Application Note

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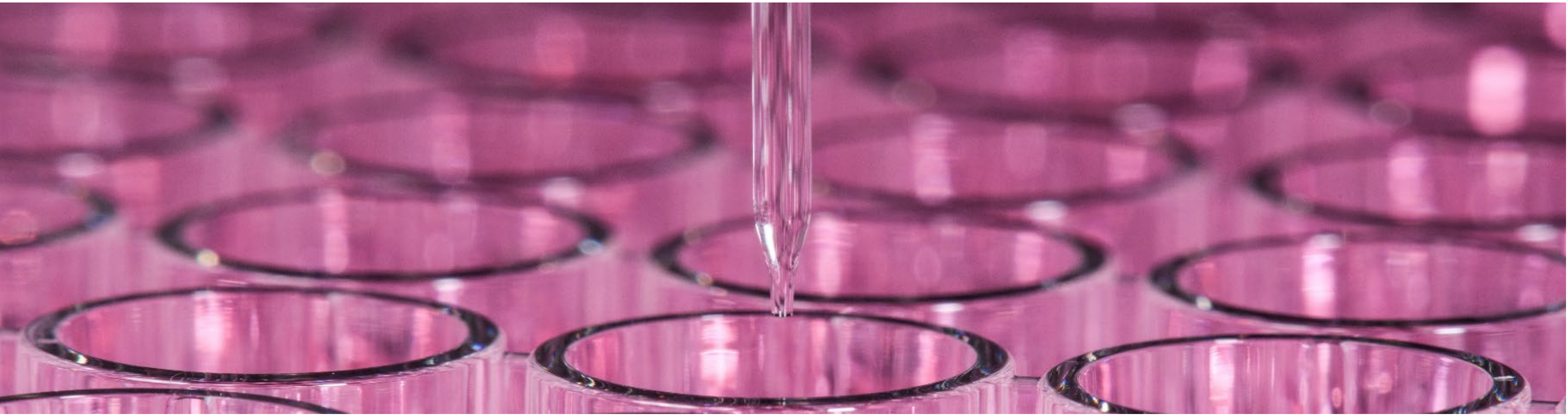

cellenONE® as automated single cell seeding platform for hiPSC subcloning

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Abstract

Traditional manual methods for hiPSC subcloning are time consuming and cannot ensure clonality. To improve hiPSC survival and cloning capacity during single cells seeding, we established an optimal workflow for hiPSC clone generation using cellenONE® F1.4 single-cell dispenser. We assessed several cell lines with varying survival rates and tested several culture conditions to identify the optimal workflow for hiPSC clone generation. Our results demonstrate that cellenONE® F1.4 automated platform is a reliable solution for accurate and efficient single hiPSC isolation that can easily be integrated as part of different workflows, such as gene editing.

Introduction

Many applications using human induced pluripotent stem cells (hiPSC), such as gene editing, subcloning and single cell based molecular methods, depend on reliable single cell isolation methods. The widely used classical methods, i.e. limiting dilution, are labor intensive, time consuming, and do not guarantee clonality. Furthermore, hiPSC are especially sensitive to single cell dissociation, which frequently triggers anoikis, a specific form of programmed cell death (1). High rates of cell death following single cell plating result in low efficiencies of clone formation. This is particularly problematic in applications requiring large numbers of clones for downstream analysis such as gene editing studies.

Therefore, an automated and gentle single cell seeding, combined with optimal hiPSC culture conditions, is critical for reducing time and labor while improving accuracy and reliability of hiPSC pipelines (2).

cellenONE® F1.4 offers a portfolio of functionalities for hiPSC workflows. Automated cell seeding reduces workload and improves reproducibility. Survival rate of plated cells is consistently high, and the fluid control imaging system allows immediate clonality assessment. Fluorescence-based cell sorting is also possible, which is especially attractive for applications using, for example, tagged proteins. Costs per run are relatively low and no additional consumables are required. Finally, the dispensing system has built-in imaging platform which allows collection of additional information, including cell size and morphology, and which can then be used in downstream analysis.

