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Optimization of iPSC Culture Protocol Using High Quality Growth Factors and Cytokines

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Abstract

Cytokines and growth factors constitute a diverse group of proteins essential for intercellular communication. Two cytokines that are a key focus for *in vitro* cell culture are fibroblast growth factor 2 (FGF-2) and transforming growth factor β1 (TGF-β1). These cytokines are used in precise concentrations for maintaining induced pluripotent stem cells (iPSCs) in their pluripotent state. However, achieving the desired efficacy of these cytokines and growth factors in an *in vitro* setting has been challenging due to issues like thermostability, leading to a short half-life and necessitating frequent media changes, which are resource intensive.

In this application note, we present data on the utilization and optimization of Sartorius Research Use Only (RUO) Cytokines and Growth Factors for iPSC culture. Our goal is to sustain pluripotency and proliferative potential while reducing the need for daily media replenishment. We assess this by analyzing crucial cell surface markers, morphological indicators, and growth patterns using advanced tools like the iQue® Advanced Flow Cytometer and the Incucyte® Live-Cell Analysis System, showcasing the practicality of Sartorius RUO Growth Factors and Cytokines in iPSC culture maintenance.

Introduction

Cytokines are a large and diverse family of proteins secreted by cells for intercellular communication. By binding to extracellular receptors, they can induce an intracellular cascade of signaling to initiate a biological response. The induction and co-ordination of a range of processes are attributed to the correct signal intensity and timing of cytokine release, from embryonic development to growth and wound healing. One group of cytokines, classified as growth factors, are responsible for the growth of a specific tissue by stimulating proliferation and differentiation. The importance of these growth factors can be seen in diseases where their strict regulation is lost, such as rheumatoid arthritis, multiple sclerosis, and COVID-19.[1,2,3] There is often a specific growth factor that drives the growth and differentiation of a defined tissue, for example epidermal growth factor (EGF) enhances osteogenesis, whilst fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) stimulate angiogenesis.[4,5]

Since these growth factors are required for maintaining cell health *in vivo*, it is essential that cells cultured *in vitro* also have access to physiological levels of growth factors. Two cytokines that are a key focus for *in vitro* cell culture are fibroblast growth factor 2 (FGF-2) and transforming growth factor β1 (TGF-β1 PLUS). FGF-2 is mitogenic, playing a significant role in physiological processes such as embryonic development, tissue repair, and cell growth, as well as pathological processes such as tumor growth, perfusion, and invasion.[6] Similarly, TGF-β1 PLUS plays a key role in differentiation and oncogenesis and is associated with Alzheimer's disease, but is also secreted by leukocytes as a key component of coordinating the immune response.[7]

Induced pluripotent stem cells (iPSCs) are an *in vitro* cell model that require precise concentrations of these growth factors to maintain their pluripotent properties. iPSCs start as somatic cells that undergo a reprogramming process, using cytokines to alter their cell fate and enable the cell to gain pluripotent properties.[8] Through further exposure to growth factors that mimic the *in vivo* differentiation process, theoretically these iPSCs can then differentiate into any somatic cell type. iPSCs are valuable for studying the differentiation process as they do not face the same ethical concerns of protocols using embryonic stem cells. The pluripotent qualities of a cell can be determined by investigating cell surface markers, such as stage-specific embryonic antigen (SSEA)-1, SSEA-4, TRA-1-60, and intracellular markers such as SOX2 and OCT-4.[9]

Despite their importance in cell culture maintenance, translating the efficacy of growth factors into an *in vitro* environment has been challenging. Sensitive cell types, such as iPSCs, require highly pure growth factors and cytokines as even trace amounts of endotoxins and other impurities can impact their genetic stability and differentiation potential. While recombinant growth factors and cytokines are free of contaminating animal proteins, thermostability can be an issue, resulting in a short half-life. This means growth factors in the cell culture media degrade faster, necessitating regular media changing; both a time and resource-intensive process.[10] Furthermore, many growth factors become compromised after three freeze-thaw cycles, complicating their use in culture.[11]

Sartorius Research Use Only (RUO) Growth Factors and Cytokines are produced using recombinant human DNA technology and do not contain any animal-derived contaminants. They are also manufactured to the highest quality standards to ensure purity and low endotoxicity. In this application note, we demonstrate optimization of culture conditions to maintain a pluripotent phenotype and the proliferative capability of iPSCs using Sartorius RUO Growth Factors and Cytokines, including thermostable varieties. We also demonstrate the utility of the iQue® Advanced Flow Cytometry Platform and Incucyte® Live-Cell Analysis System for analyzing cell surface markers, cell growth, and cell morphology.

Methods and Materials

Cell Culture

iPSCs were passaged using Accutase® to lift cells twice per week in vitronectin (10 µg/mL) coated 6-well plates at a density of 100 K/well. NutriStem® hPSC XF (Cat. No. 05-100-1A) pre-conditioned iPSCs (ATCC 1019) were cultured in NutriStem® hPSC XF media, whereas iPSC 1019 cells were cultured in commercial media. After passaging, cells were reseeded in media containing 10 µM ROCK inhibitor (Y-27632), which was removed after 24 hours.

Growth Factors for Standard iPSC Culture

To optimize the concentration of RUO Recombinant Human TGF-β1 PLUS Protein (Cat. No. CYK-0050-0010) and Recombinant Human FGF-2 Protein 154 aa (Cat. No. CYK-0100-0023) to be added to the growth factor-free media, several concentrations of each growth factor were tested. iPSCs were seeded in vitronectin (10 µg/mL) coated 24-well plates (100 K/well). Pluripotency was determined at 3 and 6 days post-seeding. A concentration range of FGF-2 was tested (3.13 – 200 ng/mL) in combination with 2 ng/mL TGF-β1 PLUS in all conditions. Similarly, a concentration range of TGF-β1 PLUS was tested (0.13 – 8 ng/mL) with 100 ng/mL FGF-2 in all conditions. NutriStem® hPSC XF GF-free (Cat. No. 06-5100-01-1A), commercial media, and RPMI 1640 were used as control media formulations. THP-1 cells were used as a non-pluripotent biological control. The cells were analyzed for marker expression using the iQue® Advanced Flow Cytometry Platform.

