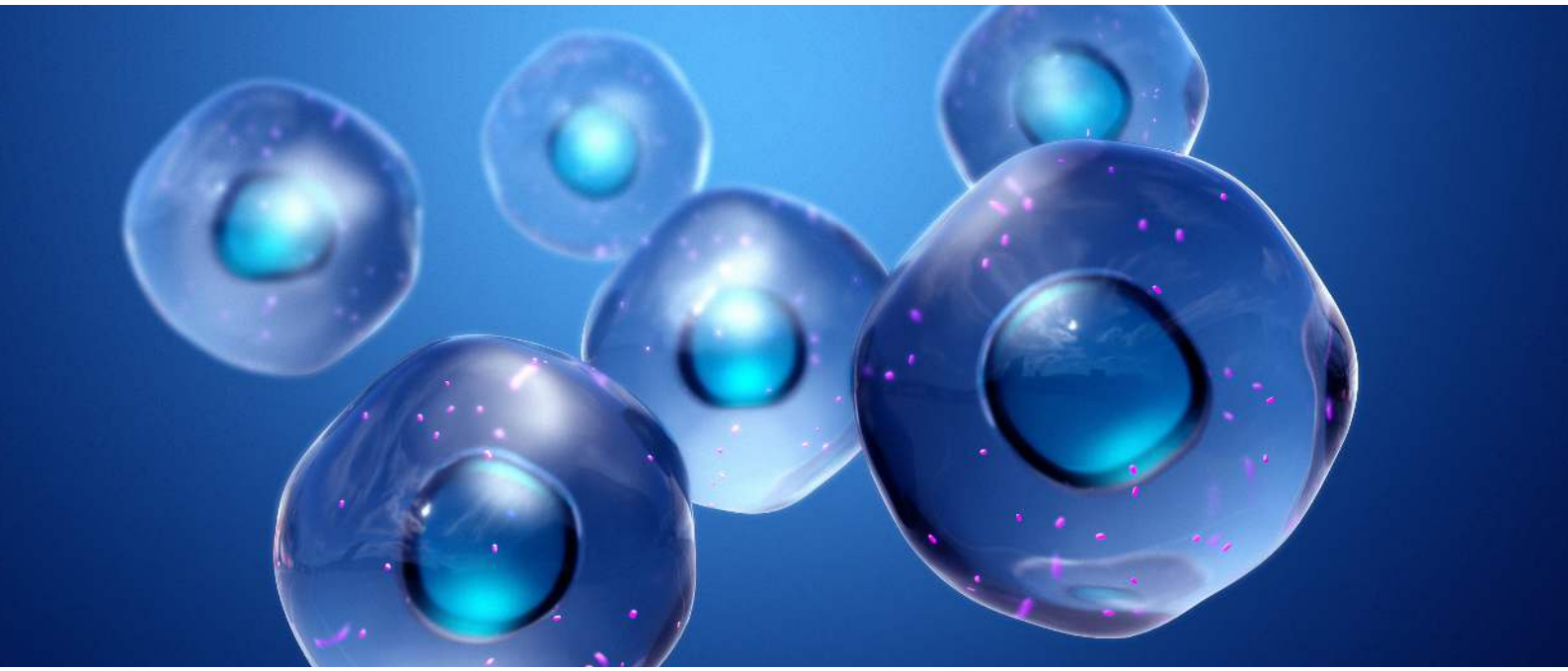
The UP.SIGHT enables gentle single cell clonal expansion of human iPSCs with high clone recovery

Christina Reinhard[1], Ruba Hammad, PhD[1], Richard Schäfer, MD[1], Fernando Aprile-Garcia, PhD[2]

[1] Freiburg iPS Core, Medical Center–University of Freiburg, Freiburg, Germany

[2] CYTENA GmbH, Freiburg, Germany



Introduction

Newly developed therapies, such as stem cell therapy, are shifting the field of medicine from treating symptoms to curing diseases altogether. Human induced pluripotent stem cells (iPSCs) have changed regenerative medicine with their capability to differentiate into different cell types and tissues[1]. Although human iPSCs are already used in autologous therapies, the next breakthrough will be to develop certified iPSCs for off-the-shelf treatment. To achieve this, the immunogenicity of heterologous iPSCs could be reduced via human leukocyte antigen (HLA) depletion, leading to the establishment of GMP-grade master cell banks for the mass production of cells, while lowering manufacturing costs of allogenic therapies[2].