In this application note we report preliminary data for hiPSC isolation, single cell seeding and clone outgrowth efficiency. Additionally, we provide analysis of different culture formats and conditions for optimization of single-cell cloning.

Materials and methods

hiPSC culture and single cell isolation conditions

Five different hiPSC lines were used for the experiments. Namely BIHi005-A, BIHi004-A (constitutively GFP expressing line), BIHi250-A, hiPSC-1 and hiPSC-2. hiPSC were maintained on growth factor reduced Geltrex (Gibco) in E8 media, which was exchanged daily. Cells were clump-passaged every 2–4 days when >70% confluency was reached using 0.05mM EDTA (Thermo Fisher). Two or three days before the single cell seeding, hiPSC were dissociated using TrypLE (Gibco) and seeded at $2x10^5$ cells per well (6 well plate, Geltrex coated) in StemFlex media (Gibco) supplemented with Clone R (Stem Cell Technologies). At day 0 (~70% confluence) cells were dissociated using TrypLE. A suspension of 200 cells/µl was prepared in DPBS (Gibco) supplemented with 10µg/ml Y-27632 (Wako Chemicals) and 0.5µg/ml propidium Iodide (Thermo Fisher) and loaded into a well of a 384-wp (sciSOURCEPLATE 384 PS, SCIENION) inside cellenONE® F1.4. Single cells were seeded in destination plates (96 -Falcon or 384 wells-Greiner or PerkinElmer CellCarrier ultra imaging plates) containing 70 and 150µl, respectively, StemFlex medium supplemented with anti-apoptotic molecule (Y-27632; Clone R – Stem Cell Technologies or RevitaCell -Gibco). Clone R and RevitaCell were used in the dilution recommended by the manufacturer. Destination plates were coated with 5µg/ml Laminin 521 (Biolamina) solution in PBS Ca^{2+}/Mg^{2+}, 5µg/ml Vitronectin (VTN-N, Thermo Fisher) in DPBS or Geltrex (Gibco) diluted 1 to 120 in DMEM-F12 (Gibco).

cellenONE® F1.4

Single cell isolation was performed using cellenONE® F1.4 which is an automated single cell isolation and nanoliter dispensing device. The isolation was done in transmission and fluorescence mode by selecting diameter, circularity, elongation, and absence of Propidium Iodide (PI) staining (orange intensity<40) as isolation parameters at 4°C. After single cell seeding, plates were incubated at 37°C, 5% CO_2 for 48h.

hiPSC clones maintenance and analysis

72h after single cell seeding, half of media was replaced with fresh Stem Flex supplemented with Clone R as described above. At day 5 (~120h) after plating, plates were imaged using Cell3iMager Duos (Screen Holdings Co., Ltd.) or Opera Phenix (PerkinElmer) using bright field and fluorescence acquisition respectively. hiPSC colonies quantification was performed using Harmony Software (PerkinElmer) at day 5 of culture. From day 5 to day 12, cells were maintained in culture and media changes were performed every other day using Stem Flex supplemented with Clone R.

Results and discussion

1. Post-isolation parameters

Single cell isolation was performed using cellenONE® F1.4 equipped with fluorescence module. The selection of initial isolation parameters was based on the previous experiments performed on 5 distinct cell lines.

The isolated particles correspond to the cells that were fitting isolation parameters and were present as single cell in the ejection zone during isolation. The example post-isolation analysis report shows that majority of isolated cells had relatively uniform distribution of cell diameter (22-27 µm) and a very small variability in circularity (~1) and elongation (~1.4) parameters (Figure 1A). Furthermore, none of the isolated particles were positive for the cell death marker PI, showing that the cellenONE® F1.4 fluorescence module allows efficient exclusion of dead cells. Off note, it could be shown that cell survival was improved when isolation was performed at 4°C (data not shown).

