview of the status of iPSCs.

Many traditional methods for culturing, monitoring and characterizing iPSCs can:

1. Be inconsistent and unreliable, resulting in seeded populations with high levels of heterogeneity, cell death and differentiation
2. Require regular disturbance of culture plates to monitor growth and confluency, with no integrated options for analysis
3. Demand large volumes of precious sample for analysis, resulting in less material for downstream applications
4. Necessitate the use of a variety of techniques to measure multiple characteristics

This application note discusses the novel solutions provided by Sartorius platforms for the culture, maintenance, and characterization of iPSCs, during research and development.

Methods

The following methods outline a flexible, in-depth workflow for growth and characterization of iPSCs using multiple Sartorius platforms.

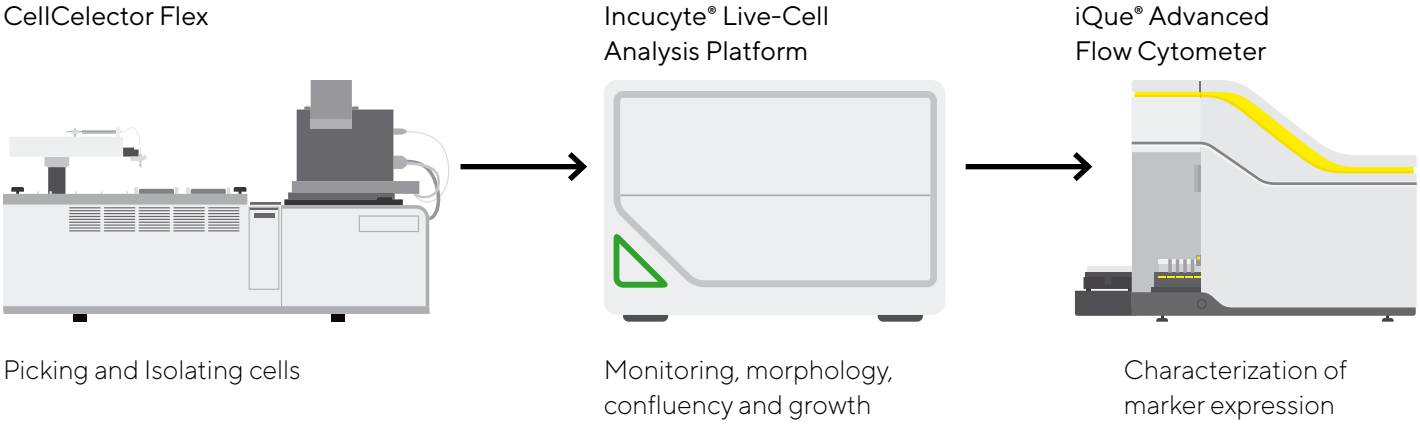


Figure 1. Schematic showcasing the use of Sartorius platforms in iPSC culture.
Using the three Sartorius instruments, CellCelector Flex, Incucyte® and iQue®, iPSCs can be picked and seeded, pluripotency tested, and growth and confluency monitored.

Cell Culture and Maintenance

Picking and seeding iPSCs

Individual cells and colonies were picked using the CellCelector Flex with the Adherent Colony Picking Module and seeded into tissue culture plates for further expansion and downstream processing. Images were taken prior to and post picking to monitor and record the effects of colony manipulation using the CellCelector Flex. Propidium Iodide (PI) staining was undertaken on iPSC colonies after seeding by adding PI at a concentration of 500 nM and incubating for 3 minutes, rinsing twice with PBS and resuspending in growth medium (mTESR Plus) for imaging.

Thawing and Culturing iPSCs

Cells (ATCC-DYS0100 cells derived from human foreskin fibroblasts) were thawed and plated onto Vitronectin XF™ (1:25 dilution in CellAdhere™ Dilution Buffer) precoated 6-well plates at a seeding density of $1x10^6$ cells/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 $1x10^5$ cells/well. Medium changes were performed daily during the week, while double volume medium changes were performed on Friday to account for no medium changes over the weekend. For the non-optimized iPSC culture, cells were grown as above except using RPMI 1640 medium supplemented with 10% FBS, L-glutamine 2 mM, Penicillin/Streptomycin 100 µg/mL.