Developing genetically engineered iPSCs requires several rounds of cloning after transfection. The traditional limiting dilution method for isolating single cells is time consuming, and fluorescence-activated cell sorting negatively affects the viability of highly sensitive iPSCs and bears the risk of contamination. In contrast, CYTENA's single cell dispensers utilize proprietary, transparent microfluidic chips and real-time imaging technology and algorithms to sort and dispense single cells into 96- or 384-well microplates with high viability and high efficiency. The disposable microfluidic chips, based on inkjet-like printing technology assures gentle cell dispensing[3,4]. Recently, methods for single cell isolation of human pluripotent stem cells using these instruments have been reported[5–7]. There, high clone recovery values of up to 80% were obtained after method optimization without loss of pluripotency.

CYTENA's latest single cell dispenser is the UP.SIGHT. Compared to previous devices, the UP.SIGHT is not only a single cell dispenser, but also provides plate imaging capabilities to enable colony tracking. On the 384 well plate format, the UP.SIGHT addresses the clonal derivation requirement by offering two independent methods of assuring monoclonality. First, through nozzle images to ensure that only droplets that contain only one cell are dispensed in each well, and second, by 3D Full Well Imaging to confirm that the cell was correctly deposited inside the well. Together, these two separate assurances of clonality provide verification of single-cell isolation during the dispensing process with a probability of clonality >99.99%, fulfilling the required regulatory expectations[8].

Here we show that gentle single cell cloning of human iPSCs employing the UP.SIGHT is technically possible, resulting in healthy undifferentiated colonies. Furthermore, we show an example of method optimization that dramatically improved clone recovery to values up to 80%.

Materials and methods

A control iPS line originally derived from human fibroblasts was maintained in culture under feeder free conditions in coated 6-well plates, with TeSR E8 culture medium[9]. Cells were passaged regularly at a 1:3 to 1:6 dilution ratio with an aggregate passaging method and split when 70% confluency was reached. Harvest for single cell dispensing was also done at 60-70% confluency, making sure cells looked healthy and not differentiated. Cells were harvested as single cells, counted and resuspended in dispensing solution at 0.5-0.8x10^6 cells/mL. Eighty uL of this single cell suspension were loaded onto the EASY.ON cartridge and mounted on the UP.SIGHT for single cell dispensing. The dispensing solution as well as the cloning medium on the plates contained ROCK inhibitors. Cells were dispensed on coated 96-well plates with cloning medium and transferred back to the incubator as soon as possible. Monitoring of colony growth was performed during the first seven days manually in a standard cell culture inverted microscope or in a semi-automated fashion on the UP.SIGHT.

Results and discussion

We performed a series of experiments to optimize the dispensing condition of iPSCs using the UP.SIGHT to reach high levels of clone recovery. We first employed the manual limiting dilution method, where we cultured 0.5 cells/well. With this manual method, we achieved, as expected[10,11], a range of 10-20% clone recovery after 10 days (data not shown). The first dispensing experiment with the UP.SIGHT led to 6% clone recovery with the same conditions (Fig 1, column A). Taking those experimental settings as a baseline, we started to sequentially improve the recovery values by a systematic step-by-step optimization of conditions. We first optimized the cell solution loaded on the UP.SIGHT cartridge. We determined that use of a saline buffer almost doubled the clone recovery compared to the use of raw basal medium (Fig 1, column B). After that, we hypothesized that letting cells recover as much as possible after dispensing would improve colony survival. iPSCs usually require regular media changes, but this early manipulation after dispensing can be harmful. We tested the effect of adding supplements to the media in the cloning plates to avoid the need of medium change during the first 7 days after dispensing. We first tested supplementation of the cloning medium with supplement formulation A (Fig 1, column C). Using the same rationale, supplementation of cloning media with supplement formulation B led to even higher recovery values, reaching up to 50% (Fig 1, columns D and E).