The cells referred to as 'fitting criteria particles' in the scatter plots correspond to cells that matched the a priori isolation parameters but were not isolated. These cells were either PI positive -dead cells- or were not alone in the ejection zone and, therefore, were rejected as monoclonality could not be assured.

cellenONE® F1.4 performs live image acquisition at the level of the dispensing from the capillary, where the content of the next droplet can be observed (Figure 1B). Therefore, post-isolation analysis of single cell events is possible. We found that, across a variety of cell lines tested, single cell isolation efficiency using cellenONE® F1.4 was ~94-100% (Figure 1C). As proof of clonality is frequently key for downstream applications, cellenONE® F1.4 provides a unique advantage over other methods, like manual limiting dilution and clone picking.

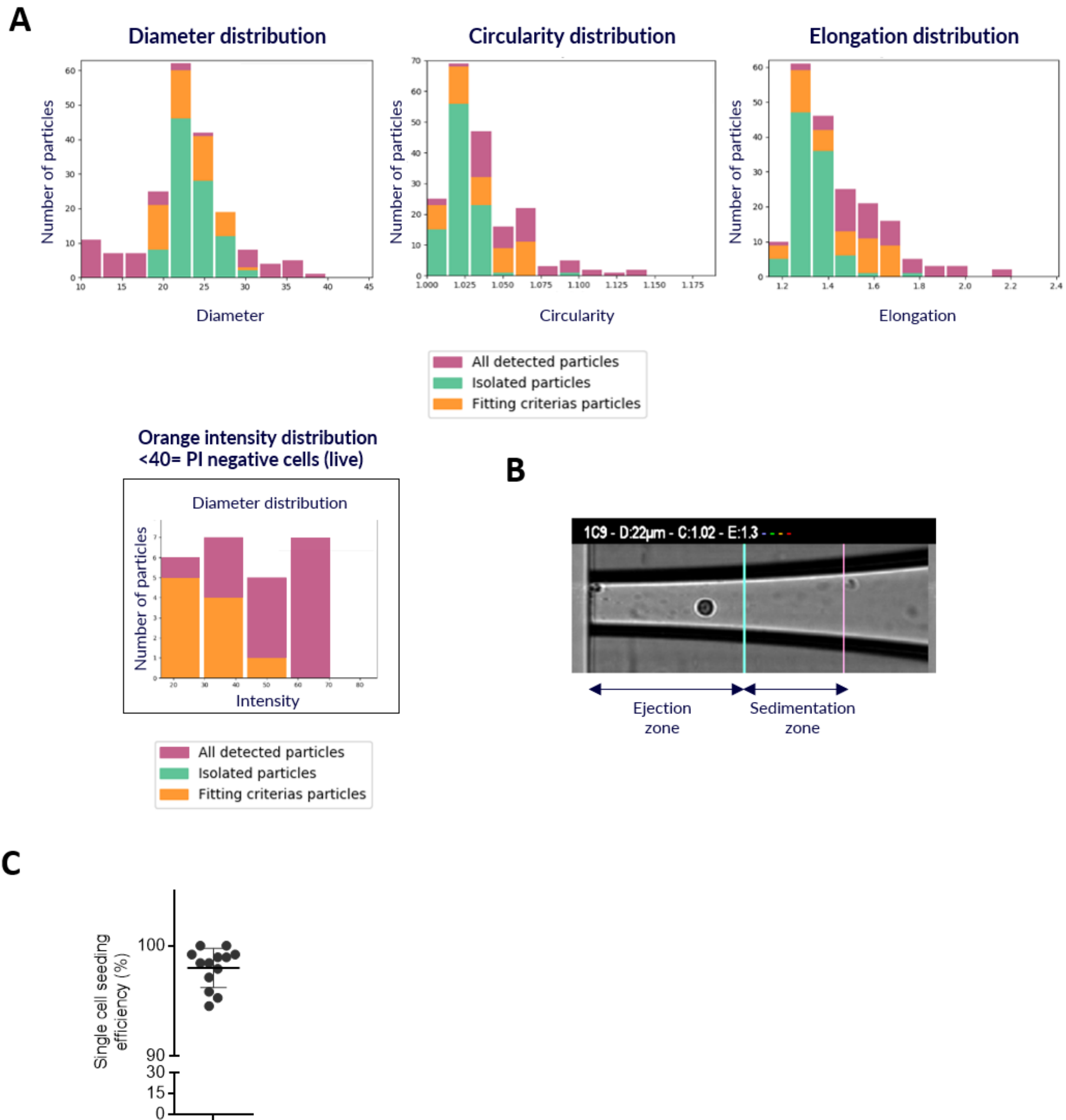

Figure 1. ***Single hiPSC isolation using cellenONE® F1.4***
A) Representative histograms automatically generated after cellenONE® F1.4 run using the cellenONE® F1.4 report application. An example of main isolation parameters is depicted: hiPSC diameter, circularity, elongation and orange intensity for propidium iodide (PI) as cell death excluding parameter. **B)** cellenONE® F1.4 report image showing an example of one isolated single hiPSC inside the ejection zone of the capillary. The well number and the parameters of the isolated cell (D: Diameter, C: Circularity, E: Elongation) are displayed on the top. **C)** Dot plot showing single cell seeding efficiency after scoring single event isolation using cellenONE® F1.4 automated acquisition during the run (n=11, mean ± SD).

2. Surface coatings and anti-apoptotic molecules