Generating Concentration Response Curves

iPSCs were seeded at 20 K/well in vitronectin coated 24-well plates and monitored over 6 days in the Incucyte® Live-Cell Analysis System. The cells were fed daily with 500 µL media containing a concentration range of TGF-β1 PLUS and FGF-2. NutriStem® hPSC XF GF-free, NutriStem® hPSC XF, commercial media, and RPMI 1640 were used as controls. The cells were then lifted using Accutase® and reseeded in a 96-well V-bottom plate, centrifuged at 300 g for 5 minutes and washed twice with PBS + 5% FBS before being treated with SSEA-1-FITC (1:50), SSEA-4-APC (1:800), TRA-1-60-PE (1:25), and iQue® B/Red Viability Dye (1:50) and incubated at room temperature in the dark for 30 minutes. The plate was then spun at 300 g for 5 minutes before analyzing on the iQue® platform.

FGF-2-G3 Long Term Comparison

Recombinant FGF2-G3 protein (Cat. No. CYK-0100-0002) is an enhanced, thermostable version of FGF-2 designed to enable longer duration between feeds. Cells were seeded at a density of 20 K/well in vitronectin (10 µg/mL) coated 24-well plates. These cells were treated with a concentration range of FGF2-G3 in combination with 2 ng/mL of TGF-β1 PLUS, without subsequent refeeds. NutriStem® hPSC XF GF-free, stabilized commercial media (enabling longer duration between feeds), and RPMI 1640 were used as control media formulations. THP-1 cells were used as a non-pluripotent biological control. After 6 days, the cells were analyzed on the iQue® platform. Cells were monitored using the Incucyte® Live-Cell Analysis System for the duration of the experiment to investigate growth dynamics and morphological changes.

Immunocytochemistry (ICC)

iPSCs were seeded at 2 K/well in a 96-well plate in ROCK inhibitor (1:1000). After 24 hours, ROCK inhibitor was removed and 100 µL NutriStem® XF was added to each well. After a further 24 hours, a concentration range of TGF-β1 PLUS was added alongside controls of NutriStem® hPSC XF, NutriStem® hPSC XF GF-free, commercial media, commercial media plus, and RPMI 1640. After 5 days, cells were washed, fixed, permeabilized, and blocked. Cells were then immunostained for markers of pluripotency (SSEA-4 Alexa Fluor™ 647, OCT-4 Alexa Fluor™ 555, and SOX2 Alexa Fluor™ 488; ThermoFisher A24881) and imaged in PBS. Phase and fluorescence images were acquired using the Incucyte® system and analyzed using integrated software.

Results

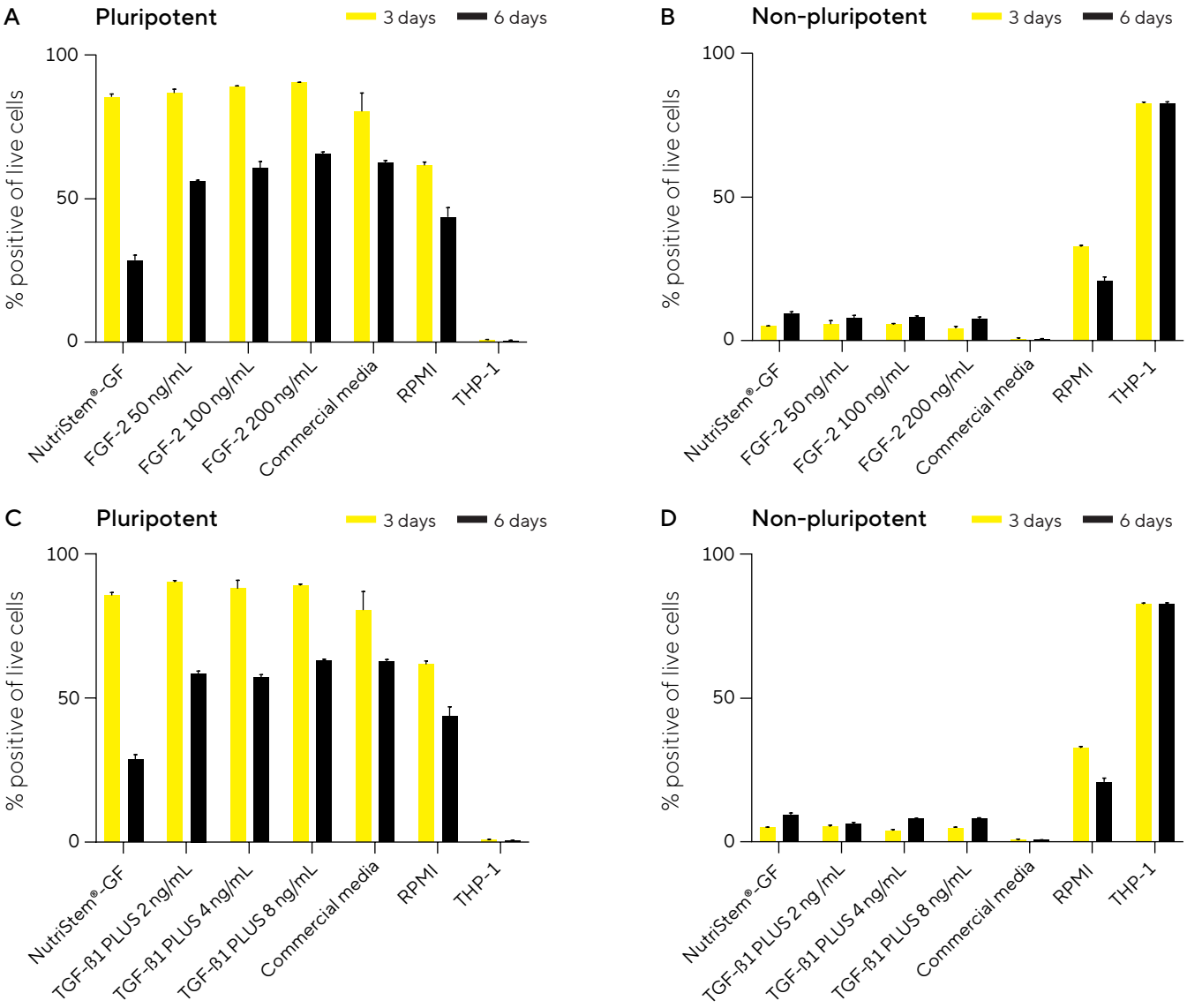
Ensuring iPSCs are cultured optimally is essential for successfully maintaining the pluripotency, health, and longevity of these highly valuable cells. The advantage of using iPSCs in research is in their differentiation capability, where disparate tissue types can be derived from a single cell. Due to their complex nature, the requirement for specific culture conditions necessitates the use of a variety of supplements in culture media to grow highly pluripotent iPSCs.