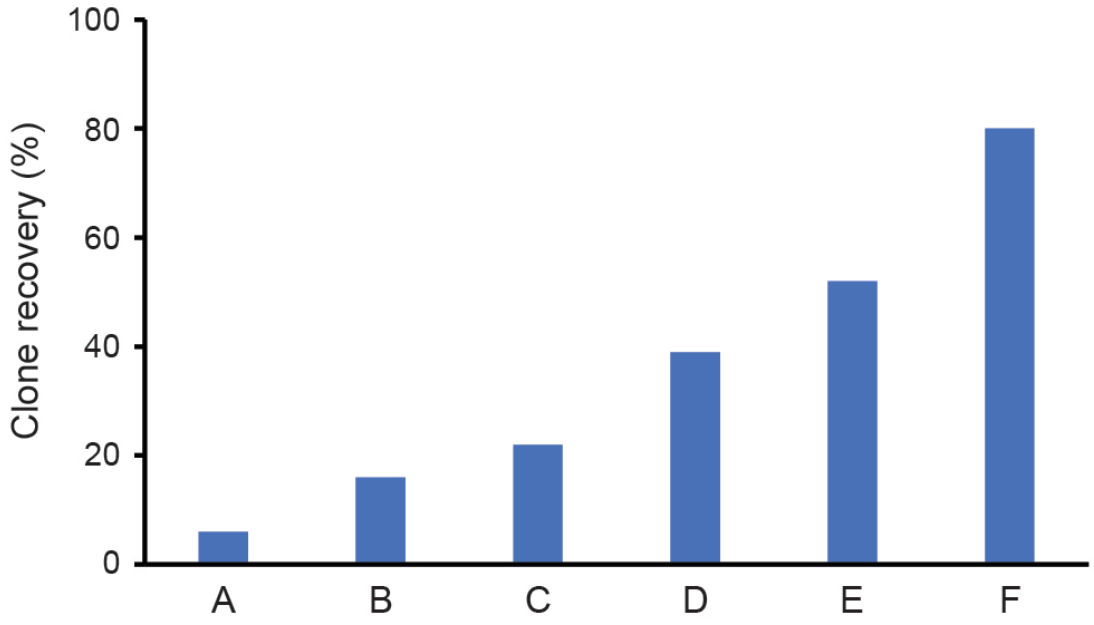


Figure 1. Optimization of dispensing to increase clone recovery values. *Results obtained across an optimization campaign are shown, depicting key sequential improvements implemented to the workflow. A: Initial dispensing conditions, most similar to standard culture conditions. B: Salt solution as dispensing solution. C: cloning medium supplemented with supplement formulation A. D-E: cloning medium supplemented with different concentrations of supplement formulation B. F: cloning medium changed to single cell cloning media with supplement formulation B. Clone recovery values are expressed as percentage of wells with growing colonies on day 7-10.*

The addition of factors that allowed us to keep the plates undisturbed during the first 7 days after dispensing noticeably increased the % of clone recovery and the growth rate of colonies. In general, we observed that under those conditions we obtained colonies of larger size and good morphology after seven days (Fig 2). Finally, we replaced the standard media in the cloning plates altogether and employed an optimised media for single cell seeding of iPSCs. These optimized dispensing conditions that include cartridge loading of saline solution plus fit-to-purpose cloning media supplemented with an optimized concentration of media supplements led to obtaining high recovery values of up to 80% (Fig 1, column F) and robust colonies (Fig 2).