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. 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 $2x10^4$ cells/well in a V-bottom 96-well plate and stained with the cocktail of antibodies described (RT in the dark for 30 minutes). To wash plates, PBS + 2% FBS (100 µl) was added, prior to centrifugation (300 x g, 5 minutes), then aspirated. Plates were shaken (3000 rpm, 60 seconds) 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 using the Incucyte® Live-Cell Analysis platform. Cultured iPSCs 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 Dye (1:1000) and measuring the cytoplasmic area (confluence mask) and the nuclear area (fluorescence mask) using basic masking to quantify pluripotency/normal iPSC morphology.

Intracellular and Surface Marker Studies

iPSC and control THP-1 cells were seeded at $2x10^4$ cells/well in a V-bottom 96-well plate and fixed, permeabilized and stained according to the protocol found in the following tech note: Intracellular Staining Assay for iQue® Platform. Pluripotency markers, SSEA-4, TRA-1-60, Oct 3/4 and Sox-2 were analyzed, while SSEA-1 expression was used as a marker for non-pluripotency. Analysis was performed on the iQue Forecyt® software after compensation had been optimized for each of the antibodies.

Results

Developing workflows for the culture and characterization of stem cells such as iPSCs is vital in producing consistent, reproducible and robust data. Using the Sartorius platforms showcased here (Figure 1), we can highlight the benefits of the approaches described for culturing iPSCs that are healthy and pluripotent while monitoring and characterizing these stem cells for key markers of health and stemness.

Picking iPSCs Using the CellCelector Flex Is Fast, Gentle and Reliable

It is important when working with any cell system, but notably stem cells such as iPSCs, to maintain good cell health. The data here highlights the delicate, gentle picking and seeding capability of the CellCelector Flex. When stained with Propidium Iodide (PI), a stain that indicates cell death, manual manipulation of iPSCs produces an

increased number of PI positive cells when compared to the CellCelector Flex, indicative of fewer healthy cells (Figure 2A). The CellCelector Flex colony also has less debris and more tightly defined borders (Figure 2B). The flexibility and power of the CellCelector Flex is exemplified by its capabilities, it is able to pick single iPSCs or whole iPSC colonies from a tissue culture plate. This provides the opportunity to select ideal colonies from cultures on a standard plate for further propagation. Additionally, portions of colonies can be selected for further culture. This is useful if a portion of the colony spontaneously differentiates. Differentiated sections can be removed or pluripotent sections can be picked for passaging or analysis (Figure 2C-F).