To further improve cell survival and clone growth after single cell isolation using cellenONE® F1.4, we tested various surface coatings and anti-apoptotic molecules. To test different coating options we used StemFlex medium, and the most widely used anti-apoptotic molecule ROCK inhibitor (ROCKi, Y27362). To compare the efficiencies of clonal outgrowths on Geltrex, Vitronectin, and Laminin we constructed and automated unbiased quantification pipeline to score number of wells with outgrowth and number of colonies per positive well. We found that Geltrex and Laminin performed similarly with ~30% of wells identified as having clonal outgrowths (Figure 2A). In comparison, Vitronectin coating had decreased ability to support colony growth (~20%). Additionally, in the plate coated with Vitronectin, we observed fewer colonies per well (1-5 hiPSC colonies) as compared to Geltrex (1-10) or Laminin 521 (1-15 hiPSC colonies) coatings (Figure 2A).

Subsequently, we used Geltrex as a constant to compare which anti-apoptotic molecule was the most suitable for our application. We compared cloning efficiencies in Clone R, RevitaCell, and ROCKi. We found that CloneR performed the best with 42% clonal outgrowths, ROCKi had slightly lower, but still acceptable efficiency of 31% (Figure 2B). RevitaCell supported outgrowth only in ~16% of clones and, therefore, was the least favorable condition.

Based on these results, we selected a combination of Laminin 521 and CloneR with StemFlex media for our further experiments.

Finally, to test whether plate format had an impact on the efficiency of clonal outgrowth we seeded cells in 96 or 384-well plates. Surprisingly, we found that clonal expansion was more successful in 384-well plate (~43%) as compared to 96-well plate (~17%), suggesting that smaller surfaces and lower media volumes may favor hiPSC clone growth (Figure 2C).