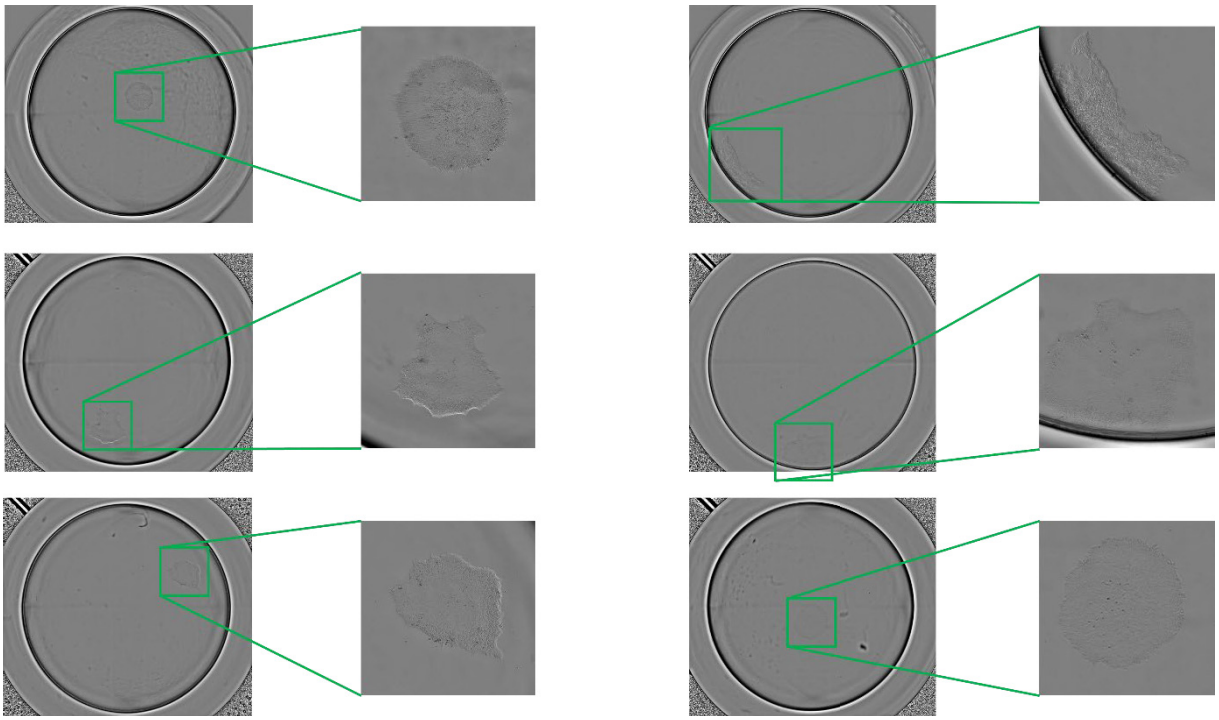


Figure 2. Colony growth and morphology. *Example images of colonies at day 7 after single cell dispensing on 96 well plates. Imaging was performed with the UP.SIGHT.*

It is important to highlight that during these experiments we also tested other parameters that did not significantly influence clone recovery. For example, we tested different ROCK inhibitor formulations, plate coatings and plate types. Nevertheless, we cannot claim that these variables would not have an influence in clone recovery when used under other experimental conditions, as for example reported before[5].

The plate imaging capabilities of the UP.SIGHT were also put to test during these experiments. We were able to confirm that the image quality is good enough to assess the morphology of the colonies to determine if cells maintained their typical iPSCs morphology after single cell dispensing (Fig 2). Furthermore, automated imaging on the UP.SIGHT has the advantage that is a faster procedure compared to classic microscopes: image acquisition of a full well plate (either 96 or 384 well plate) requires less than 10 minutes, severely reducing the time the plate remains outside the incubator, in particular for 384 well plates. This is of particular interest when performing initial screening on full well plates, allowing to perform imaging at multiple time points quickly (Fig 3).

