The Stem Cell Encyclopedia

What We Have Learned About High-Efficiency Single Cell Cloning of Human Pluripotent Stem Cells

Introduction

Pluripotent stem cells (PSCs) hold the potential to transform medicine as cellular therapeutics and models of disease with their unique ability to differentiate into any somatic cell type. PSCs encompass embryonic stem cells (ESCs), which are isolated from a blastocyst post in-vitro fertilization, and induced pluripotent stem cells (iPSCs), which are generated by overexpressing pluripotency genes in somatic cells.

The first human PSCs (hPSCs) were cultured on a feeder layer of mitotically inactivated mouse embryonic fibroblasts, which provided a matrix to promote growth, and in a culture medium supplemented with fetal bovine serum. However, feeder cells and serum both contribute to an undefined culture system with high lot-to-lot variability. The first commercial extracellular matrix (ECM) and medium designed for feeder cell-free hPSC culture were Matrigel® (Corning®) and mTeSR™1 (STEMCELL™ Technologies), respectively. Since then, a variety of ECMs and media have been designed for feeder-free hPSC culture, including chemically defined Vitronectin[1] and Essential 8[2]. Various culture reagents to support hPSC viability during passaging and single cell cloning have been developed as well, including gentle dissociation reagents like EZ-LiFT™ (Sigma-Aldrich®) and supplements like Y-27632 Rho-associated protein kinase (ROCK) inhibitor.

While hPSCs hold great therapeutic promise, *in vitro* culture remains a challenge as hPSC lines require meticulous culturing to maintain pluripotency and genetic stability. Furthermore, there is significant variation between hPSC lines in terms of stability, growth, and culturing preferences. These differences are particularly apparent in iPSC lines, as they are developed from donor samples with diverse genetics backgrounds, including variation in DNA methylation, relative abundance of protein and mRNA, morphology, and differentiation potential[3].

CellRaft® Technology provides an integrated platform for gene editing, differentiation, and single cell cloning of hPSCs, as well as reprogramming somatic cells. It utilizes time-course imaging, software-guided selection, and automated isolation of monoclonal hPSC colonies for downstream expansion and analysis. The CellRaft® Array can be used with a wide variety of media, extracellular matrices, and supplements, allowing each user to optimally support the growth of their unique hPSC line. Here, we discuss current recommendations and factors to consider for culturing and single cell cloning of hPSCs using CellRaft Technology.

Key Highlights:

1. Single cell cloning of hPSCs is time-consuming and often results in low cloning efficiency, lack of proven clonality, unwanted differentiation, and loss of pluripotency.
2. Viability of hPSCs during single cell cloning is improved by the CellRaft Array, which provides flask-like culture conditions while maintaining spatial segregation of monoclonal colonies.
3. CellRaft Technology provides an automated workflow that is compatible with a variety of hPSC coatings and media for the propagation of hundreds of clones.

Factors to Consider When Culturing hPSCs on the CellRaft Array

Array Size

The CellRaft Array is available in 100x100 µm and 200x200 µm sizes. These dimensions refer to the size of each CellRaft, which is the microscale polystyrene growth surface that will support the development of a single monoclonal colony. The 200 µm Array supports efficient hPSC colony growth and provides sufficient seeding density for high single cell cloning efficiency (Figure 1). Due to the larger growth area for colony formation, the 200 µm Array is recommended over the 100 µm Array format. A 200 µm single-reservoir Array contains 10,000 individual CellRafts, allowing for the generation of hundreds to thousands of monoclonal hPSC colonies on one consumable.

Dissociation Reagent

Successful single cell cloning on the CellRaft Array relies on seeding the Array with a single cell suspension of hPSCs. Some ROCK inhibitors, like Y-27632, require adaptation to single cell passaging while others, like CloneR2, do not. If using a ROCK inhibitor that requires adaptation, cells should be passaged as single cells at least twice before seeding the Array. Enzymatic dissociation reagents like TrypLE™ (Gibco™), Accutase, or EDTA work well for obtaining a single cell suspension.