The following data highlights the efficacy of Sartorius RUO Growth Factors and Cytokines as supplements in media for the successful culture of iPSCs.

FGF-2 and TGF-β1 PLUS Supplementation Maintains Pluripotency in iPSCs

We grew iPSCs in NutriStem® hPSC XF GF-free with or without supplementation with FGF-2 and TGF-β1 PLUS over 3 and 6 days with daily feeds. Using the iQue® Advanced Flow Cytometer, we analyzed changes in cell surface pluripotency marker expression associated with optimized growth conditions. The capabilities of the iQue® Advanced Flow Cytometer allow for multiple markers to be analyzed in the same sample well, greatly increasing the speed of data collection and saving precious sample. Comparisons of pluripotency (SSEA-1 –, SSEA-4 +, TRA-1-60 +) and non-pluripotency (SSEA-1 +) marker expression profiles (Figure 1) highlight a decrease in pluripotency marker expression when iPSCs are cultured in NutriStem® XF GF-free for 6 days.

Figure 1: *Quantification of iPSC Marker Expression During Stem Cell Maintenance.*



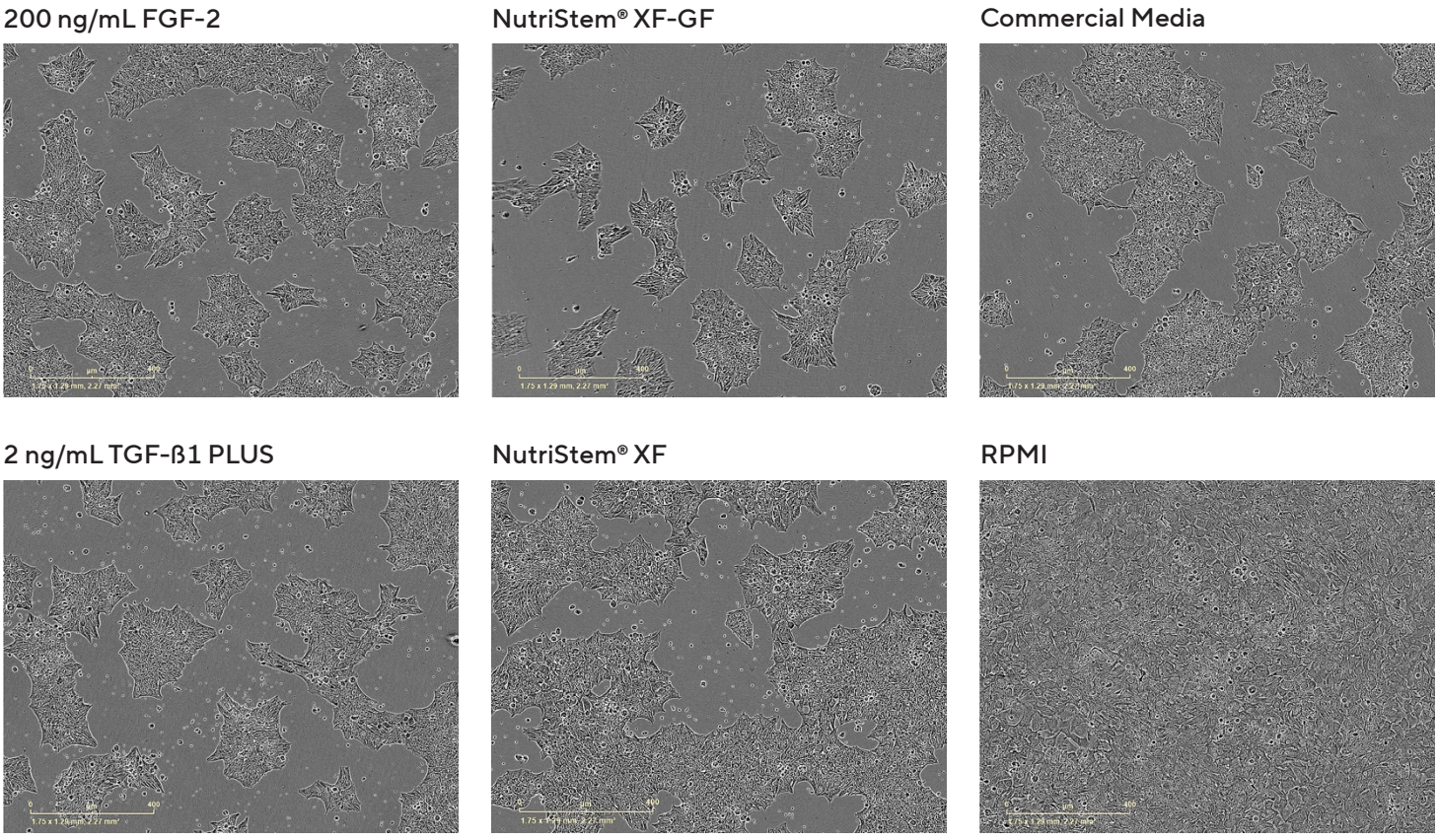
Note: iPSCs were cultured in NutriStem® hPSC XF GF-free (NutriStem®-GF) supplemented with concentration ranges of Sartorius RUO FGF-2 and TGF-β1 PLUS. Cells were harvested after 3 and 6 days and analyzed on the iQue® Advanced Flow Cytometer. (A) Pluripotent (SSEA-1 -, SSEA-4 +, TRA-1-60 +) and (B) Non-pluripotent (SSEA-1 +) expression profile analysis of iPSCs supplemented with FGF-2. (C) Pluripotent and (D) Non-pluripotent expression profile analysis of iPSCs supplemented with TGF-β1 PLUS. Data presented as mean ± SEM, n = 3.