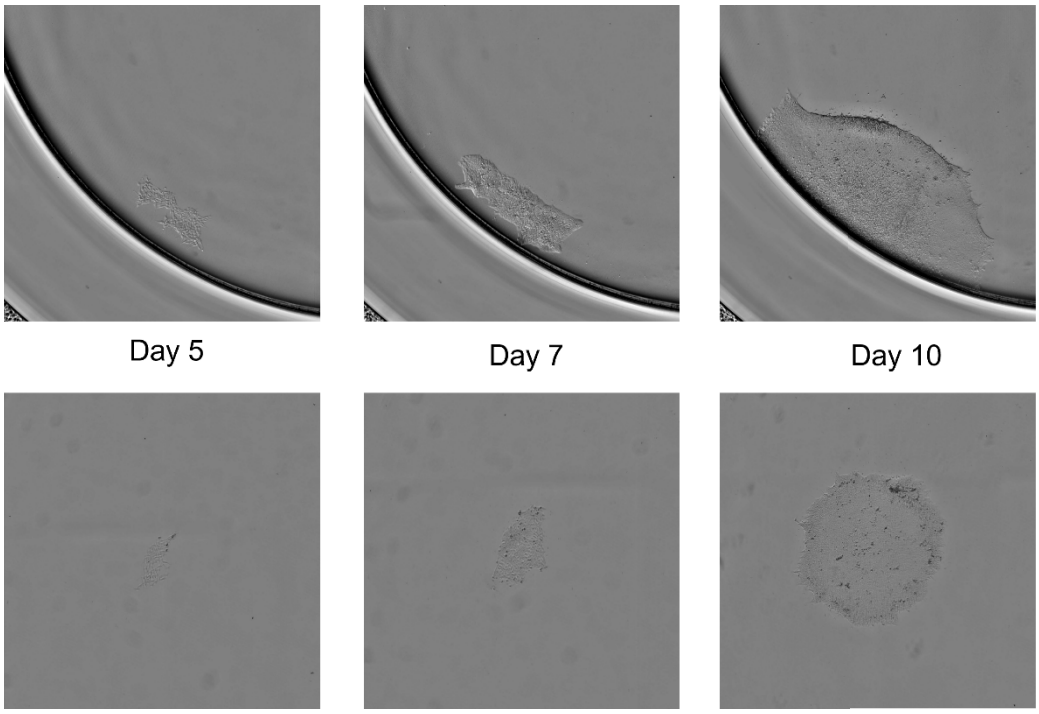


Figure 3. Colony monitoring with the UP.SIGHT. *Two examples of colony growth across time. Imaging was performed with the UP.SIGHT.*

Conclusion

Here we show that dispensing iPSCs with the UP.SIGHT results in colonies of good shape and morphology, with high clone recovery values. In order to maximize clone recovery, optimization is recommended. The key parameters to optimize are the quality and growth state at which the cells are at the time of harvest before dispensing, the condition in which the cell suspension is loaded in the cartridge and the cloning media and its supplements to ensure cells are kept as undisturbed as possible during the first days after seeding. The fast plate imaging capabilities of the UP.SIGHT allows for fast screening and morphology monitoring of the growing colonies.

In conclusion, the UP.SIGHT is a multipurpose instrument that allows efficient, high-quality and timesaving dispensing of iPSCs and early monitoring to pick the right clones.

References

1. El-Kadiry, A.E.H., Rafei, M., and Shammaa, R. (2021). Cell Therapy: Types, Regulation, and Clinical Benefits. Front Med (Lausanne) 8. 10.3389/fmed.2021.756029.

2. Jarrige, M., Frank, E., Herardot, E., Martineau, S., Darle, A., Benabides, M., Domingues, S., Chose, O., Habeler, W., Lorant, J., et al. (2021). The future of regenerative medicine: Cell therapy using pluripotent stem cells and acellular therapies based on extracellular vesicles. Cells 10, 1–29. 10.3390/cells10020240.