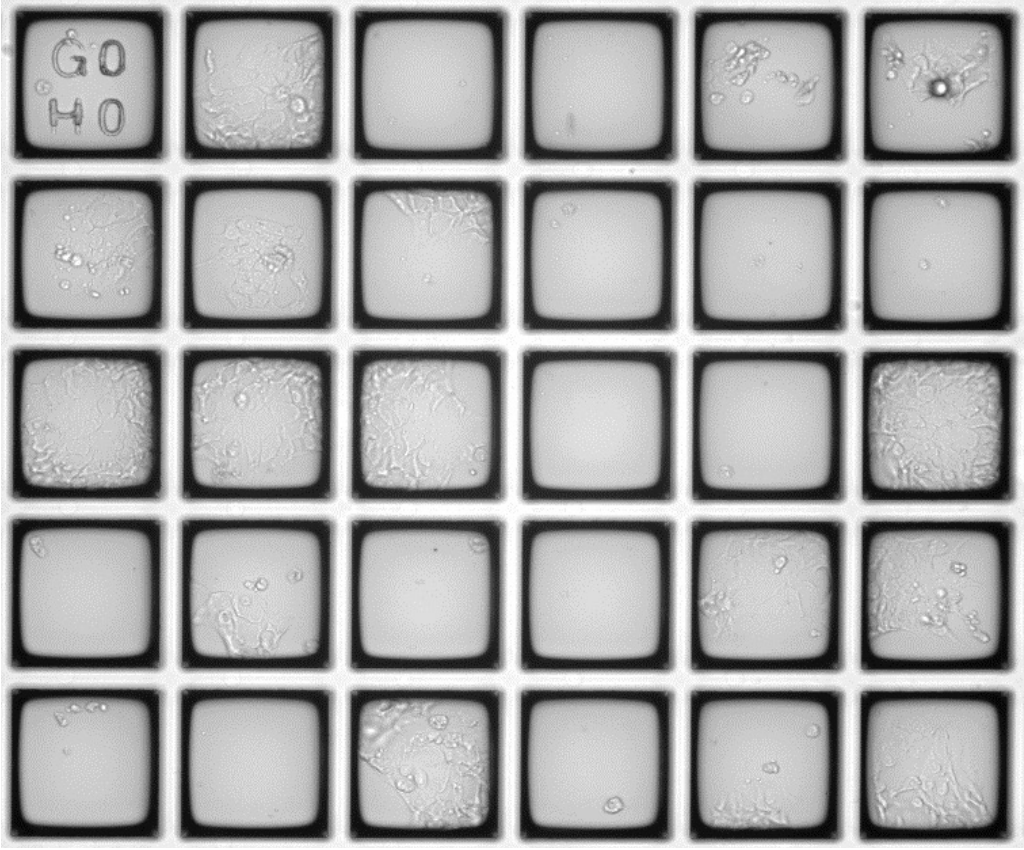

Figure 1. DYS0530 iPSCs (ATCC®) after 4 days of culture on a Vitronectin-coated 200 µm CellRaft Array seeded in mTeSR Plus supplemented with CloneR™2 (STEMCELL Technologies). Each image is of a releasable 200x200 µm CellRaft that allows for clonal propagation in shared media. Using the CellRaft AIR® System, CellRafts containing monoclonal colonies can be automatically transferred to collection plates where the colonies can expand further for downstream applications.

Media

Single cell cloning of hPSCs on the CellRaft Array has been validated with most hPSC media, including mTeSR Plus (STEMCELL Technologies), StemFlex (Gibco), Cellartis® DEF-CS™ (Takara Bio), Essential 8™ (Gibco), and PluriStem™ (Sigma-Aldrich), and custom media. In general, stem cell-specific media used for routine culture can be used on the CellRaft Array.

Extracellular Matrix

Culture of hPSCs on the CellRaft Array has been validated with most hPSC extracellular matrices, including hESC-Qualified Matrigel, Growth Factor Reduced Matrigel, Synthemax® II-SC (Corning), iMatrix-511 (Takara Bio), iMatrix-511 SILK (Matrixome), hESC-Qualified Geltrex™ (Gibco), VTN-N Vitronectin (Gibco), CellAdhere™ Laminin-521 (STEMCELL Technologies), and Cultrex (Bio-Techne®) (Figure 2). In general, any extracellular matrix that can be used to coat tissue culture-treated cultureware can be used to coat the CellRaft Array. However, coating on the array is typically done overnight compared to several hours on standard tissue culture dishes. For optimal viability, cells should be adapted to the desired matrix that will be used to coat the Array for at least two passages before seeding the Array.

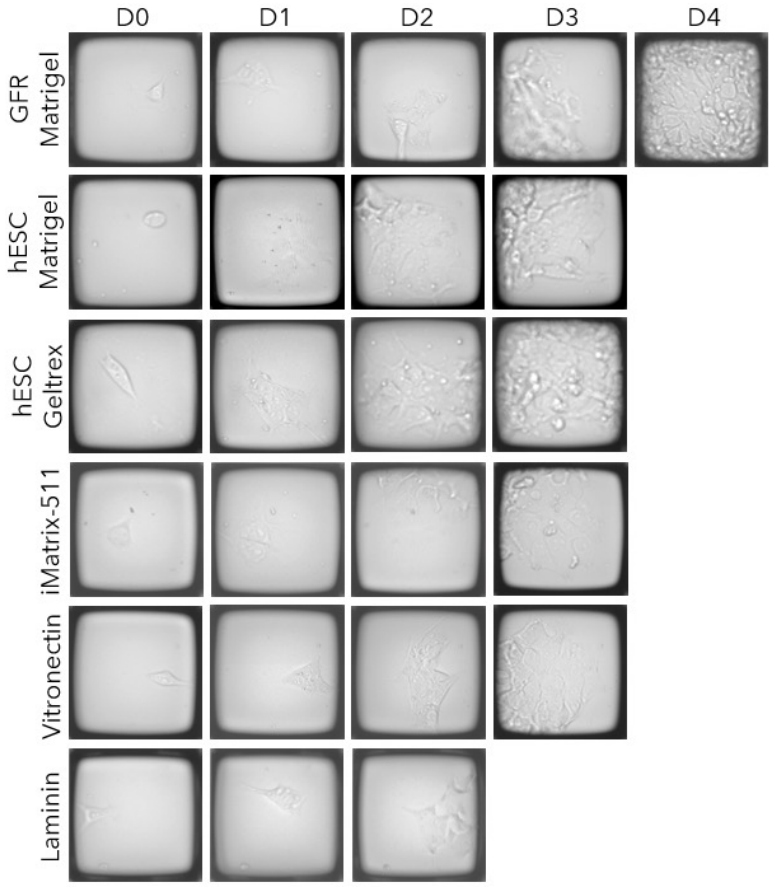

Figure 2. Clonal propagation of DYS0530 iPSCs on the CellRaft Array on Growth Factor-Reduced Matrigel, hESC-Qualified Matrigel, hESC-Qualified Geltrex, iMatrix-511, VTN-N Vitronectin, and Laminin-521.