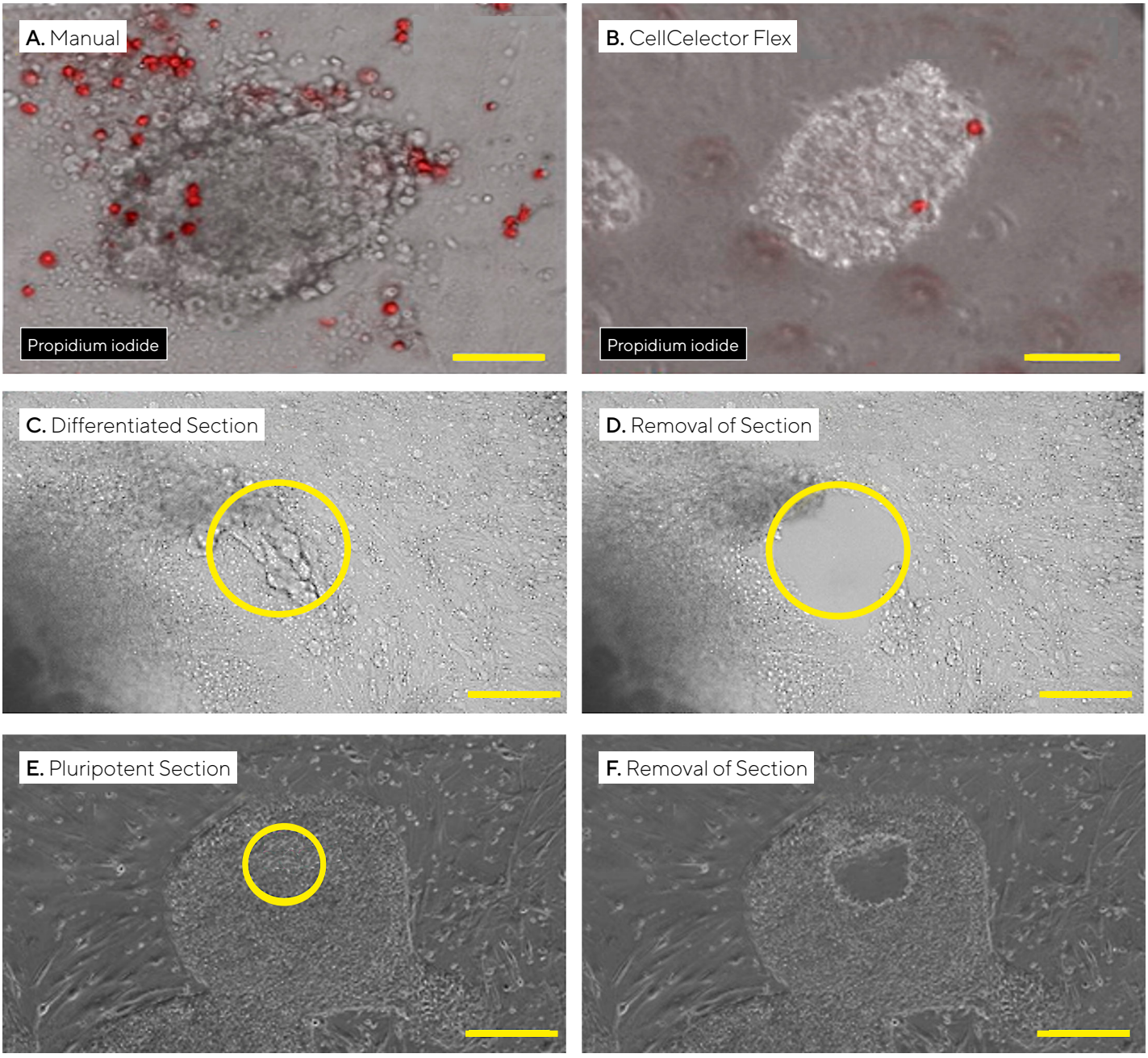


Figure 2. Picking iPSCs using the CellCelector Flex is accurate, fast, gentle and reliable.
Micrographs taken using the CellCelector platform highlighting iPSC colonies selected by the system. (A) Manually and (B) CellCelector picked and seeded iPSC colony stained with propidium iodide (PI) to identify cell death. (C) Micrograph depicting an area of differentiation in a stem cell colony prior to picking with the CellCelector. (D) The same area of the culture plate shown in (C) after removal. (E) Micrograph of a large iPSC colony grown on a feeder layer, prior to picking a section of pluripotent cells. The bottom right of the colony has indications of spontaneous differentiation. (F) The colony in (E) after picking using the CellCelector Flex, the area of pluripotent cells targeted by the machine has been collected for further culture. Scale bar equals 500 µm.