In previous experiments, iPSCs cultured in RPMI presented a loss in pluripotency marker expression and a gain in SSEA-1 expression, so this condition was used as a non-optimized control. High expression of SSEA-1 combined with low levels of pluripotency marker expression were seen in non-pluripotent iPSC controls; grown in RPMI, or the THP-1 non-pluripotent immortalized cell line. Supplementation with FGF-2 (50 - 200 ng/mL) showed a marked, concentration-dependent increase in pluripotency marker expression (Figure 1A), while SSEA-1 expression remained constant (Figure 1B). We observed a similar trend when supplementing with TGF-β1 PLUS; pluripotency marker expression maintained at 6 days in cells supplemented with concentrations as low as 2 ng/mL (Figure 1C). SSEA-1 expression also remains low throughout the study timeline (Figure 1D).

iPSCs Supplemented with FGF-2 and TGF-β1 PLUS Display Pluripotent Morphological Phenotypes

In addition to phenotypic marker analysis using the iQue® Advanced Flow Cytometer, it is also important to assess the morphological characteristics of iPSCs to provide more insight into the condition and growth of cultures. Using the Incucyte® Live-Cell Analysis System, we were able to compare different media for their impact on the growth and morphology of the live iPSCs directly from the incubator, without perturbing the cells (Figure 2). High-definition phase contrast images of iPSCs grown in NutriStem® XF-GF show low confluency in the plate, with visible spontaneous differentiation. Following addition of FGF-2 or TGF-β1 PLUS, iPSC morphology changed, with higher confluency, increased colony density, and no spontaneous differentiation. The morphology was comparable to cells grown in NutriStem® XF. The RPMI control illustrated that the morphological presentation associated with a loss of pluripotency; the colony density and compactness was reduced, and the cellular cytoplasmic area was increased.

Figure 2: *Morphological Analysis of iPSCs During Stem Cell Maintenance.*



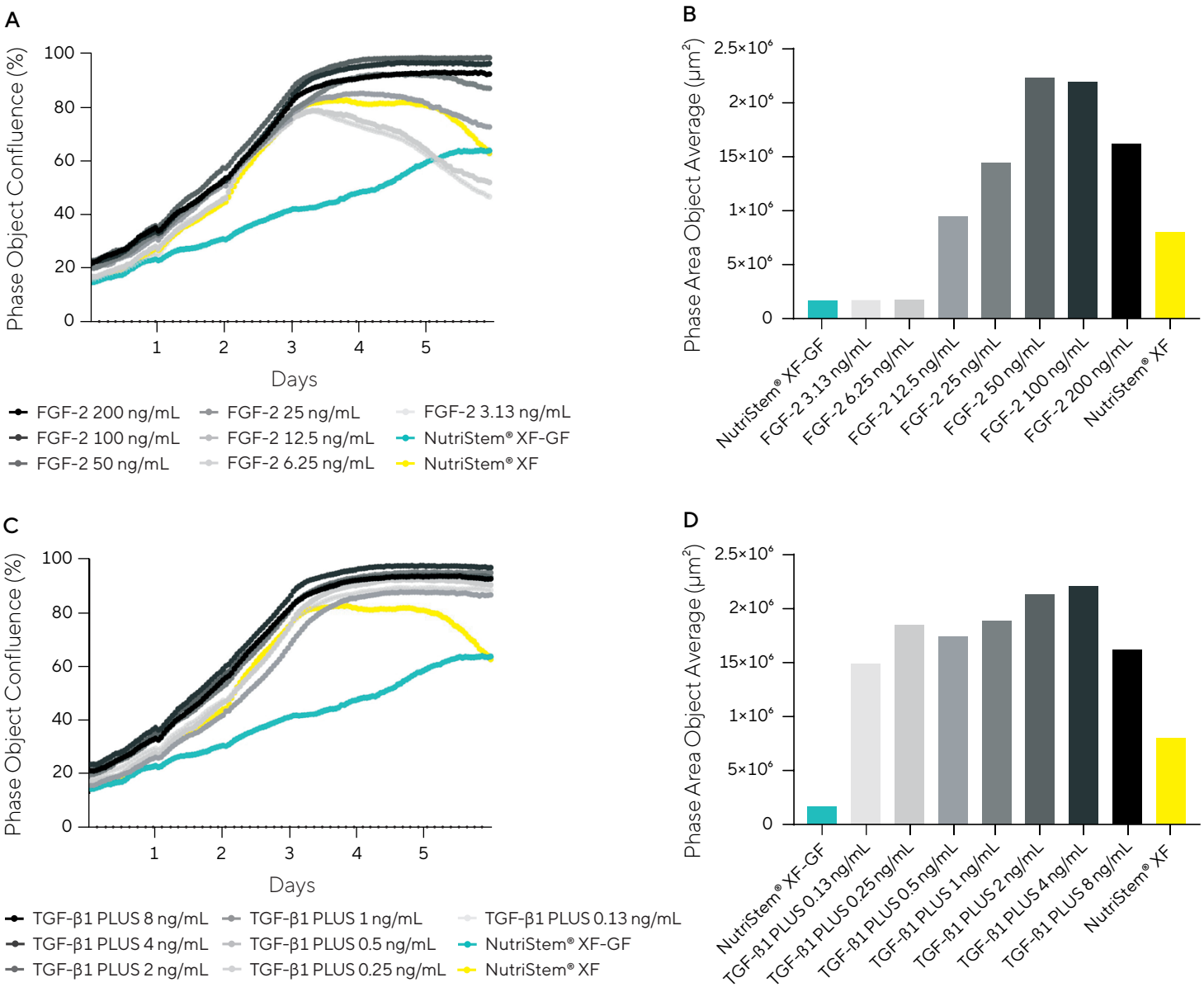
Note: iPSCs were cultured in NutriStem® XF-GF supplemented with a range of concentrations of Sartorius RUO Recombinant Human FGF-2 and TGF-β1 PLUS. Cells were monitored over 6 days and images acquired using the Incucyte® Live-Cell Analysis System. Representative 10X phase contrast images taken at 2 days and 10 hours post-treatment.