Each hPSC line has unique preferences for matrices and matrix concentrations. For all hPSC lines, using a higher matrix concentration on the Array than is used in routine bulk culture typically improves viability of single cells. To optimize cloning of an hPSC line on the CellRaft Array, we recommend utilizing a quad-reservoir CellRaft Array to test different matrix concentrations. We typically test four increasing concentrations starting with the manufacturer-recommend concentration or the concentration routinely used to maintain the hPSCs in bulk culture (Figure 3).

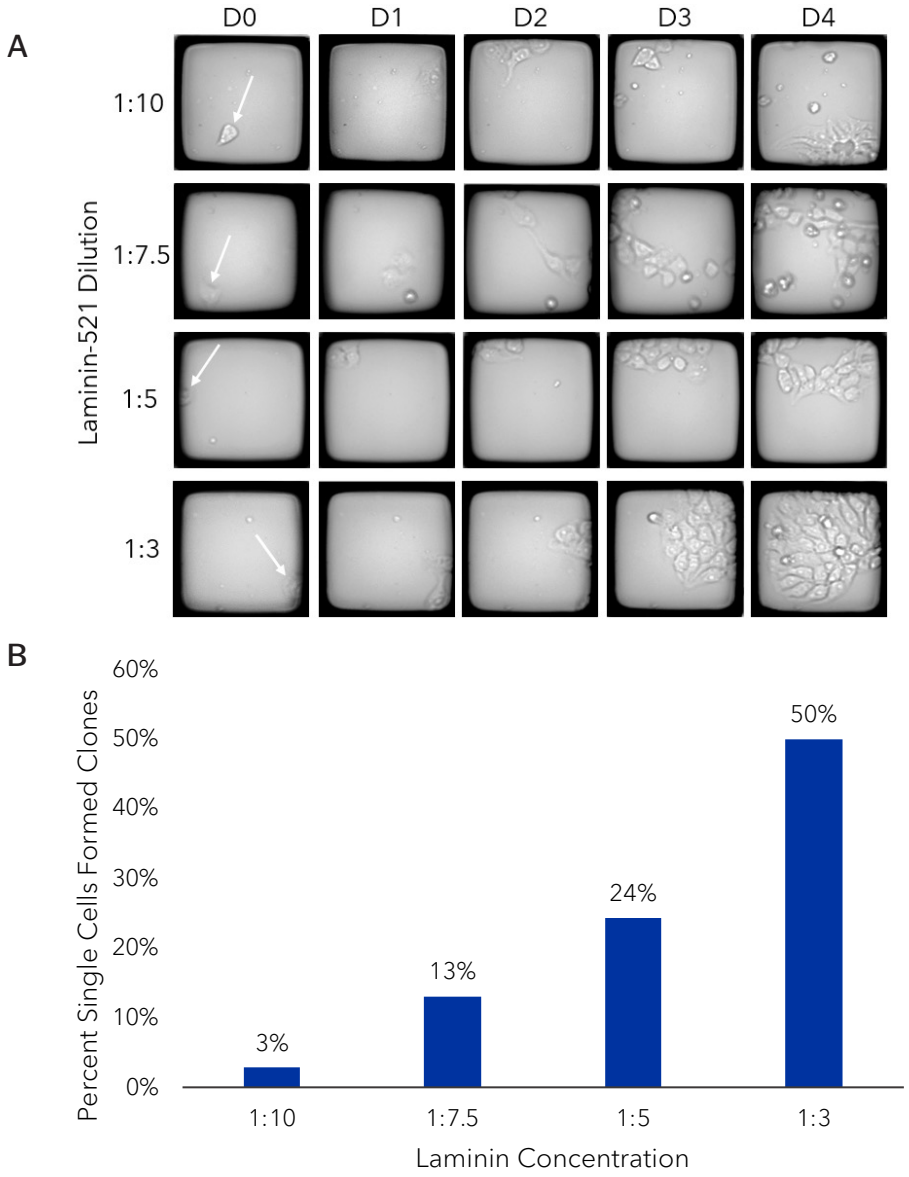

Figure 3. Clonal propagation and single cell cloning efficiency of ß-actin GFP iPSCs (Sigma-Aldrich, IPSC1030) on a 200µm CellRaft Array coated with increasing concentrations of Laminin-521. A. Time course images of single cells forming monoclonal colonies imaged daily on the CellRaft AIR System. B. Percent of single iPSCs that formed clones. While iPSCs were maintained in bulk culture at the manufacturer-recommended Laminin dilution of 1:10, coating the Array with a 1:3 dilution significantly improved single cell cloning efficiency from 3% to 50%.

ROCK Inhibitor Supplementation

ROCK inhibitors are widely used during single cell cloning of hPSCs to prevent dissociation-induced apoptosis or anoikis, and enhance single cell viability. ROCK inhibitor should be added to the culture medium when seeding a single cell suspension of hPSCs on the CellRaft Array. We have tested the ROCK inhibitor-containing survival supplements CloneR, CloneR2 (STEMCELL Technologies), RevitaCell (Gibco), Y-27632, Thiazovivin (STEMCELL Technologies), and CEPT Cocktail (Biotechne), and have found that CloneR2 and CEPT Cocktail result in the highest single cell cloning efficiency (Figure 4).