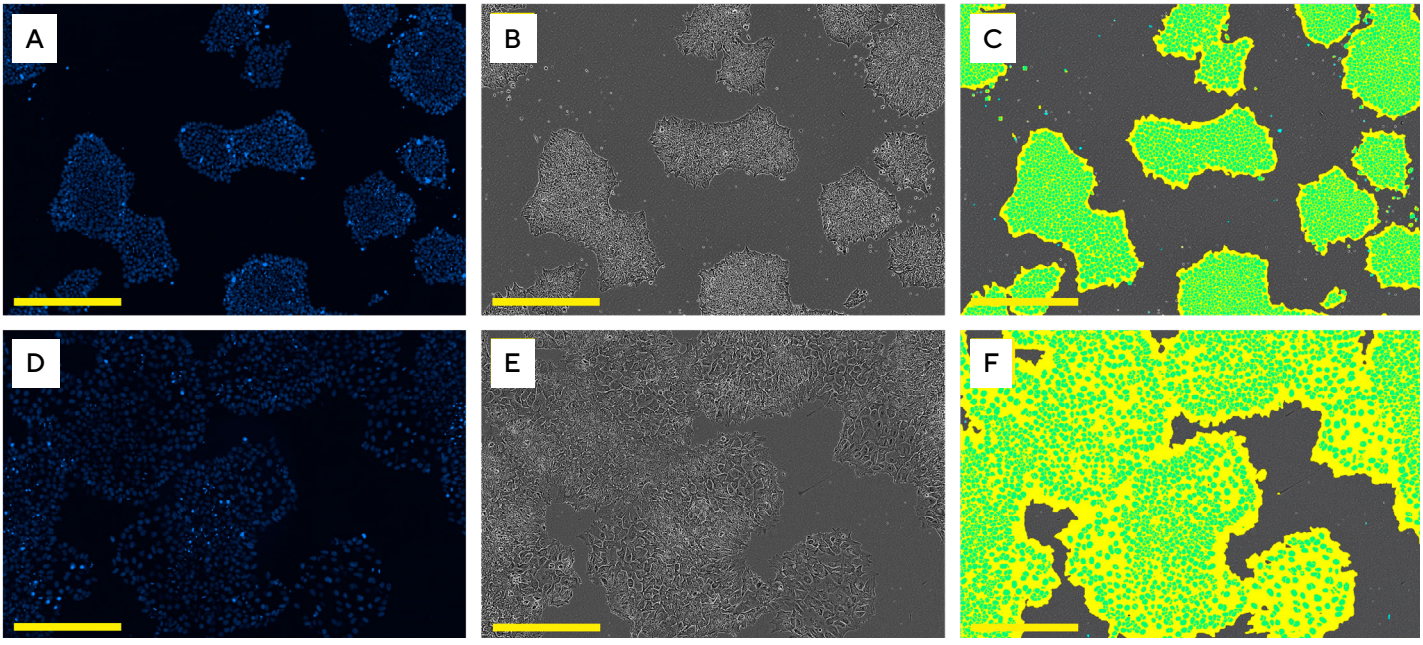
Monitoring Morphology and Pluripotent Potential During iPSC Culture

The CellCelector Flex can be used within the same workflow as another Sartorius platform, the Incucyte® Live-Cell Analysis platform. This system provides tools for monitoring cells during culture within the incubator, so changes in morphology can be recorded and analyzed without requiring removal of culture plates. In the following case, losses in morphological indicators of pluripotency can be observed, recorded, and subsequent analysis can be performed to quantify these changes.

Incucyte® images of iPSCs after 2 days in culture, show a marked difference in morphology between the optimized and non-optimized culture conditions. iPSCs grown in optimized conditions form tightly packed colonies with clearly defined edges, that 'glow' under phase images (Figure 3B), by contrast, non-optimized iPSCs are much more spread out and no longer form tightly packed colonies, they are beginning to resemble fibroblast cells (Figure 3E). Nuclear staining using Incucyte® Nuclight Rapid Red Dye also highlights the separation of the cells when grown in non-optimized conditions (Figure 3D), nuclei are much more spread out and lose the tight distribution found in optimized conditions (Figure 3A). Quantification of these morphological differences was performed using the Incucyte® Adherent Cell-by-Cell scan at 10X magnification and nuclear and cytoplasm area measurements were made using the Basic Analyzer and AI Confluence analysis (micrographs in Figure 3C, F) using the following equation to provide a nuclear/cytoplasm ratio, a standard measurement used when studying iPSCs.

