CELL Microsystems®

CASE STUDY

Gene Editing Success: A Case Study in Cloning Rare Edited iPSC Lines

Introduction

CRISPR/Cas9 technology has the power to manipulate gene expression in cells at a base pair level. Having the ability to edit cells at specific locations invites the opportunity to study the root cause and effects of mutations on physiological processes and ultimately use that understanding to develop novel treatments for diseases. To enable this, researchers often develop reporter cell lines, commonly engineered to induce a visually identifiable phenotype using fluorescence, to study genes involved in diseases. To ensure stability and homogeneity of phenotype and response once a reporter is established, monoclonal colonies are derived using the edited cells. However, when the CRISPR edit results in cells that are rare, poorly fluorescent, slow growing, or non-viable, establishing a model cell line becomes incredibly challenging and inefficient, leading to stalled scientific progress.

This case study highlights this paradigm and how novel approaches can overcome the biological challenges of traditional CRISPR editing workflows, particularly in difficult-to-use cell types. An academic scientist using a CRISPR knock-in strategy to tag a lowly expressed, imprinted gene with green fluorescent protein (GFP) in a diseased induced pluripotent stem cell (iPSCs) line had failed to derive a tagged line after a year of effort. Much of the difficulty stemmed from the complex folding of the C-terminus that GFP was fused to, making it hard to visualize and sort positives using traditional methods like fluorescence-activated cell sorting (FACS) or droplet dispensing. The researcher was searching for a way to improve their chance of success, which led them to Cell Microsystems and CellRaft Technology.

Key Highlights:

1. Direct-to-array workflow after electroporation enabled rapid screening of edited iPSCs weeks faster than traditional methods.
2. CellRaft Technology delivered a monoclonal edited iPSC fluorescent reporter line where FACS and NamoCell failed.
3. Stable edited iPSCs could differentiate into 2D forebrain neurons and 3D cerebral organoids after use of CellRaft Technology.