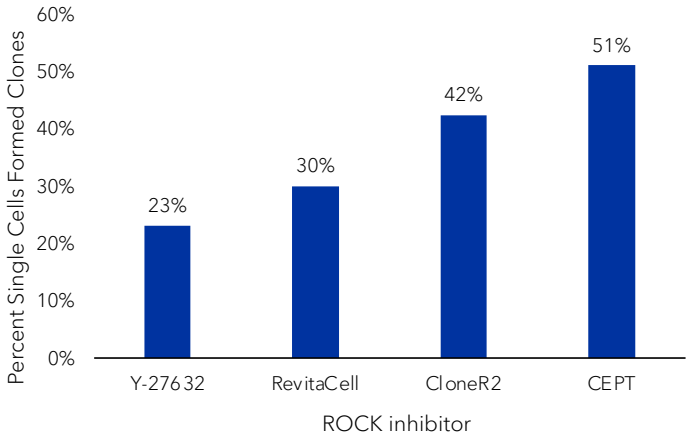

Figure 4. Single cell cloning efficiency of DYS0530 iPSCs on a CellRaft Array with four different ROCK inhibitor-containing supplements. The CEPT Cocktail resulted in the highest single cell cloning efficiency of iPSCs with 51% of single cells forming colonies.

Seeding Density

The optimal seeding density on the Array for a given cell line maximizes the number of CellRafts that are seeded with a single cell. This ensures sufficient density to condition the shared media and maintain high single cell cloning efficiency. A quad-reservoir Array can be utilized to test four different densities to determine the optimal seeding density for each unique hPSC line (Figure 5).

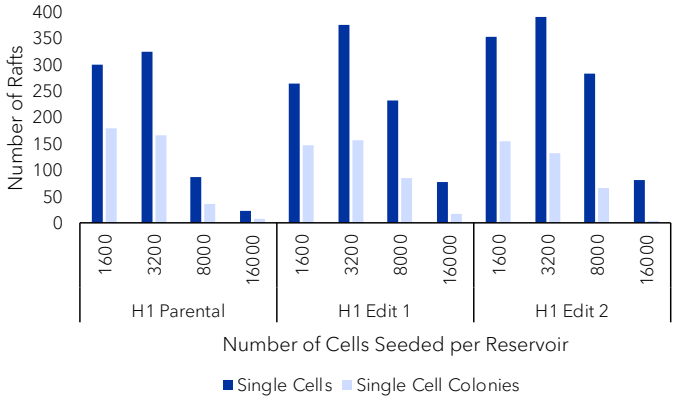

Figure 5. Evaluation of optimal seeding density for parental and edited H1 human embryonic stem cell lines. Parental and edited H1 cells were seeded on 200 µm quad-reservoir Arrays at different seeding densities and the number of CellRafts containing a single cell on the day of seeding and a monoclonal colony three days post-seeding was quantified. The optimal seeding density was determined to be 1600-3200 cells per reservoir for all three cell lines.

Timing and Frequency of Scans

The CellRaft Array can be serially scanned on the CellRaft AIR System to monitor cell viability, morphology, fluorescence, and clonality, and CellRaft Cytometry™ software can be subsequently used to identify single cells and colonies in brightfield. Fluorescent detection and quantification can also be performed to detect live fluorescent tags and/or stains.

A single scan takes approximately 10 minutes, after which the CellRaft Array is returned to the incubator for further culture. hPSCs seeded on the Array with ROCK inhibitor adhere rapidly (15-30 minutes post-seeding). Once adhered, hPSCs can flatten quickly and become very low contrast (Figure 6). Scanning the array approximately 15-30 minutes after seeding will significantly increase the accuracy of CellRaft Cytometry in identifying single cells. The Array should also be scanned 2-4 hours post-seeding to ensure that cells have adhered and maintained clonality.

After initial scans on the day of seeding to identify single cells, the Array can be scanned as often as desired. Our recommended workflow is to scan Arrays once per doubling time to monitor viability, clonality, and growth rate; however, more scans can be performed if desired.

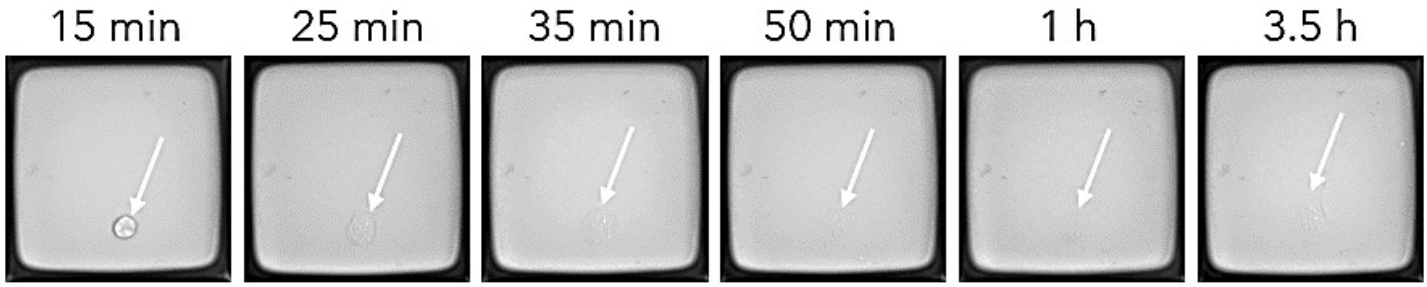

Figure 6. Time-course flattening of DYS0530 iPSCs onto the CellRaft Array starting 15 minutes post-seeding. At 15 minutes, the cell has settled into the microwell of the Raft but is still high contrast and easily identified in brightfield by CellRaft Cytometry. After only 25 minutes post-seeding, the cell begins to flatten and can be difficult to see.

Maintenance on Array

hPSCs require regular media changes to maintain cell viability and pluripotency. We have observed that seeding cells with the manufacturer-recommended concentration of ROCK inhibitor, followed by either feeding with fresh media and ROCKi or slowly diluting out the ROCKi with daily half media changes on the Array, can result in the highest number of viable monoclonal colonies on the Array (Figure 7, reservoir B2 and A1).