Growth Rate and Confluency of iPSCs Supplemented With FGF-2 and TGF-β1 PLUS is Concentration-Dependent

The acquisition of kinetic data on the Incucyte® Live-Cell Analysis System enabled easy quantification of iPSC growth via confluency and phase object area analysis (Figure 3). Here we showed the difference in iPSC growth when cultured with varying concentrations of FGF-2 and TGF-β1 PLUS (Figure 3). The data shows that in higher concentrations of FGF-2 (50 – 200 ng/mL), iPSC growth is similar with a plateau at 3 days, which remains stable (Figure 3A). In contrast, data for lower FGF-2 concentrations (3.13 – 25 ng/mL), show a dramatic drop in confluency post three days, indicating a lack of growth and potential cell death. Analysis of average phase object area (size of iPSC colonies) over time provided more granular indication of iPSC growth patterns.

For example, lower concentrations of FGF-2 (3.13–25 ng/mL) produced iPSCs that form smaller colonies after 5 days, compared to higher concentrations of supplemented FGF-2 (25–200 ng/mL) (Figure 3B). TGF-β1 PLUS supplementation at a range of concentrations (Figure 3C), had minimal impact on the growth of iPSCs, indicating this protein is not as critical as FGF-2 for iPSC growth at this concentration range. Analysis of average phase object area, however, revealed that with decreasing concentrations of TGF-β1 PLUS, iPSCs form smaller colonies over time. Although growth is minimally affected, colony size varies depending on TGF-β1 PLUS concentration (Figure 3D). Based on this data, we recommend the concentrations of 100 ng/mL FGF-2 and 2 ng/mL TGF-β1 PLUS for optimal growth and maintenance of iPSCs.

Figure 3: *Growth Analysis of iPSCs During Stem Cell Maintenance.*



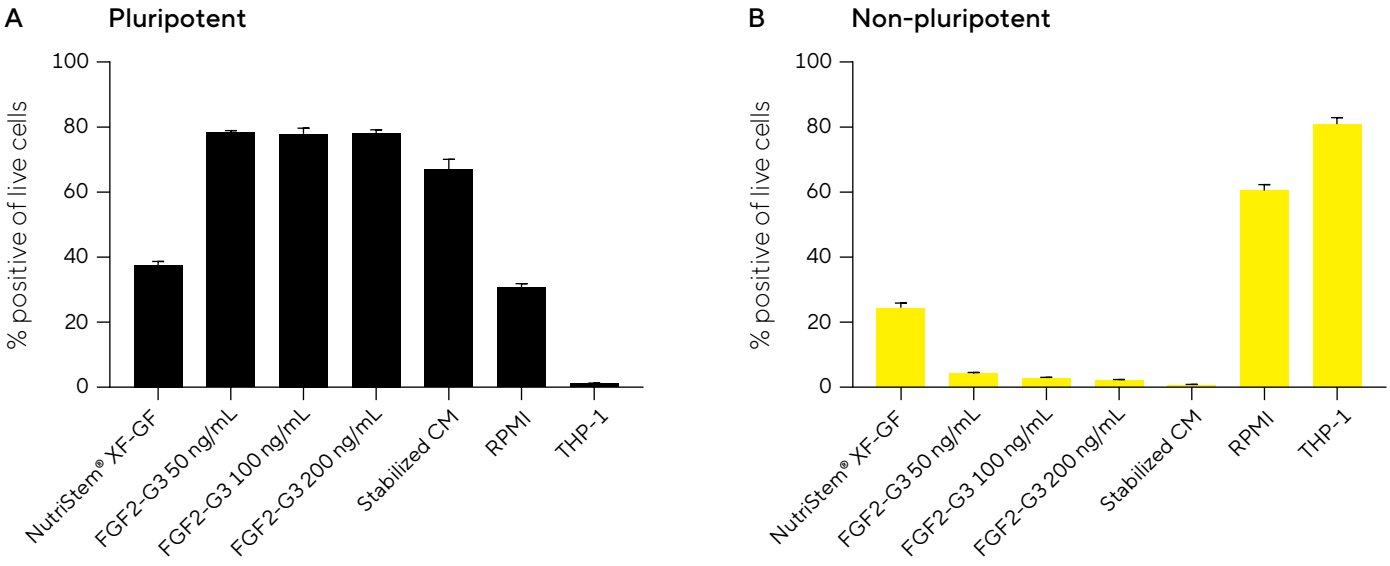
Note: iPSCs were cultured in NutriStem® XF-GF supplemented with a range of concentrations of Sartorius RUO Recombinant Human FGF-2 and TGF-β1 PLUS. Cells were monitored over 6 days and analyzed using the Incucyte® Live-Cell Analysis System. Confluency analysis of iPSCs supplemented with (A) FGF-2 at decreasing concentrations and (C) TGF-β1 PLUS at decreasing concentrations. Phase Area Object Average analysis at 5 days for (B) FGF-2 and (D) TGF-β1 PLUS.

