Application Note 001

Expanding Human Induced Pluripotent Stem Cells (hiPSCs) in a Benchtop Cell Culture Automation System (*Emmet*).

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Abstract

Induced pluripotent stem cells (iPSCs) have many desirable characteristics, including self-renewal and the ability to differentiate into multiple cell types. This makes them ideal starting materials in drug discovery, toxicology testing, and regenerative medicines. However, current manual iPSC culture workflows introduce process variability, technical difficulties and unit economics not viable for mass market manufacture, all of which are barriers to scaling the production of iPSCs and their differentiated progeny. Unicorn Bio has developed a benchtop instrument (*Emmet*) to automate and standardize iPSC culture to solve these challenges.

Introduction

The Challenges of iPSC Culture

Culturing pluripotent stem cells, whether embryonic (ES) or induced (iPSC), is technically challenging, requiring highly trained scientists to perform repetitive and manual workflows. This introduces inherent process variability - from the angle at which an operator holds a pipette to determining confluence - and multiple points for contamination and culture failure. Operator variability is compounded by stem cells being in a dynamically unstable cell state, frequently suffering from loss of pluripotency, spontaneous differentiation, and cell death.

Manual iPSC workflows require tedious media exchanges and (more often than not) weekend working rotas, making them prohibitively expensive to scale for mass market production. More fundamentally, talented individuals should be designing and analyzing experiments to solve interesting problems, not moving liquids between plastic containers at 8 pm on a Saturday.

Automation and industrialization of iPSC workflows is the clear solution. However, in the almost two decades since iPSCs were discovered, none of the automation platforms repurposed from adjacent applications have been widely adopted by the stem cell community

Meet *Emmet*: a Benchtop System to Automate Your iPSC Culture

Emmet is a benchtop instrument that standardizes and automates iPSC culture in TC flasks: from coating flasks and seeding cells to performing media exchanges, passaging, differentiating, and harvesting cells in a comparable way to gold standard manual TC techniques.

This application note provides an overview and analysis of hiPSCs expanded using *Emmet*.

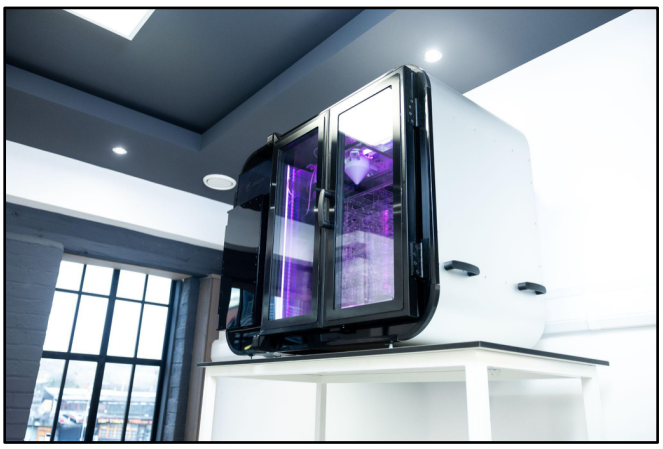

Image 1: *Emmet* in a Unicorn Bio tissue culture (TC) lab.

Methods

Culture of hiPSCs on *Emmet*

hiPSCs were passaged using accutase onto cultrex coated T25, T75, and T225 tissue culture flasks. A single T25 on *Emmet* was seeded with 200,000 cells/cm^2 in 5 ml mTeSR™ Plus (Stemcell Technologies). Media exchanges were performed daily. On day four, cells were passaged from one T25 flask into two T75 flasks (1:6 passage ratio). Daily media exchanges of 15 ml were performed. On day eight, cells were passaged from one T75 flasks into three T225 flasks (1:9 passage ratio). Daily media exchanges of 45 ml were performed. Cells were harvested from the three confluent T225 flasks on day eleven. All cell cultures on *Emmet* were mirrored with a manual control.

Cell Counts and Confluence Analysis of hiPSCs

Cell counts were taken (NucleoCounter® NC-200™) from both the manual control and *Emmet* cultured flasks at the first seed (day 0), first passage (day four), second passage (day eight), and harvest (day eleven).