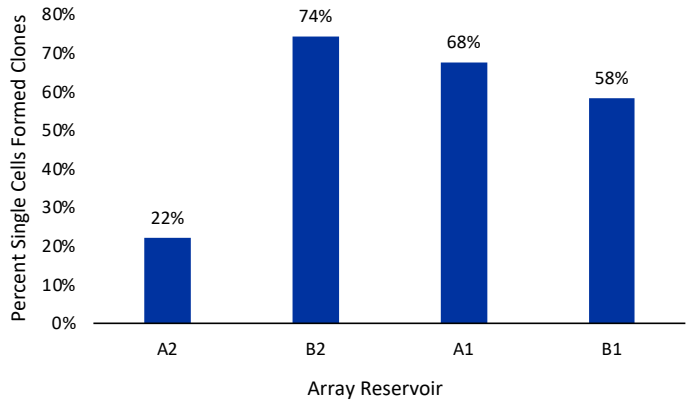

Figure 7. Effect of feeding schedule and ROCK inhibitor supplementation on KYOU-DXR019B iPSC (ATCC) proliferation on a 200 µm quad-reservoir Array. iPSCs were seeded on day 0 in 500 µL mTeSR Plus with 10 µM Y-27632 ROCK inhibitor per reservoir. Reservoir A2: full media change using mTeSR Plus only (no additional ROCK inhibitor) on days 1, 2, 4, and 5; Reservoir B2: full media change using mTeSR Plus with 10 µM ROCK inhibitor on days 1, 2, 4, and 5; Reservoir A1: topped up to 1 mL with mTeSR Plus only (no ROCK inhibitor) on day 1, half changes of media on days 2 and 4, and a full change of media on day 5 and Reservoir B1: no media changes throughout 5 days on the Array. The conditions in reservoirs B2 and A1 resulted in optimal clonal proliferation.

Fluorescence on the CellRaft Array

In addition to brightfield, the CellRaft AIR System has the capability for imaging in three fluorescent channels (see Table 1) for software-guided selection of fluorescently-tagged hPSCs for single cell cloning. hPSCs can also be live-stained directly on the CellRaft Array with cellular stains or conjugated antibodies (Figure 8).

Table 1. Fluorescence Detection on CellRaft AIR System

Channel	Representative Dyes	Excitation	Emission
Blue	Hoechst, SYTO 41	378-401 nm	412-453 nm
Green	FITC, Alexa488	460-490 nm	497-548 nm
Red	Texas-Red	559-591 nm	602-805 nm

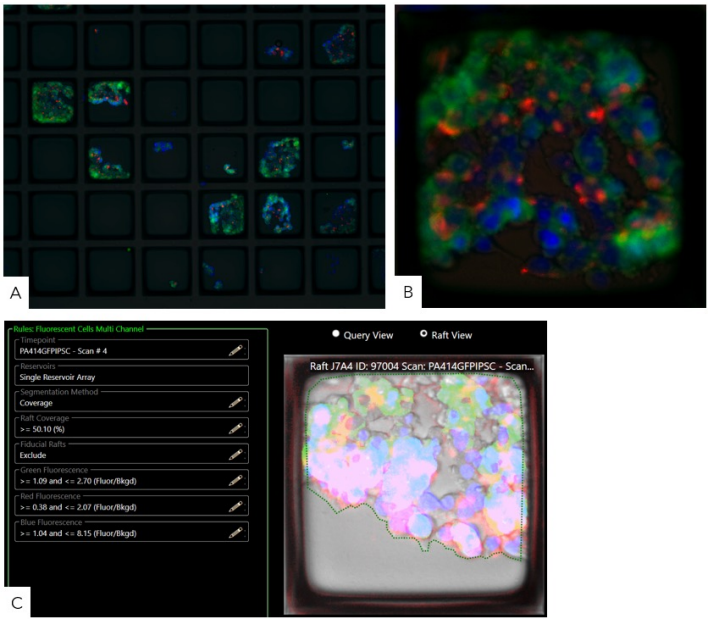

Figure 8. β-actin GFP iPSC colonies on a 200 µm CellRaft Array live stained with anti-TRA-1-60 Alexa Fluor 594 and NucBlue™ Hoechst 33342 Stain (Invitrogen™). β-actin -GFP hiPSCs were seeded on a 200S Array and allowed to proliferate for 5 days before live staining directly on the CellRaft Array. A. Composite field of view of CellRafts, B. Composite of an individual CellRaft, and C. Use of CellRaft Cytometry to identify CellRafts containing colonies expressing all three fluorescent markers.

Colony Size at Isolation

A unique advantage of CellRaft Technology is the ability to deposit a clone into a well of a 96-well plate after it has grown into a monoclonal colony, significantly improving outgrowth and clone health. hiPSCs were isolated at different timepoints to test the impact of colony confluency on outgrowth. hiPSCs were isolated at the 1 cell, 2 cell, 4 cell, small colony (<30% coverage), and large colony (>30% coverage) stages. Higher coverage resulted in a higher percentage of clones expanding off-raft after isolation to a collection plate (Figure 9). While it is recommended that the isolation day be characterized for each cell line, in general, isolating when the CellRafts are 50-75% covered is ideal for optimal outgrowth and isolation efficiency.