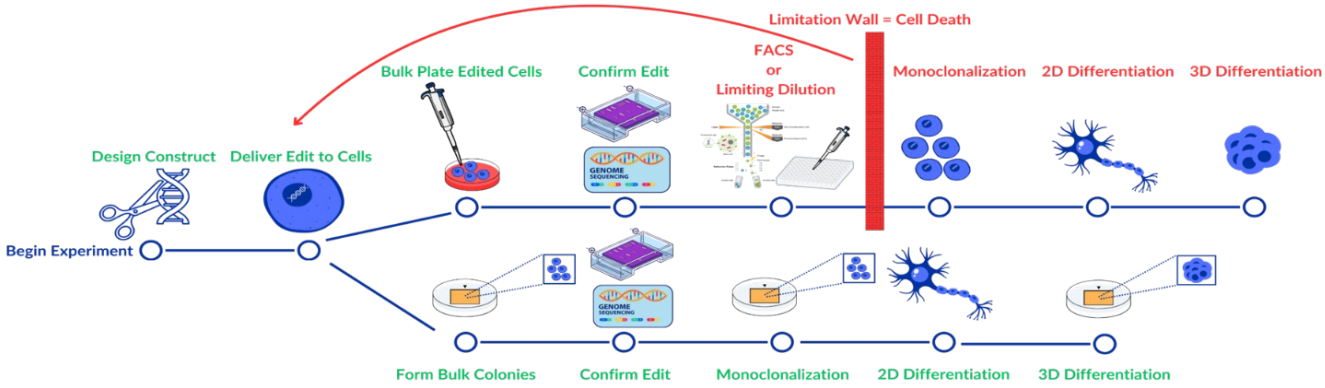


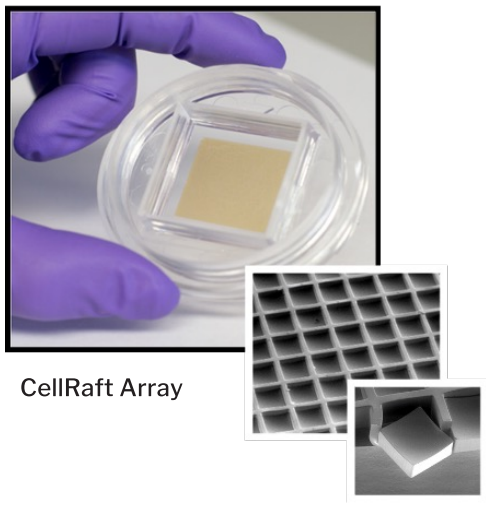
Figure 1. Graphical Abstract of Editing Cells for Differentiation Workflows. Cells were electroporated with the CRISPR construct and plated in bulk in either a standard plate or on a CellRaft array. The edit was confirmed in the cell population then seeded on a CellRaft Array or FAC sorted for monoclonalization. In the standard workflow using FACS, cells reached a limitation wall where they ultimately died, and the researcher had to repeat the experiment in an attempt to derive the desired cell line. In the CellRaft Array workflow, the desired gene edit was successfully confirmed during monoclonalization and the experiment continued further into 2D and 3D differentiation.

Scientific Problem

To generate an edited stem cell line, CRISPR/Cas9 technology was used to knock-in a fluorescent reporter gene fusion using electroporation. iPSCs were previously sorted using a low-pressure cell sorter. However, the gene had very low levels of expression that could not be detected. Additionally, the stress of the editing and sorting process resulted in cell death. The researcher entered the Clone Challenge program at Cell Microsystems to increase their chances of successfully cloning positive iPSCs using CellRaft Technology.

Experimental Design

As shown in Figure 1, iPSCs were electroporated using the Neon™ Transfection System (Invitrogen™) and seeded onto a 200µm single-reservoir CellRaft® Array. Cells were imaged over time using the CellRaft AIR® System to generate a full clonality report of time course scans. CellRaft Cytometry™ software was utilized to find CellRafts containing viable colonies using advanced brightfield algorithms. Viable cell colonies were then automatically isolated and deposited into 96-well collection plates using the CellRaft AIR System. Cells were expanded off the CellRaft and were duplicated into additional plates for screening. gDNA was extracted and the target sequence was amplified using qPCR. To visualize the target region, gel electrophoresis was run along with a positive control to identify edited populations..



CellRaft Array

The positive heterogenous populations of cells were screened on a 200µm Single CellRaft Array to create monoclonal colonies that were isolated using the CellRaft AIR System. Monoclonal colonies were expanded in the 96-well collection plates and the presence of the knock-in reporter gene was again confirmed in the clones via qPCR screening of gDNA and Sanger Sequencing. Since the reporter is not active in the pluripotent state, a STEMdiff™ Forebrain Neuron Differentiation Kit (StemCell™ Technologies) was used to differentiate the clonal and parental iPSCs into forebrain neurons. After 11 weeks, neurons were stained for TUJ1, a neuronal lineage marker, and DAPI before being imaged under a microscope. In addition, the STEMdiff™ Cerebral Organoid Differentiation Kit (StemCell™ Technologies) was used to differentiate the clonal and parental iPSCs into cerebral organoids. After 11 weeks, whole organoids were cleared or cryo-sectioned and then stained for TUJ1 and DAPI before being imaged under a microscope.

Results

Immediately after the patient-derived iPSCs were electroporated with the CRISPR/Cas9 knock-in construct, the cells were seeded directly onto the CellRaft Array to maximize viability and minimize the time necessary to recover and proliferate. Using CellRaft Cytometry Software, CellRafts that contained a healthy colony were identified. To expand the healthy electroporated cells, polyclonal colonies were isolated into 96-well plates using the CellRaft AIR System (Figure 2). To determine if the isolated colonies were positive for the edit of interest, gDNA was extracted, amplified, and analyzed for the gene of interest. Of the 10 colonies tested, 2 contained a correctly spliced band (Figure 3).