Use of Thermostable FGF2-G3 Facilitates iPSC Culture Without Daily Feeding

iPSCs are high maintenance and require daily media refreshing to maintain a pluripotent phenotype. Thermostable growth factors enable reduced media change frequency due to increased compound stability, which eliminates the need for inconvenient weekend media changes. To test this, we grew iPSCs in NutriStem® hPSC XF GF-free with supplementation (2 ng/mL TGF-β1 PLUS, varying concentrations of FGF2-G3) for 5 days without media changes and analyzed marker expression on the iQue® platform at day 6. The Sartorius RUO FGF2-G3 is an enhanced, thermostable version of FGF-2 designed to enable longer duration between feeds.

Pluripotency marker expression was higher in all cells treated with FGF2-G3 compared to all other treatment types, with a consistent level across the concentration range (~70%) outperforming an alternative commercial media (Figure 4A). Comparisons of SSEA-1 expression show very low levels in iPSCs cultured with FGF2-G3 from 50–200 ng/mL, lower than NutriStem® hPSC XF GF-free and NutriStem hPSC XF (Figure 4B). Control cells grown in RPMI, or THP-1 cell controls, presented high expression of SSEA-1 and low levels of pluripotency marker expression as seen previously. This data indicates that thermostable Sartorius RUO FGF2-G3 maintains function in culture for longer periods, mitigating the need for daily media changes.

Figure 4: *Long Term iPSC Culture Without Media Change.*



Note: iPSCs were cultured in NutriStem® XF-GF supplemented with thermostable FGF2-G3 at a range of concentrations for 5 days without media changes and pluripotency markers were analyzed on the iQue® Advanced Flow Cytometer on day 6. (A) Pluripotent (SSEA-1, SSEA-4 +, TRA-1-60 +) and (B) Non-pluripotent (SSEA-1 +) marker expression profile. Stabilized CM (Stabilized Commercial Media enabling extended duration between feeds). Representative data from one of 3 experiments. Data presented as mean ± SEM, n = 3.

Morphological Analysis of iPSCs Supplemented With FGF2-G3

We monitored the iPSCs supplemented with thermostable FGF2-G3 on the Incucyte® Live-Cell Analysis System over the 5-day study to investigate morphological effects. Representative images of cells cultured in FGF2-G3 PLUS supplemented NutriStem® hPSC XF GF-free showed formation of very tightly condensed colonies with defined edges and minimal cell death (Figure 5A). While iPSCs grown in NutriStem® hPSC XF GF-free without supplementation presented reduced growth with more cell death (Figure 5B). Cells grown in commercial media produced very tight colonies with defined edges, but with some spontaneous differentiation (data not shown).

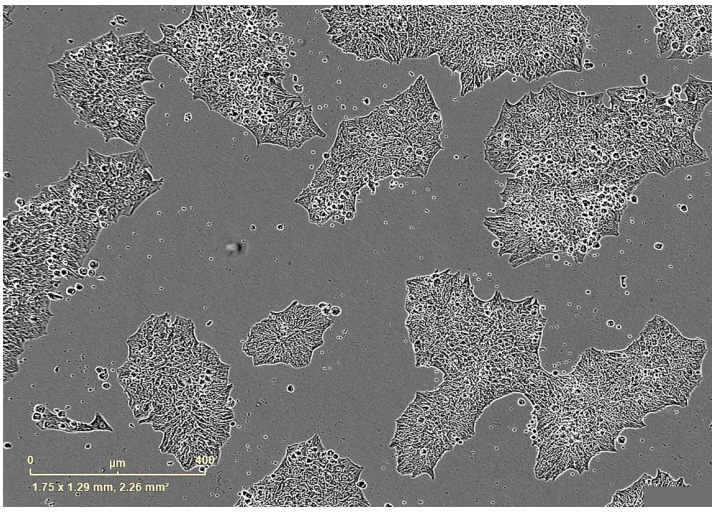
Analysis of the Incucyte® images for iPSCs grown in FGF2-G3 supplemented media for 5 days without daily media refeeds highlighted changes in growth compared to alternative media types.

For example, iPSCs grown in NutriStem® hPSC XF GF-free supplemented with FGF2-G3 exhibited increased growth over NutriStem® hPSC XF and NutriStem® hPSC XF GF-free cultured iPSCs (Figure 5C). NutriStem® hPSC XF outperformed NutriStem® hPSC XF GF-free, but the differences were less marked at the end of the time course, suggesting degradation of key growth factors, such as FGF-2. Culturing iPSCs in alternative commercial media designed for weekend feeding provided similar growth to FGF2-G3 supplementation, however, these cells plateaued sooner, suggesting exhaustion of growth factors required for continued growth (data not shown).

