SINGLE-CELL CLONING OF HUMAN INDUCED PLURIPOTENT STEM CELLS
AUTOMATION AND PROTOCOL OPTIMIZATION

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ABSTRACT

CiRA Foundation's Facility for iPS Cell Therapy is a Cell Processing Center responsible for manufacturing of clinical-grade iPS cells, and our newest project is generation of HLA-genome-edited iPS cell lines, designed to reduce the risk of immune rejection, by targeting HLA-A, HLA-B and CIITA genes for CRISPR/Cas9-based knock-out. One of the challenges in the manufacturing of genetically engineered iPS cells is single-cell cloning, specifically, choice of the optimal cell dispensing technology, confirmation of clonality, dependable imaging technique and assuring survival of single iPS cell in culture.

Here we report our progress in development of the single-cell cloning process suitable for manufacturing of clinical-grade iPS cells with adherence to cGMP regulations.

First, we evaluate several single-cell dispensing instruments – CellCelector™ (ALS Automated Lab Solutions), F.SIGHT™ and UP.SIGHT™ (Cellink/Cytena) and VIPS™ (Solentim) – with regard to reliability of single cells identification and monoclonality verification. Next, we compare the technologies based on dispensing single cells into individual wells of the multiwell plate with systems utilizing nanowell arrays for single-cell isolation and clonal expansion.

Finally, we are optimizing culture protocols, specifically, comparing various supplements to ensure single cell survival: Y-27632 ROCK inhibitor, CEPT cocktail (NCATS), CloneR™ reagent (STEMCELL Technologies) and RevitaCell™ supplement (Invitrogen), choosing plate coating method for optimal cell attachment and identifying culture plate type for highest imaging quality.

MATERIALS AND METHODS

CELLS

The iPS cells used in this study were 201B7 line generated at our facility from dermal fibroblasts, and 201B7-derived fluorescent lines expressing either GFP, mCherry (gift from Dr. Woltjen) or BFP (engineered from GFP line).

CULTURE SYSTEM

Cells were cultured on iMatrix-511 substrate (Nippi) in StemFit AK03N medium (Ajinomoto) and passaged with TrypLE™ Select enzyme (Invitrogen). In order to avoid frequent medium replacements after single-cell dispensing, the culture medium was supplemented with StemBeads® FGF2 (StemCultures), slow-release FGF2 microspheres. Cells were cultured at 37°C in humidified atmosphere with 5% CO_2.

CYTOPROTECTION

During passaging the culture medium was supplemented with either Y-27632 ROCK inhibitor, CEPT cocktail [chroman 1, emricasan, polyamine supplement and trans-ISRIB] (NCATS), CloneR™ reagent (STEMCELL Technologies) or RevitaCell™ supplement (Invitrogen).

CULTURE VESSELS

For single-cell dispensing experiment we used standard TC-treated 96-well (Corning 3596 & 3603), CellBIND® surface treated 96-well (Corning 3340) and 384-well (Corning 3770), and half area 96-well (Corning 4680 & Greiner Bio-One 675096) microplates.

IMAGING

The microplates were imaged by either Keyence BZ-X810 All-in-One Fluorescence Microscope or Incucyte® S3 Live-Cell Analysis System.

SINGLE CELL DISPENSING

Proof-of-concept experiments (results presented at ISSCR2020) we performed with CellCelector™ (ALS Automated Lab Solutions), for preliminary protocol optimization experiments we used F.SIGHT™ (Cellink), and the results presented here were obtained with UP.SIGHT™ (Cellink) instrument.

The UP.SIGHT™ instrument is capable of dispensing single cells into 96- 384- or 1536-well microplates. The operating software analyzes cell morphology to isolate single cells according to set parameters such as size, roundness and fluorescence intensity. It utilizes disposable EASY.ON cartridges.

RESULTS

SINGLE CELL DISPENSING EFFICIENCY

Single cell dispensing with UP.SIGHT™ was very fast and reliable. On average, the 96-well microplate dispensing took about 3 minutes. Nozzle imaging confirmed single cell dispensing into close to 100% wells.

SUCCESS RATE

We found that for the successful colony outgrowth from single cell the most critical parameter was the condition of the cells during harvest. In order to achieve optimal cell recovery after single-cell dispensing, we considerably modified our culture and cell harvest protocols, i.e. initial seeding density, passage timing, cell harvest technique and target vessel coating protocol.

PLATES

Among tested vessels, the best result were produced with CellBIND® surface treated microplates.