3. Gross, A., Schoendube, J., Zimmermann, S., Steeb, M., Zengerle, R., and Koltay, P. (2015). Technologies for Single-Cell Isolation. Int J Mol Sci 16, 16897–16919. 10.3390/IJMS160816897.

4. Gross, A., Schöndube, J., Niekrawitz, S., Streule, W., Riegger, L., Zengerle, R., and Koltay, P. (2013). Single-Cell Printer: Automated, On Demand, and Label Free. J Lab Autom 18, 504–518. 10.1177/2211068213497204.

5. Chen, Y., Tristan, C.A., Chen, L., Jovanovic, V.M., Malley, C., Chu, P.H., Ryu, S., Deng, T., Ormanoglu, P., Tao, D., et al. (2021). A versatile polypharmacology platform promotes cytoprotection and viability of human pluripotent and differentiated cells. Nat Methods 18, 528–541. 10.1038/s41592-021-01126-2.

6. Vallone, V.F., Telugu, N.S., Fischer, I., Miller, D., Schommer, S., Diecke, S., and Stachelscheid, H. (2020). Methods for Automated Single Cell Isolation and Sub-Cloning of Human Pluripotent Stem Cells. Curr Protoc Stem Cell Biol 55. 10.1002/cpsc.123.

7. Krol, R.P., Yamashita, M., Tsukahara, M., and Takasu, N. (2021). Single-cell cloning of human induced pluripotent stem cells automation and protocol optimization. In International Society for Stem Cell Research.

8. Scherzinger, J., Türk, D., and Aprile-Garcia, F. (2022). An optimized and validated workflow for developing stable producer cell lines with >99.99% assurance of clonality and high clone recovery. bioRxiv, 2022.12.16.520697. 10.1101/2022.12.16.520697.

9. Craig-Mueller, N., Hammad, R., Elling, R., Alzubi, J., Timm, B., Kolter, J., Knelangen, N., Bednarski, C., Gläser, B., Ammann, S., et al. (2020). Modeling MyD88 Deficiency In Vitro Provides New Insights in Its Function. Front Immunol 11, 3327. 10.3389/FIMMU.2020.608802/BIBTEX.

10. Rodin, S., Antonsson, L., Hovatta, O., and Tryggvason, K. (2014). Monolayer culturing and cloning of human pluripotent stem cells on laminin-521–based matrices under xeno-free and chemically defined conditions. Nature Protocols 2014 9:10 9, 2354–2368. 10.1038/nprot.2014.159.

11. Kuo, H.H., Gao, X., DeKeyser, J.M., Fetterman, K.A., Pinheiro, E.A., Weddle, C.J., Fonoudi, H., Orman, M. V., Romero-Tejeda, M., Jouni, M., et al. (2020). Negligible-Cost and Weekend-Free Chemically Defined Human iPSC Culture. Stem Cell Reports 14, 256–270. 10.1016/J.STEMCR.2019.12.007.

CYTENA, A BICO COMPANY

CYTENA spun off from the University of Freiburg, Germany, in 2014 with its patented single-cell dispensing technology. Today, as part of BICO, the world's leading bioconvergence company, CYTENA continues building on that groundbreaking technology to develop high-precision instruments for isolating, dispensing, imaging and handling biological cells. Its award-winning devices are manufactured in Germany and used at prestigious academic and pharmaceutical labs around the world to automate workflows in numerous application areas, including stable cell line development, single-cell omics, high-throughput screening and drug discovery. CYTENA's breakthrough innovations for the lab combine advanced automation, state-of-the-art software engineering and the latest insights in cell biology to maximize efficiencies in the life sciences and create the future of health. Learn more at cytena.com.

info@cytena.com | +49 761 21632 000 | www.cytena.com