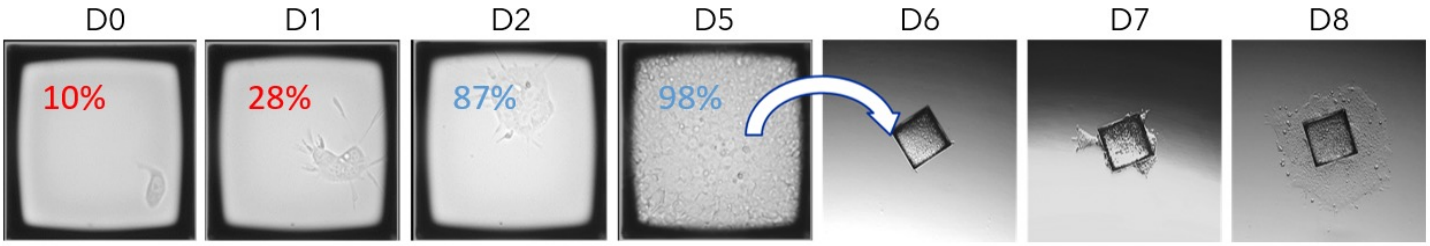

Figure 9. Percent outgrowth of KYOU-DXR019B hiPSCs isolated from a CellRaft Array at different timepoints. D0 is defined as the day of seeding cells on the Array. Percentages are given as the percent of isolated Rafts that had cells growing off-Raft 6 days post-isolation. Isolation on day 5 at 100% Raft coverage resulted in 98% outgrowth. Isolation at the single cell stage on day 0 resulted in only 10% outgrowth, comparable to cloning by limiting dilution of the same cell line.

Post-Isolation Expansion and Maintenance

For isolation of desired monoclonal colonies from the CellRaft Array for further expansion, a standard 96-well collection plate should be coated with the same extracellular matrix at the same or higher matrix concentration as was used to coat the Array. Once coated, the 96-well plate is loaded with 100 µL of the same media with the same survival supplement that was used to culture the hPSCs on the Array. If the hPSC line is primed for embryoid body (3D) formation, mix extracellular matrix into the media for the isolation plate to help prevent 3D growth. Once the desired CellRafts are successfully transferred to the collection plate, a media addition is required to maintain viability and pluripotency of hPSCs. As hPSC media contains ingredients like fibroblast growth factor that degrade quickly at 37 °C, hPSCs in collection plates should have 100 uL media without survival supplement added at Day 3 post-isolation. If the colony is not passaged at Day 5, a full media change should be performed daily starting at Day 5. As the CellRaft itself will not adhere to the collection plate, care should be taken to avoid aspirating the CellRaft when feeding plates.

After isolation and expansion off the CellRaft, cells can be passaged per normal off-array protocol. The CellRaft is a biologically inert polystyrene growth surface and will eventually be removed through cell passaging or media changes.

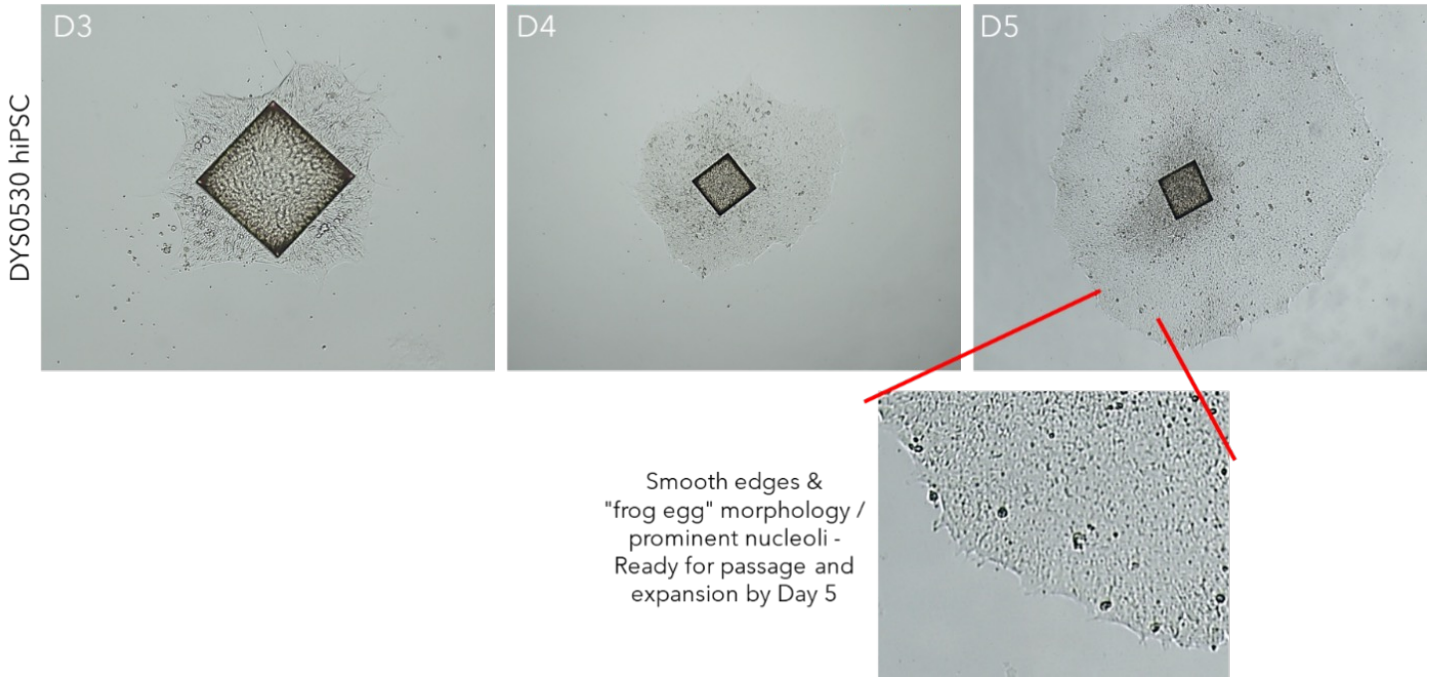

Figure 10. Time-course outgrowth of hiPSC clone expanding off-Raft 3 days, 4 days, and 5 days post-isolation to collection plate. When hPSCs reach the colony size shown in the third image, cells exhibit smooth edges with distinct morphology associated with pluripotent colonies. At this point, colonies should be dissociated and transferred to a new well of a 96-well plate to prevent differentiation. Clones can then be expanded stepwise from the 96-well plate.