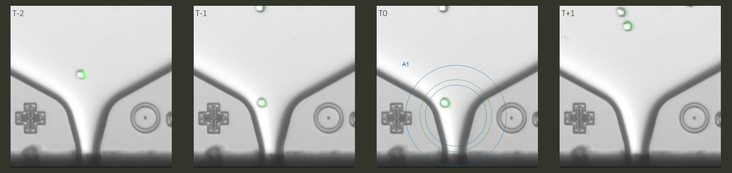
CYTOPROTECTION

Surprisingly, the choice of cytoprotective reagent played much smaller role that optimal cell condition. The best cloning efficiency were achieved when culture medium was supplemented with combination of CloneR™ reagent and CEPT cocktail: the colonies outgrowth regularly exceeded 75% of wells in 96-well microplates. Very good results were produced also with CEPT cocktail alone: regularly over 30%, and CloneR™ reagent: over 70% (single experiment). With standard Y-27632 ROCK inhibitor the efficiency was usually between 20 and 30% (in the most successful experiment over 70%). The lowest efficiency was observed with RevitaCell™ supplement: below 20% (single experiment).

In order to directly compare the effect of cytoprotective reagents we performed an experiment with differently supplemented medium in alternating rows of 384-well microplate (CEPT cocktail was used during harvest and dispensing). The results were: CEPT cocktail 64 of 96 wells (66%), CloneR™ reagent 58 of 96 wells (60%), Y-27632 ROCK inhibitor 52 of 96 wells (54%), and RevitaCell™ supplement 20 of 96 wells (21%).

IMAGING

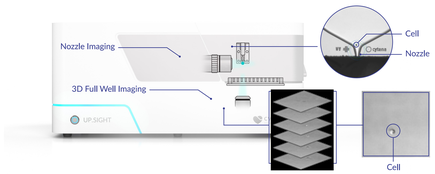
We were able to perform very convenient and reliable imaging of the single-cell cloning microplates with Incucyte® S3 instrument. The whole well scanning at adjustable time intervals allowed us to trace the cloning progress starting from dispensed single cell, through subsequent cell divisions, colony formation and outgrowth.



EXAMPLE OF UP.SIGHT™ NOZZLE IMAGES DURING CELL DISPENSING

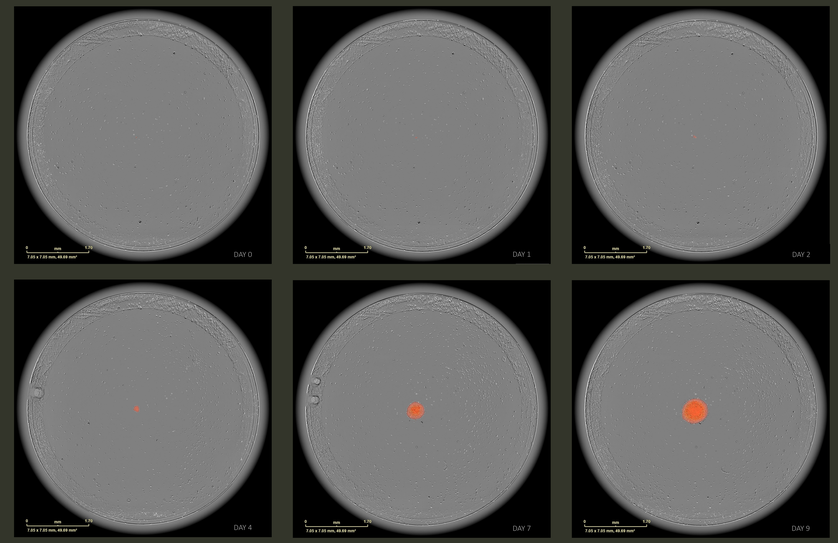
Series of images (overlay of brightfield and fluorescent) taken during dispensing of GFP-expressing iPSCs, T0 indicates recognition of the cell, T+1 shows the

UP.SIGHT™ INSTRUMENT



EXAMPLE OF INCUCYTE S3 IMAGING OF THE COLONY OUTGROWTH

Series of whole images (overlay of brightfield and fluorescent) taken during the colony outgrowth of mCherry-expressing iPSCs on the 96-well microplate.



CONCLUSIONS

1. The most critical parameter for a successful single-cell cloning experiment is the optimal condition of harvested cells.
2. The UP.SIGHT™ instrument very reliably dispenses single cells with minimal damage to the cells.
3. Corning CellBIND® surface offers superior cell recovery over standard TC-treatment.
4. Standard cytoprotective reagents allow for single-cell cloning efficiency sufficient for downstream applications.
5. Incucyte® S3 instrument allows very convenient imaging of single-cell cloning experiments.

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