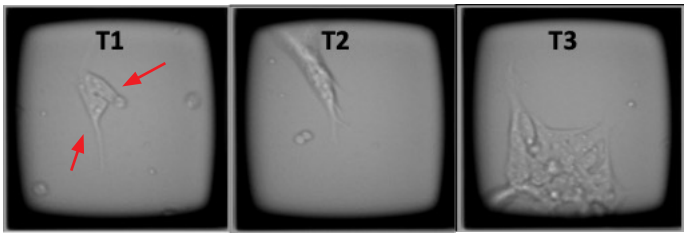


Figure 2. Image-Based analysis of CellRafts identified positive polyclonal populations. 200µm Single CellRaft Array images of Raft ID: E1D4 deposited into well B3. At timepoint 1, there are two cells (red arrows) noticed in the first scan indicating this to be a polyclonal colony.

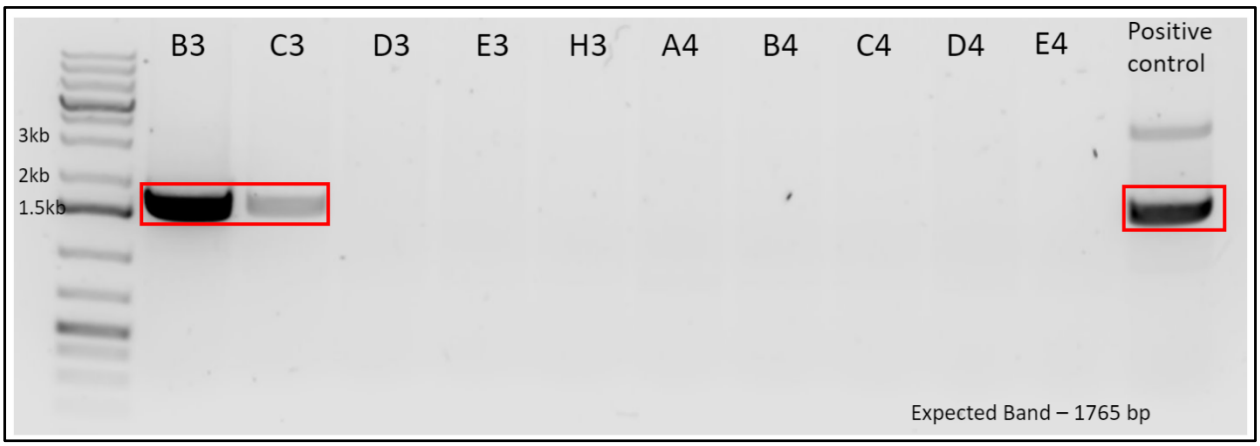


Figure 3. Gel Electrophoresis identified two positive polyclonal colonies for the reporter gene edit. Expected band should appear at 1765 bp and is visualized in the positive control as well in B3 and C3.

Since polyclonal cell lines can lead to heterogeneity of response and population drift, the researcher wanted to generate a stable monoclonal population. To create monoclonal colonies with the edit of interest, the polyclonal cells were expanded and seeded on a CellRaft Array. After serially scanning the CellRaft array daily for four days, CellRaft Cytometry software was used to identify CellRafts containing monoclonal iPSC colonies. Using the CellRaft AIR System, 80 monoclonal colonies were isolated and expanded (Figure 4). To confirm which of the monoclonal isolated colonies contained the desired edit, 17 colonies were selected and screened as previously described. All 17 monoclonal colonies displayed bands spliced in the appropriate location, demonstrating that the cells had successfully incorporated the tag (Figure 5). To understand the expression of the gene of interest (GOI), 3 positively identified monoclonal colonies were screened using qRT-PCR, and the expression of the knock-in gene of interest was positively expressed compared to wildtype cells (Figure 6). To confirm the GOI was in the correct sequence with no abnormal splicing or irregular insertions, the clones were Sanger sequenced and validated to confirm the fluorescent reporter sequence.