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

The graph in Figure 3G illustrates the reduction in this ratio in the non-optimized conditions, from 0.6 to 0.4. The more iPSC like, and thus pluripotent, a cell is, the higher the nuclear/cytoplasm ratio.



G.

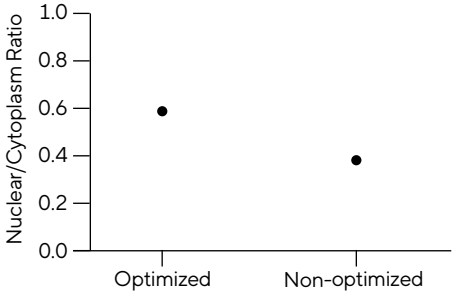


Figure 3. Monitoring morphology and pluripotent potential during iPSC culture. Incucyte® images of iPSCs grown under optimized (mTESR Plus) and non-optimized (RPMI) conditions. (A, D) Fluorescent images of iPSCs stained with Nuclight Rapid Red Dye comparing nuclear density between conditions. (B, E) Phase contrast images of the same iPSCs showing morphological differences between the two variables. (C, F) Analysis masking on the Incucyte® depicting confluency and nuclear masking that can be used to determine the nuclear/cytoplasm ratio illustrated in (G). Scale bar equals 400 µm.

Changes in iPSC Marker Expression Analyzed with the iQue® Flow Cytometry Platform

To investigate further the losses in pluripotency in iPSCs when cultured in non-optimal conditions, surface marker expression of specific pluripotency markers can be analyzed with the iQue® Flow Cytometry Platform, requiring as little as 10 µL per sample.

iPSCs grown in non-optimized conditions show rapid loss of pluripotency marker expression compared to optimized conditions (Figure 4). This indicates a loss in pluripotency correlating with the data collected on the Incucyte® platform (Figure 3). After 2 days in culture (Figure 4A), analysis of non-optimized conditions shows a decrease in expression of pluripotency markers SSEA-4 (97.3 ± 0.8%), TRA-1-60 (89.8 ± 0.9%), and the pluripotent population (34.6 ± 0.3%), with a further decrease 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 compared with optimized conditions (Figure 4B). In contrast, for the optimized iPSCs, no marked differences in expression profile over the time course of these studies was observed (95 ± 0.4% for pluripotent markers and less than 1.8 ± 0.5% for SSEA-1). (Figure 4A, B). In addition, the increase in non-pluripotent marker SSEA-1 expression (57.5 ± 0.7%) is clear as early as 2 days post treatment (Figure 4A) and remains high throughout culture.

In Figure 4C, (dot plots taken directly from iQue Forecyt® software) there is a clear shift in SSEA-1 expression between the optimized (1.63% SSEA-1 positive) and non-optimized conditions (57.5 % SSEA-1 positive) (upper two dot plots). The lower plots further illustrate the shift away from pluripotent 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.