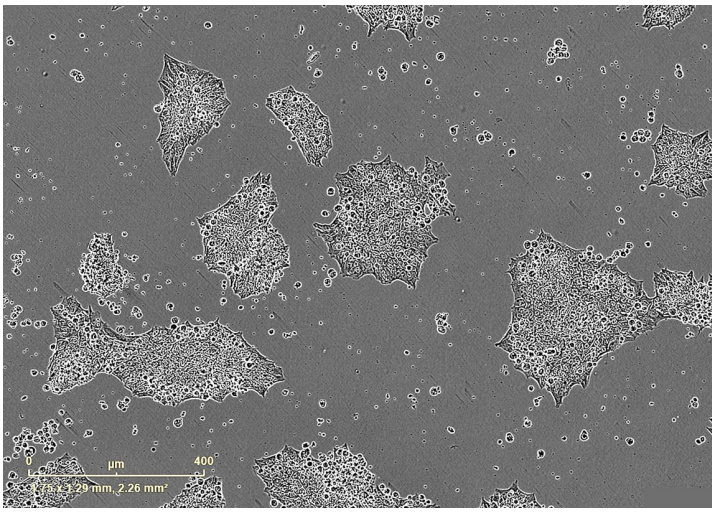
Interestingly, lower concentrations of FGF2-G3 supplementation (50 and 100 ng/mL) achieved the same effect as the highest concentration (200 ng/mL) in analyses on both the iQue® and Incucyte® platforms, suggesting that the potency of this growth factor allows for lower dosing, thus saving reagent.

Figure 5: *Morphological Visualization and Long-Term Growth Analysis of iPSCs Supplemented With FGF2-G3 Without Media Changes.*

A 200 ng/mL FGF2-G3



B NutriStem® XF-GF



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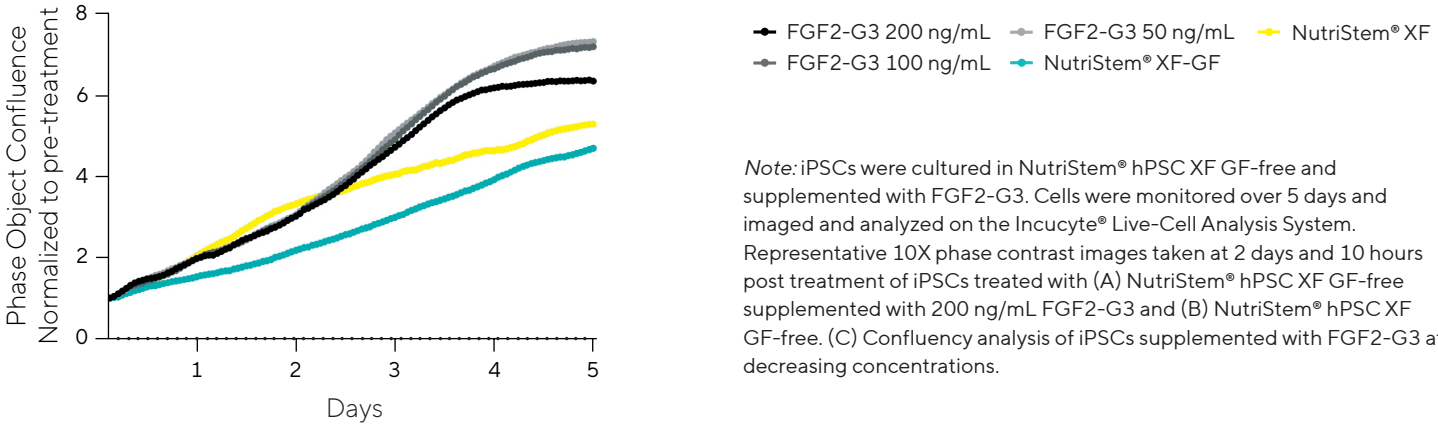


Note: iPSCs were cultured in NutriStem® hPSC XF GF-free and supplemented with FGF2-G3. Cells were monitored over 5 days and imaged and analyzed on the Incucyte® Live-Cell Analysis System. Representative 10X phase contrast images taken at 2 days and 10 hours post treatment of iPSCs treated with (A) NutriStem® hPSC XF GF-free supplemented with 200 ng/mL FGF2-G3 and (B) NutriStem® hPSC XF GF-free. (C) Confluency analysis of iPSCs supplemented with FGF2-G3 at decreasing concentrations.