Characterization and Analysis of hPSCs Cloned on the CellRaft Array

Hallmarks of healthy hPSCs include characteristic hPSC morphology, hPSC-specific surface markers, indefinite self-renewal, genome integrity, and potential to differentiate into the three primordial germ layers. hiPSCs cloned on the CellRaft Array were positive for TRA-1-60 and alkaline phosphatase activity (Figure 11), and hiPSCs reprogrammed from primary fibroblasts and subsequently cloned on the CellRaft Array demonstrated all five of these hallmarks. These data highlight that culture on the CellRaft maintains hiPSC phenotypes identically to traditional culture dishes and methodologies (Figures 12-13).

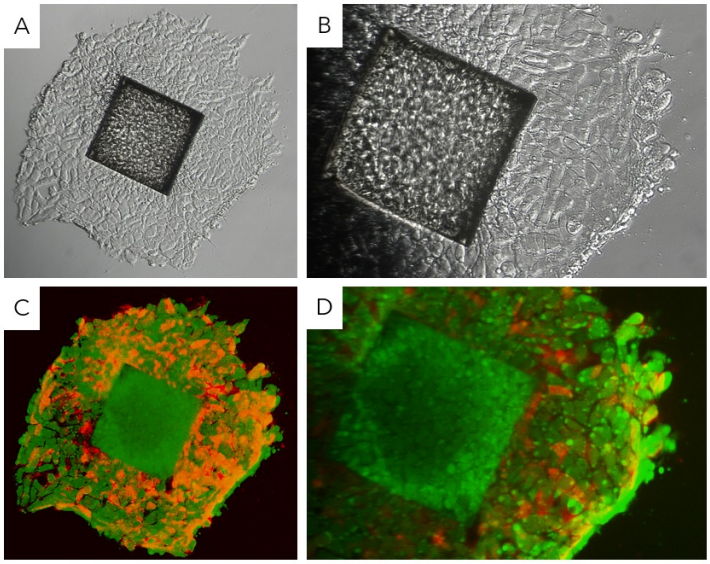

Figure 11. 200 µm CellRaft in collection plate with expanding hiPSCs stained with anti-TRA-1-60 AlexaFluor 594 and Alkaline Phosphatase Live Stain (Thermo Fisher). A. Brightfield, 10X and B. 20X magnification. TRA-1-60 (red) and alkaline phosphatase (green) staining, C. 10X and D. 20X magnification.

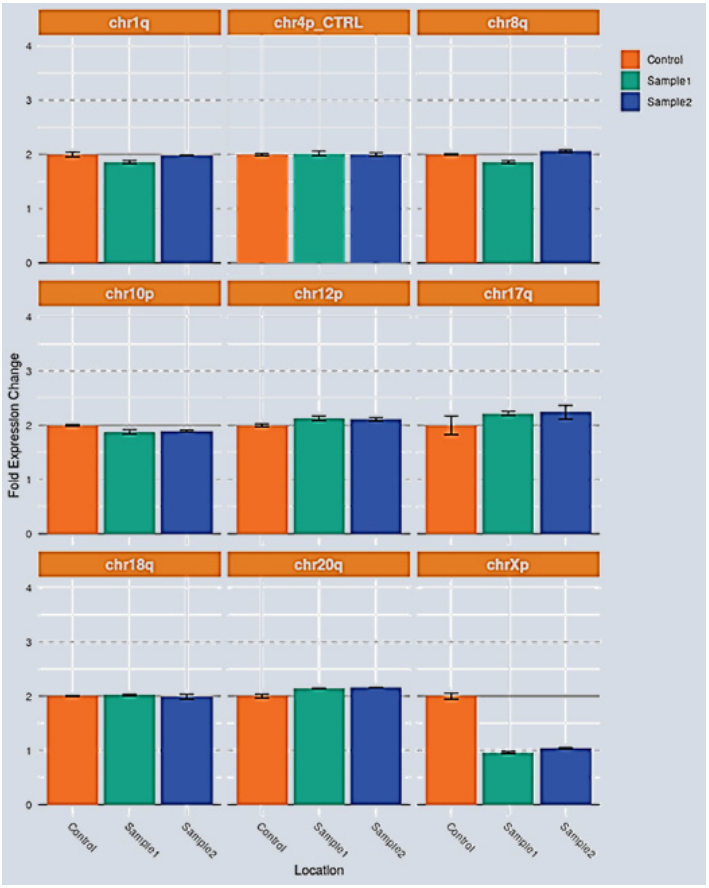

Figure 12. Genetic analysis qPCR readout of Control (Female), Sample 1 (Male iPSC clone), and Sample 2 (Male iPSC clone) using the hPSC Genetic Analysis Kit (STEMCELL Technologies). BJ fibroblasts were reprogrammed and cloned using the CellRaft AIR System to generate monoclonal iPSC populations then analyzed using qPCR to ensure normal karyotype.