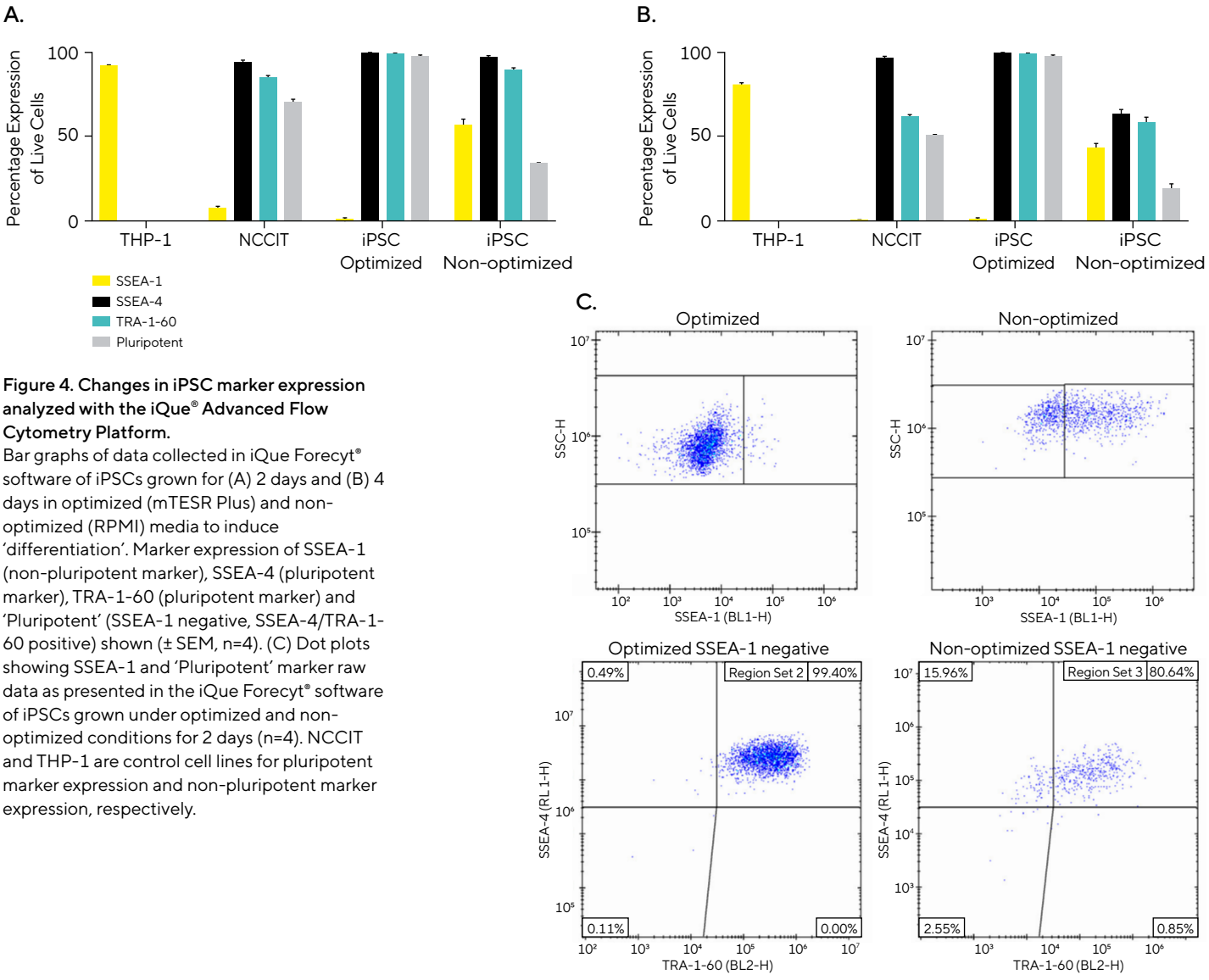


Figure 4. Changes in iPSC marker expression analyzed with the iQue® Advanced Flow Cytometry Platform.
Bar graphs of data collected in iQue Forecyt® software of iPSCs grown for (A) 2 days and (B) 4 days in optimized (mTESR Plus) and non-optimized (RPMI) media to induce 'differentiation'. Marker expression of SSEA-1 (non-pluripotent marker), SSEA-4 (pluripotent marker), TRA-1-60 (pluripotent marker) and 'Pluripotent' (SSEA-1 negative, SSEA-4/TRA-1-60 positive) shown (± SEM, n=4). (C) Dot plots showing SSEA-1 and 'Pluripotent' marker raw data as presented in the iQue Forecyt® software of iPSCs grown under optimized and non-optimized conditions for 2 days (n=4). NCCIT and THP-1 are control cell lines for pluripotent marker expression and non-pluripotent marker expression, respectively.