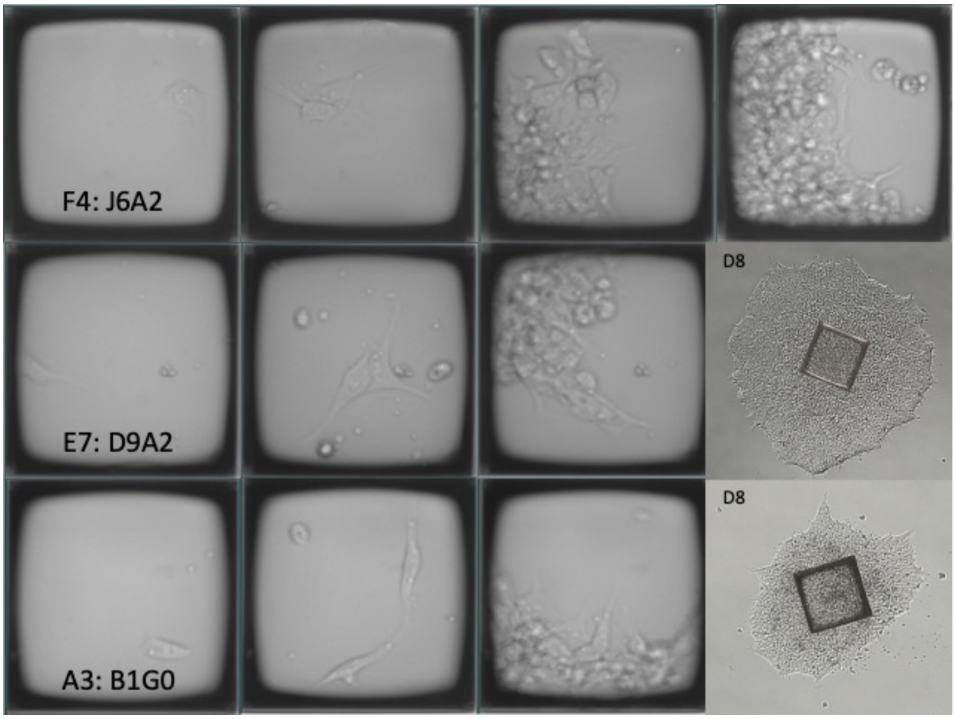


Figure 4. Image-Based analysis of CellRafts identified positive monoclonal populations. Time course images of 3 representative CellRafts containing monoclonal colonies along with outgrowth images in the 96-well plate.