Images of the hiPSCs were taken daily at pre-defined points on each TC flask: 18 images per T25 & T75 and 30 images per T225 flask. Images were analyzed using Ilastik and Image J to quantify the confluence at each pre-defined point in each flask. This data was run through R Studio to estimate the overall confluence of each flask.

Flow Cytometry Analysis for Pluripotency

After harvesting from both *Emmet* and the manual control, hiPSCs were assessed for the expression of pluripotency markers using flow cytometry (Cytek®, Aurora). hiPSCs were stained using a viability dye, fixed using paraformaldehyde, and incubated with antibodies for SSEA-4, TRA-1-60, OCT4, and SOX2. The cells were suspended in PBS with 2% FBS.

Embryoid body (EB) Formation and qPCR Gene Expression Analysis of Germ Layer Markers

After hiPSCs had been harvested from *Emmet* or manual culture, they were seeded at a density of 10,000 cells per well in a 96-well plate, in 100µl of differentiation media containing rock inhibitor. The plates were spun, and embroid bodies (EBs) were grown over 15 days. The EBs were harvested, and RNA extraction was performed using the NEB Monarch Total RNA miniprep kit. Using the Applied Biosystems high-capacity cDNA reverse transcription kit, 2µg cDNA was made from 2µg RNA. 25ng cDNA was used per well of a 96-well PCR plate. The following primers were used: OCT4 (pluripotency), T (mesoderm), NEUROD1 & PAX6 (ectoderm), FOXA2 & SOX17 (endoderm). qPCR was performed using the Quantstudio 1 real-time PCR system. Delta-delta CT analysis was performed.

Results

Developing a machine to standardize and automate iPSC culture is essential to removing technical and economic bottlenecks stymying the field. Using fluidic and mechanical systems designed to mimic how scientists culture cells, *Emmet* expanded iPSCs as well as a trained stem cell scientist while reducing operator time by 82.6% across the eleven-day hiPSC expansion workflow. The following data highlights the successful culture of hiPSCs and their ability to retain pluripotency and differentiation capacity after expansion in *Emmet*.

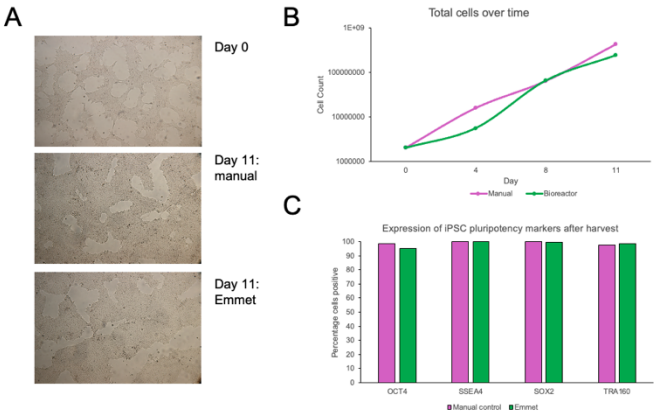

Figure 1: hiPSC expansion in *Emmet* and manual control. A) Brightfield microscopy images of hiPSCs. B) Growth curves of hiPSCs grown on *Emmet* and in manual culture. C) Flow cytometry analysis of pluripotency markers (OCT4, SSEA4, SOX2, TRA-1-60).

Expanded hiPSCs Maintained Pluripotency

hiPSCs expanded on Emmet retained normal colony morphology (figure 1A), and the number of hiPSCs produced by *Emmet* was consistent with the manual control throughout (figure 1B).

hiPSCs expanded both in *Emmet* and in the manual control had similar expression levels of pluripotency markers OCT4, SOX2, SSEA4, and TRA-1-60 (figure1C). This indicates that both expanded hiPSC populations retained their pluripotency.