Surface and Intracellular Marker Staining Provides Solutions for High-Throughput Cellular Characterization

Using the iQue® Flow Cytometry Platform to monitor intracellular markers in addition to surface markers further characterizes the pluripotency of cells.

Using THP-1 cells as a non-pluripotent control, iPSCs were fixed, permeabilized and stained for the surface markers SSEA-1, SSEA-4 and TRA-1-60, in addition to the intracellular markers Oct 3/4 and Sox 2 (Figure 5). Dot plot data taken directly from iQue Forecyt® software, clearly show the expression of pluripotency markers SSEA-4, TRA-1-60, Oct 3/4 and Sox 2 in iPSC cells (black) and the non-pluripotent marker, SSEA-1, only expressed in the THP-1 control cell line (yellow) (Figure 5A). The heatmap in Figure 5B illustrates this expression pattern in a plate view configuration, where black is high expression and yellow is low expression, exemplifying the flexibility of data presentation in the iQue Forecyt® software. Analysis of this data as a bar graph in Figure 5C further highlights the contrasting expression profiles of the two cell types. The ability to characterize a range of marker expression in cell lines, including iPSCs, via a flexible multiplexed workflow, exemplifies the power and utility of Sartorius platforms such as the iQue® Advanced Flow Cytometer.

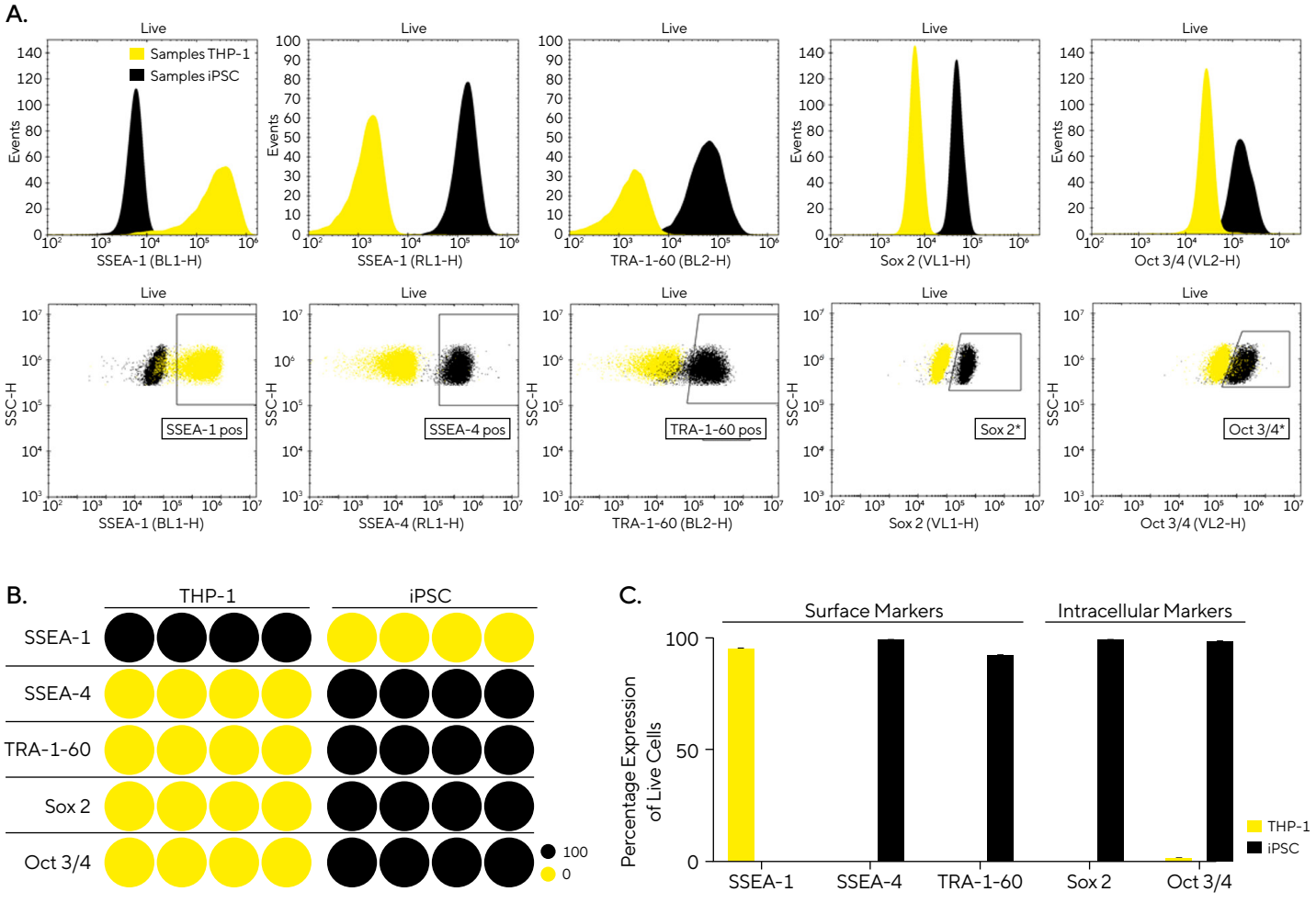


Figure 5. Surface and intracellular marker staining provides solutions for high throughput cellular characterization.
SSEA-1 was used as a marker of normal, non-pluripotent cells, while SSEA-4, TRA-1-60, Sox 2, and Oct 3/4 were all used to characterize pluripotent cells. (A) Histograms and dot plots created in the Forecyt software system for iQue®, showing the expression of various surface and intracellular markers in iPSC and control cells (n=4). (B) Heatmap from iQue Forecyt® illustrating the expression of the same markers, representing the plate map and expression profile per well. (C) Bar graph showing marker expression data in 3rd party software (± SEM, n=4).

Conclusions