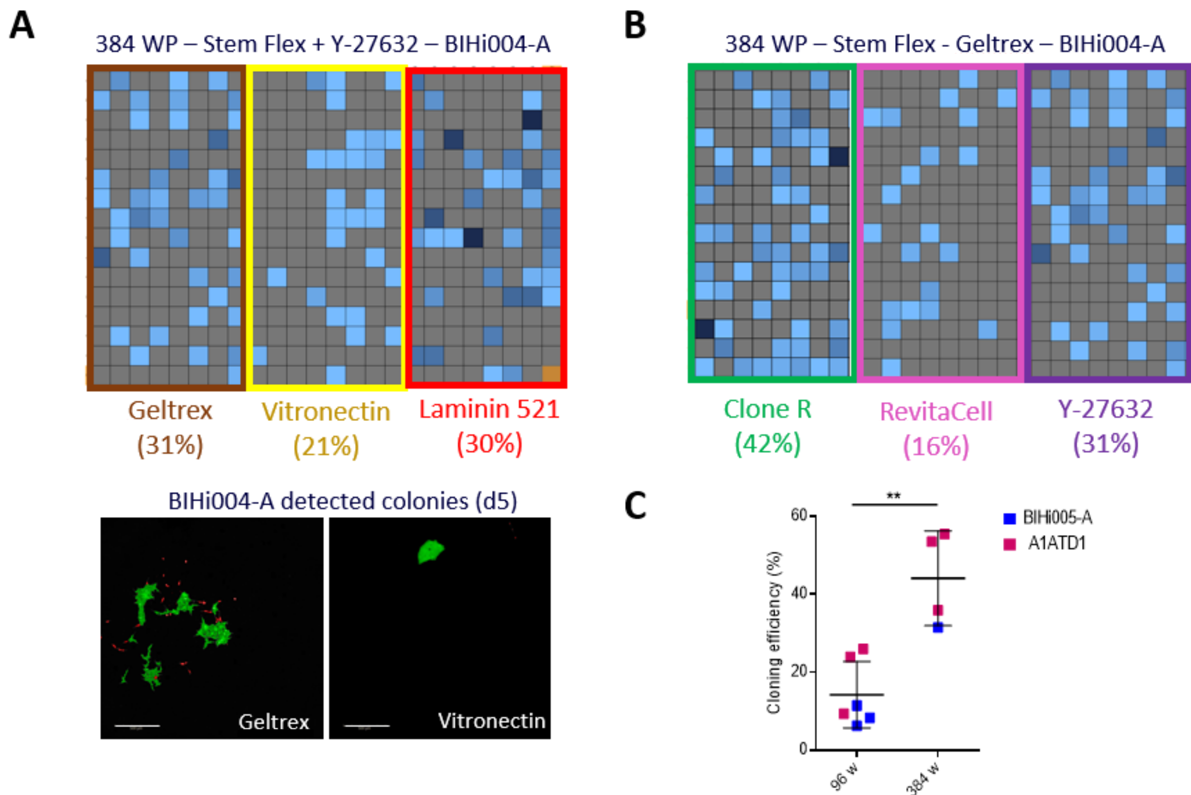

Figure 2. ***Cloning efficiency is affected by hiPSC culture conditions after single cell seeding with cellenONE® F1.4***
A) Heatmap depicting number of wells with hiPSC clonal outgrowth (expressed below as percentage (%)= n° wells with positive outgrowth at day 5 / n°wells with single cell at day 0); dark blue indicates higher number of colonies per well, all derived from 1 single cell. The heatmap shows performance comparison between different plate coatings (left panel). Representative pictures showing in green segmented elements considered hiPSC colonies for unbiased automated quantification at day 5. **B)** Heatmap constructed as described in panel A to compare performance of different anti-apoptotic molecules. **C)** Graph showing differential hiPSC outgrowth efficiency according to size well for 2 different hiPSC lines. Each dot represents a technical replicate.
Values are expressed as mean ± SD. Statistic analysis: non-parametric Mann-Whitney test. Scale bars= 500µm