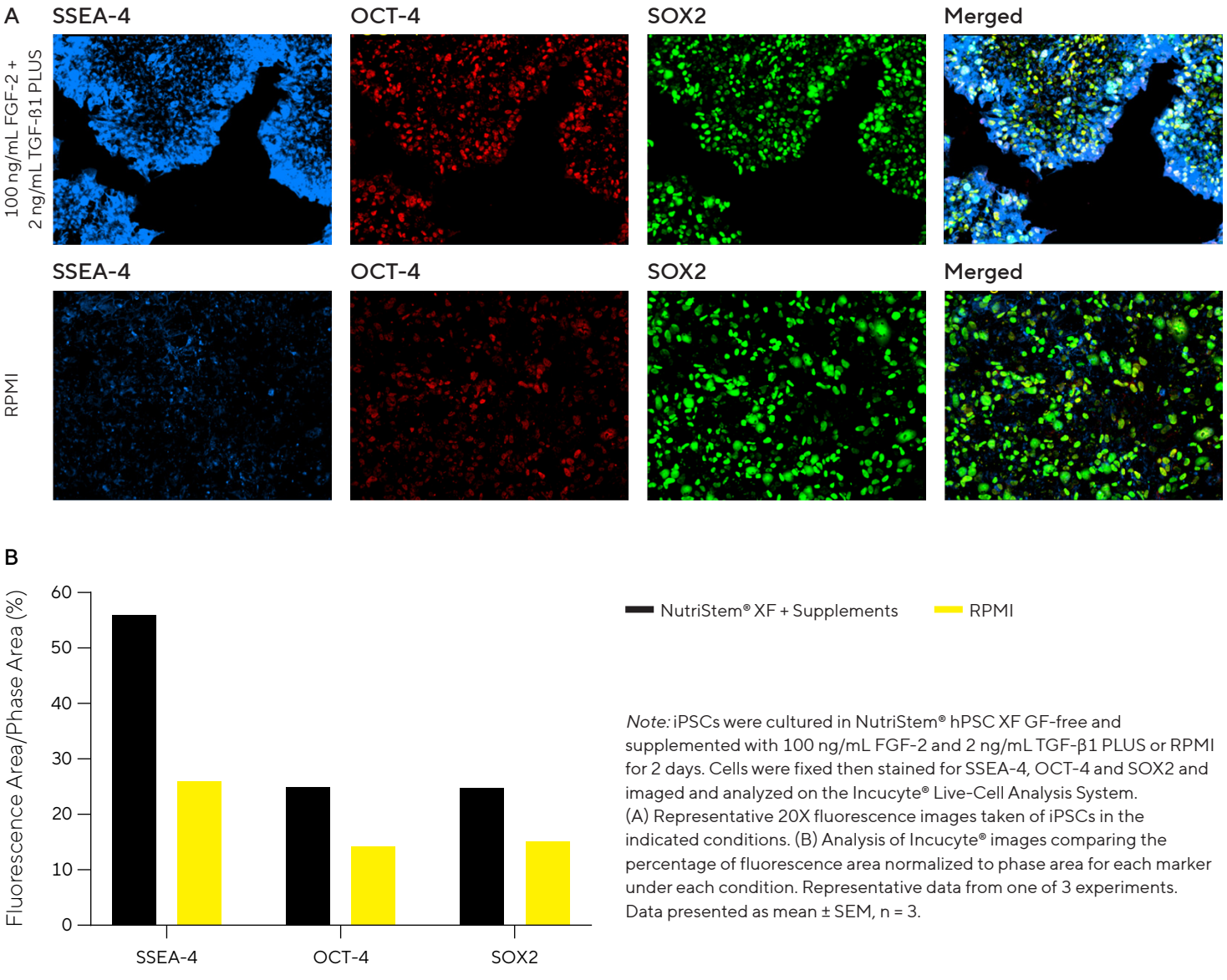
Determining Pluripotency Marker Expression in iPSCs Using Immunocytochemistry

To gain further insight about the efficacy of Sartorius RUO Growth Factors and Cytokines, we cultured iPSCs in RPMI and NutriStem® hPSC XF GF-free supplemented with 100 ng/mL FGF-2 and 2 ng/mL TGF-β1 PLUS followed by staining to observe expression of key markers for pluripotency in different culture conditions. Our data showed that when supplemented with Sartorius RUO Growth Factors and Cytokines, iPSCs expressed high levels of pluripotency markers: the cell surface marker SSEA-4, and sub-cellular markers SOX2 and OCT-4. However, cells grown in RPMI displayed much lower levels of expression, as expected (Figure 6A). The Incucyte® Live-Cell Analysis System facilitated our investigation of the marker expression pattern in iPSCs grown under different conditions.

SSEA-4 expression, for example, highlights the differences in distribution in pluripotent iPSCs grown with FGF-2 and TGF-β1 PLUS compared to RPMI. Low levels of expression were evident in RPMI cultured iPSCs, while a high level of expression throughout the surface of the cell was clearly visible in growth factor supplemented iPSCs.

Quantification of fluorescence area normalized to phase area to account for differences in cell area revealed global differences in marker expression across each condition (Figure 6B). SSEA-4 expression was approximately 30% higher in supplemented iPSCs compared to RPMI, while OCT-4 and SOX2 expression was approximately 10% higher in supplemented iPSCs. These data support previous analysis of marker expression on the iQue® Advanced Flow Cytometer platform, indicating an increased level of pluripotency in iPSCs supplemented with Sartorius RUO Growth Factors and Cytokines compared to other media conditions.

Figure 6: *Immunocytochemistry Analysis of Pluripotency Marker Expression in iPSCs Supplemented With FGF-2 and TGF-β1 PLUS.*



Note: iPSCs were cultured in NutriStem® hPSC XF GF-free and supplemented with 100 ng/mL FGF-2 and 2 ng/mL TGF-β1 PLUS or RPMI for 2 days. Cells were fixed then stained for SSEA-4, OCT-4 and SOX2 and imaged and analyzed on the Incucyte® Live-Cell Analysis System. (A) Representative 20X fluorescence images taken of iPSCs in the indicated conditions. (B) Analysis of Incucyte® images comparing the percentage of fluorescence area normalized to phase area for each marker under each condition. Representative data from one of 3 experiments. Data presented as mean ± SEM, n = 3.

Conclusion

iPSCs are rapidly becoming the cell type of choice for a multitude of research and clinical fields; they are highly proliferative and can be differentiated into all somatic tissues in the human body. However, the requirements for successful culture of these cells are both resource and time-intensive. It is also of paramount importance to maintain pluripotency during culture and finding the correct media formulation is essential to achieving this goal. The data shown here demonstrate the efficacy of Sartorius RUO Growth Factors and Cytokines as supplements in iPSC media formulations to preserve pluripotency, support growth, and increase the interval between feeding, helping to remove the requirement of daily media exchanges. Sartorius RUO Growth Factors and Cytokines are free from animal-derived contaminants, are manufactured to the highest quality standards ensuring purity and low endotoxicity, providing excellent solutions to challenging cell culture conditions. In addition, using the iQue® Advanced Flow Cytometer Platform in conjunction with the Incucyte® Live-Cell Analysis System, allows simple monitoring and quantification of growth and pluripotent phenotype of iPSC cells during cell culture and media formulation development.

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