iPSCs are increasingly used in many areas of research, requiring specific conditions for optimal growth, to maintain pluripotency, viability, and propagation potential. These requirements are often expensive and methods for monitoring iPSC status can be complex and time intensive, requiring multiple complicated techniques and solutions.

Using various Sartorius platforms throughout an iPSC culture workflow, we have shown how we can successfully pick and seed iPSCs, monitor their morphological status and characterize their pluripotency using the CellCelector Flex, the Incucyte® Live-Cell Analysis platform and the iQue® Advanced Flow Cytometer. The key advantages of using this combined workflow over conventional methods are:

1. Consistency and reliability, the CellCelector Flex can reproducibly pick specific iPSC colonies for further testing or culture, maintaining high levels of cell health
2. The ability to monitor delicate iPSC line culture morphology and growth characteristics without removing plates from the incubator
3. Minimal sample volumes required to characterize precious cell types, with minimal attrition for downstream requirements
4. Multiplexing experiments providing flexibility for the characterization of multiple metrics, such as surface and intracellular marker expression, using the same platform

The data presented here showcases the advantages of using a streamlined workflow combining multiple Sartorius systems for the culture, monitoring and characterization of iPSCs for several applications from drug development, disease modeling and clinical therapy research.

References

1. Takahashi K, Yamanaka S. (2022) **Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors.** *Cell.* 2006 Aug 25;126(4):663-76. doi: 10.1016/j.cell.2006.07.024. Epub 2006 Aug 10. PMID: 16904174.

North America
Phone +1 734 769 1600
Email: lps.opm.na@sartorius.com

Europe
Phone +44 1763 227400
Email:
orderhandling.lps.ne@sartorius.com

Japan
Phone +81 3 6478 5202
Email: sartoriusap@sartorius.com

China
Phone +86 21 6878 2300

Rest of Asia Pacific and other countries around the world:
Phone +65 6872 3966

Find out more: www.sartorius.com/incucyte

Find out more: www.sartorius.com/ique

For questions, email: AskAScientist@sartorius.com

Culture-Maintain-characterize-iPSCs-App-Note-2307-en-L-Sartorius Status: 07|2023