3. Workflow across different hiPSC lines

Using the above culture conditions (Laminin 521, StemFlex media + CloneR, in 384-well plate), we defined a workflow to perform single cell seeding with cellenONE® F1.4 followed by colony maintenance until day 10-12 of culture (Figure 3A and B). To test whether this workflow could be universally applied across different hiPSC lines, we analyzed clonal efficiencies of 4 distinct hiPSC lines. We analyzed outgrowth efficiency of the colonies with proven clonality on day 5 after seeding. We found that there are major differences in cloning capacities of different hiPSC lines (Figure 3C). For example, BU3 NG and BIHi250-A lines produced clones with <20% efficiency, whereas BIHi004-A and BIHi005-A cloning efficiencies were ~60%. This variability between hiPSC highlights the importance of optimizing culture conditions and minimizing cellular stress during the single cell seeding procedure.

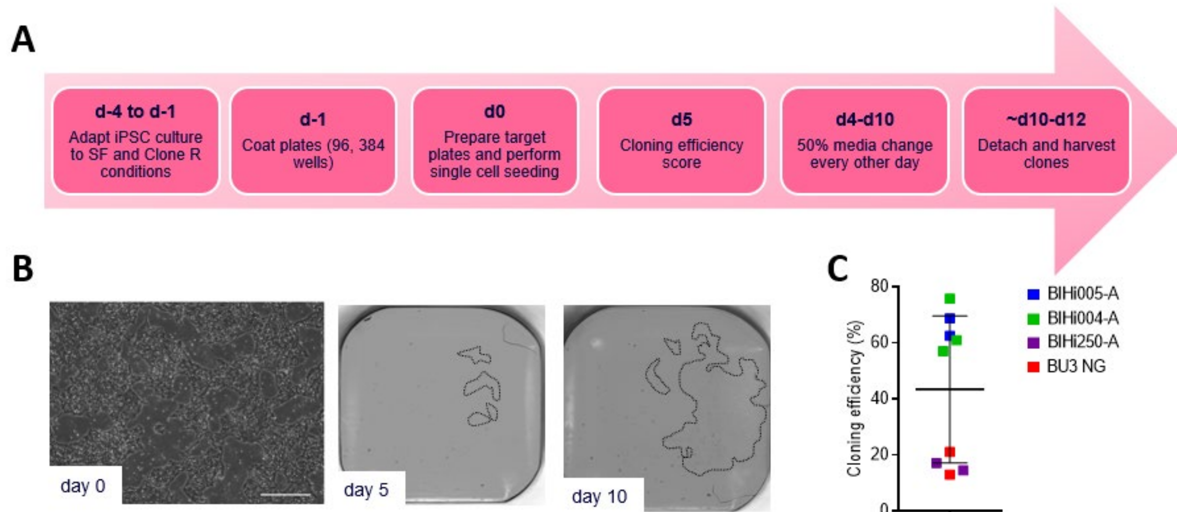

Figure 3. cellenONE® F1.4 embedded in hiPSC automated subcloning workflow
A) General timeline to generate hiPSC monoclonal expansion after single cell seeding. **B)** Brightfield pictures show hiPSC culture before single cell dissociation at day 0 and follow up of clone growth in 384 well plate. **C)** Graph showing hiPSC cloning efficiency at day 5 after single cell seeding in 384 well plates. Each dot represents technical replicates. *Values are expressed as mean ± SD. Statistic analysis: non-parametric Mann-Whitney test. Scale bar= 500μm.*

Conclusions and future direction

Success in hiPSC subcloning requires gentle and optimized conditions and has to be robust and reliable. The data presented here suggests that the combination of specific coating (Laminin 521), media (StemFlex) and anti-apoptotic small molecule (CloneR) conditions enhances hiPSC survival and growth after single cell isolation using cellenONE® F1.4.

Our preliminary observation regarding a better cloning efficiency using smaller plate formats and therefore higher throughput is promising for further scaling up.
cellenONE® F1.4 automated platform for single cell seeding offers unmatched characteristics such as proof of clonality, gentle dispensing, high speed, possibility of fluorescence selection and potential to be embedded into a fully automated pipeline.

References

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