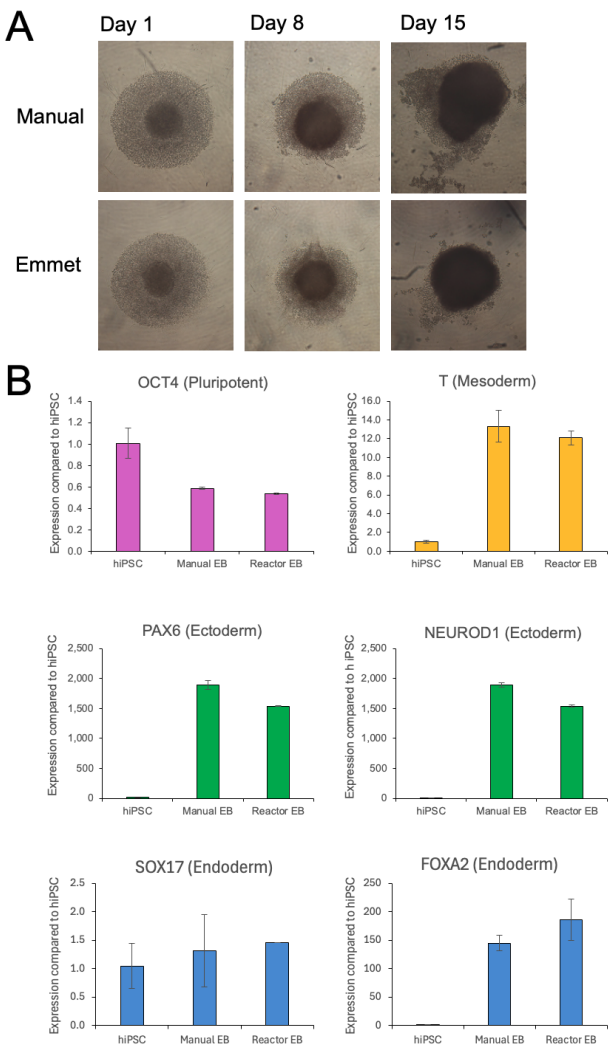

Figure 2: Functional analysis of hiPSC differentiation capacity. A)Images of embryoid bodies formed from hiPSCs cultured manually and in *Emmet*. B) Gene expression analysis of the pluripotency (OCT4), mesoderm (T), ectoderm (NEUROD1, PAX6), and endoderm (FOXA2, SOX17) lineage markers.

Expanded hiPSCs Maintained Tri-Lineage Differentiation Capacity

hiPSCs cultured on *Emmet* and the manual control were both successfully differentiated into EBs after 15 days (figure 2A). PCR with cDNA from both EBs was performed, indicating a reduced level of pluripotency markers (OCT4) compared to pluripotent hiPSCs.

Both EB's were positive for markers from all three germ layers: mesoderm (T), ectoderm (NEUROD1, PAX6), and endoderm (FOXA2, SOX17) (figure 2B). These results indicate that both hiPSCs expanded on *Emmet* and the manual control maintained tri-lineage differentiation capacity.

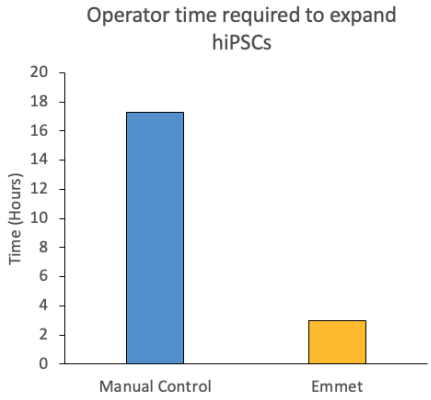

Figure 3: Operator time to complete hiPSC expansion. Total time to complete hiPSC expansion workflow on *Emmet* and the manual control.

Emmet Reduces Operator Time Required to Expand hiPSCs

Logs were kept of operator time spent on manual and *Emmet* hiPSC expansion protocols, which were both performed by the same operator.

The manual hiPSC expansion required 17.25 hours of time, compared to 3 hours of time for the *Emmet* hiPSC expansion, a reduction in total time needed of 82.6% (figure 3).

Discussion

Emmet expanded hiPSCs had similar morphology and expression of pluripotency markers as hiPSCs expanded via manual cell culture while requiring appx. 80% less time to culture than using manual culture methods. hiPSCs cultured on *Emmet* maintain their ability to differentiate into embryoid bodies and are positive for all three germ layer markers. *Emmet* is suitable for expanding hiPSCs in TC flask-based workflows and de-coupling hiPSC culture from manual labor.