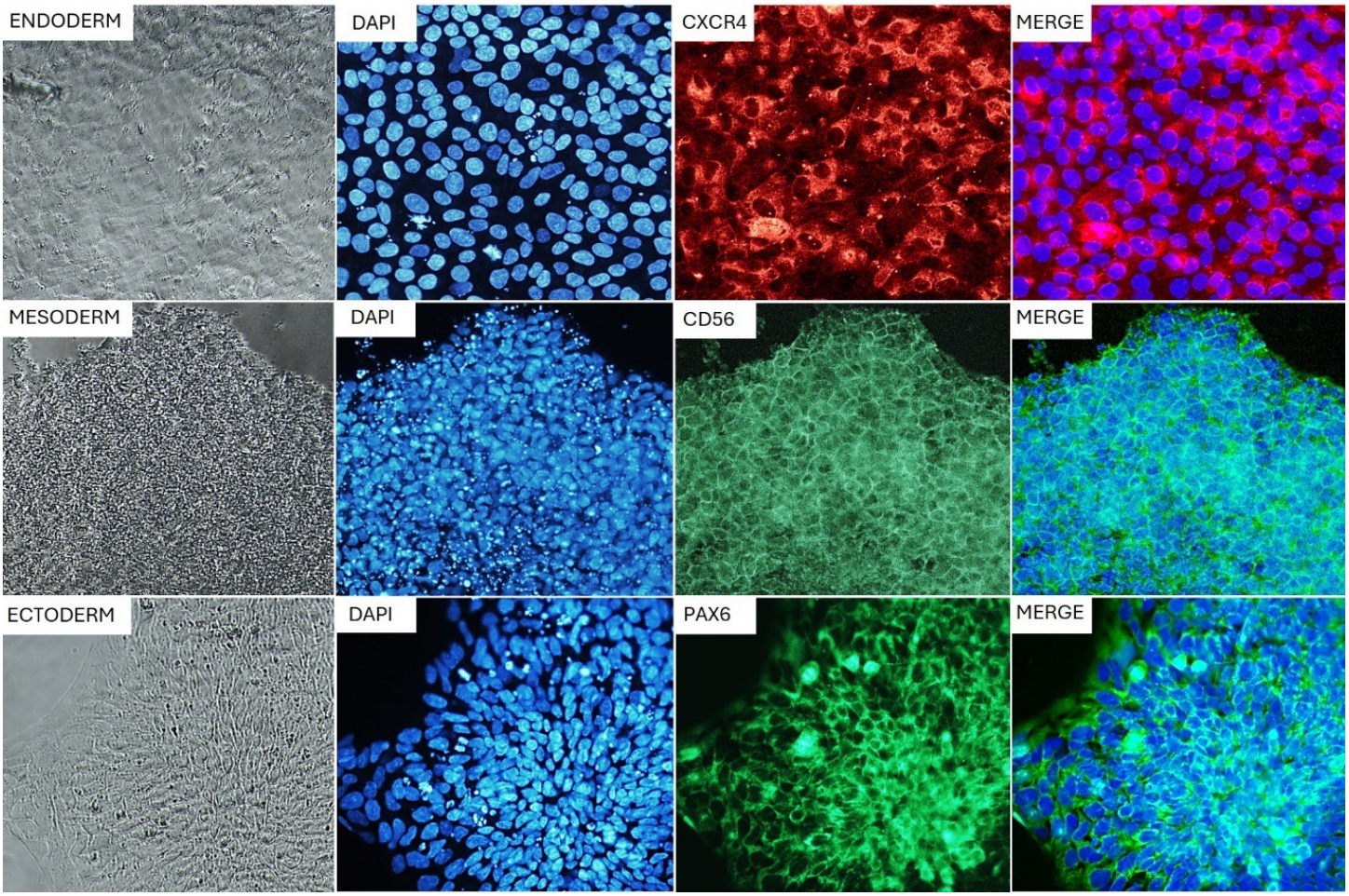

Figure 13. Staining of trilineage differentiation markers of hiPSCs reprogrammed from primary fibroblasts and cloned using the CellRaft AIR System. Differentiation was performed using the STEMdiff™ Trilineage Differentiation Kit (STEMCELL Technologies). Differentiated cells were fixed using a 4% paraformaldehyde solution, blocked using 1% bovine serum albumin, and then stained for endoderm with anti-CXCR4, AlexaFluor 594 conjugated (Bioss Antibodies), mesoderm with anti-human CD56/NCAM, FITC conjugated (STEMCELL Technologies), and ectoderm with rabbit anti-human PAX6 unconjugated primary with anti-rabbit FITC conjugated secondary antibodies (20X magnification).

Conclusions

CellRaft Technology provides a solution for complex workflows, including gene editing, differentiation, and single cell cloning with sensitive cells like hPSCs. Additionally, the CellRaft AIR System provides a solution to manual, labor-intensive processes like reprogramming somatic cells into iPSCs, with time-course imaging, software-guided selection, and automated isolation of monoclonal hPSC colonies for downstream expansion and analysis.

With this flexibility, the CellRaft Array can be used with a wide variety of media, extracellular matrices, and supplements, allowing each user to optimally support the growth of their unique hPSC line. While this encyclopedia covered many current recommendations and factors to consider for culturing and single cell cloning of hPSCs using the CellRaft Array, it is important to note that every hPSC cell line may respond differently and need its own optimized protocol.

Using the factors listed below, an easy and reliable method to obtaining your desired cells can be developed with CellRaft Technology:

- Array Size
- Dissociation Reagent
- Media
- Extracellular Matrix
- ROCK Inhibitor Supplementation
- Seeding Density
- Timing and Frequency of Scans
- Maintenance on Array
- Fluorescence on the CellRaft Array
- Colony Size at Isolation
- Post-isolation Expansion and Maintenance

To seamlessly implement hPSC workflows with the CellRaft AIR System in your lab and create an optimized protocol for these variables, download our hPSC-specific standard operating procedures from cellmicrosystems.com/knowledge-base to guide you in your assay development.

References

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For more information on the presented data or CellRaft Technology, visit cellmicrosystems.com

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