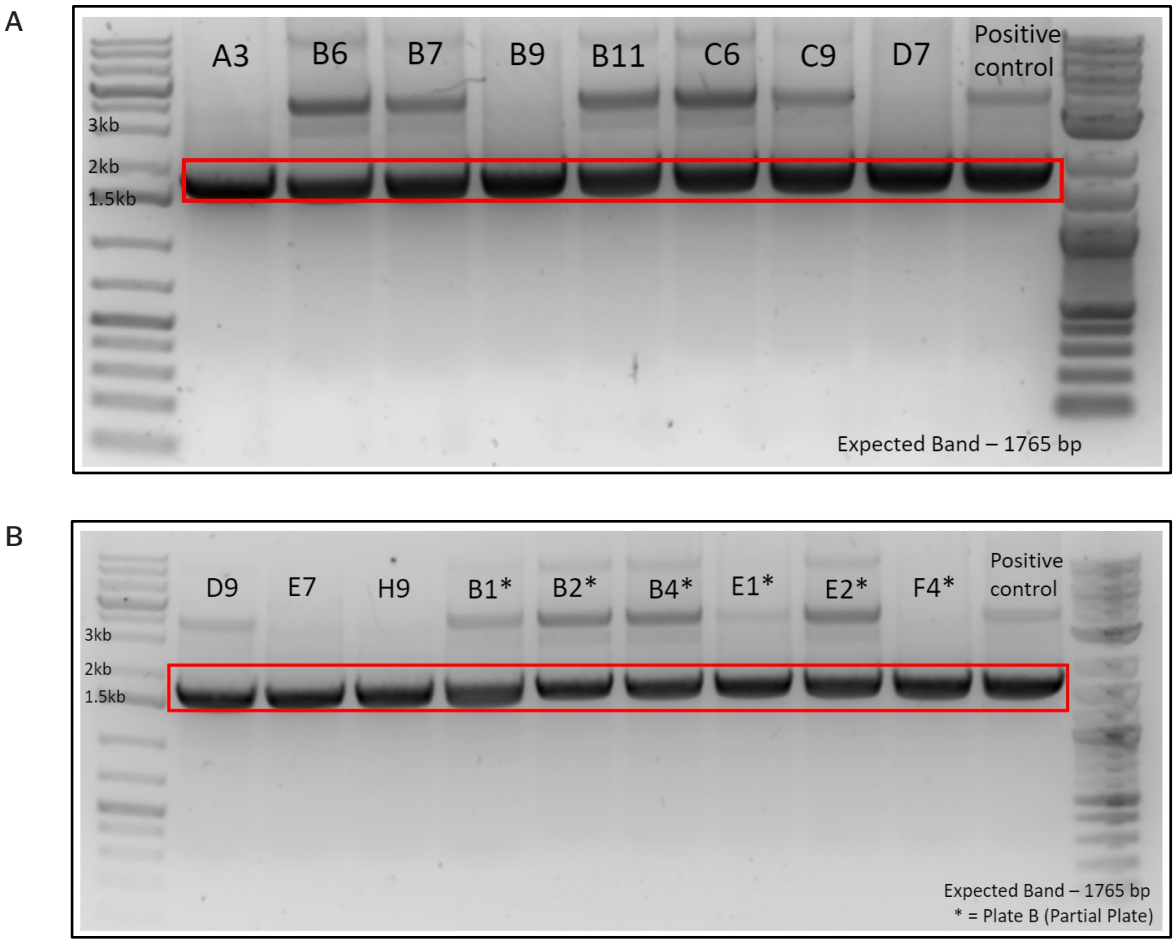


Figure 5. Gel Electrophoresis identified 17 positive monoclonal colonies for the gene edit. (A, B) Gel Electrophoresis of 17 potential colonies displaying positive editing of gDNA target sequences and the positive control lanes.

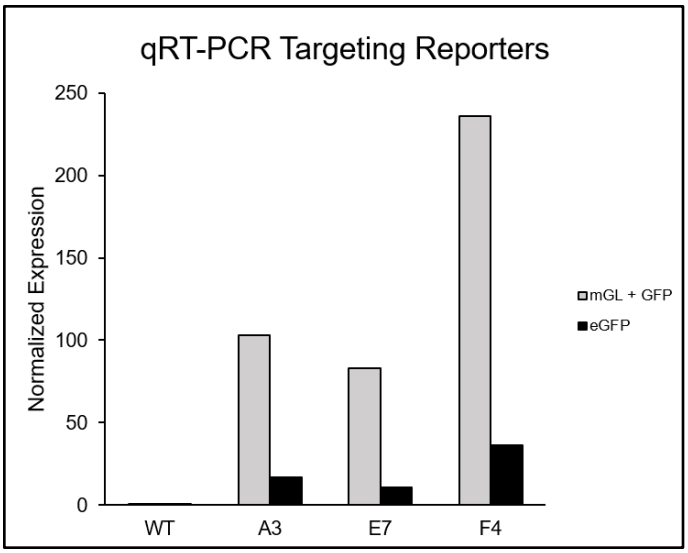


Figure 6. RT-qPCR confirms expression of GOI in reporter lines compared to wildtype. RT-qPCR of positive clones displaying GFP expression normalized to the wildtype (WT) colony.

After confirming that the three candidate reporter lines expressed the correct gene edit, it was necessary to confirm expression of GFP after gene activation. Since the reporter is not highly active in the pluripotent state, the cells were stained using an antibody against the reporter and visualized under ICC enhancement (Figure 7). To visualize the reporter after differentiation and drug treatment, the three clones were differentiated into forebrain neurons, treated for 72 hours with compound, and then stained for the neuronal marker TUJ1 and GFP. As shown in Figure 8, the clones were able to successfully differentiate into TUJ1 positive neurons, and treatment with the drugs activated expression of the knocked-in reporter gene (Figure 8).

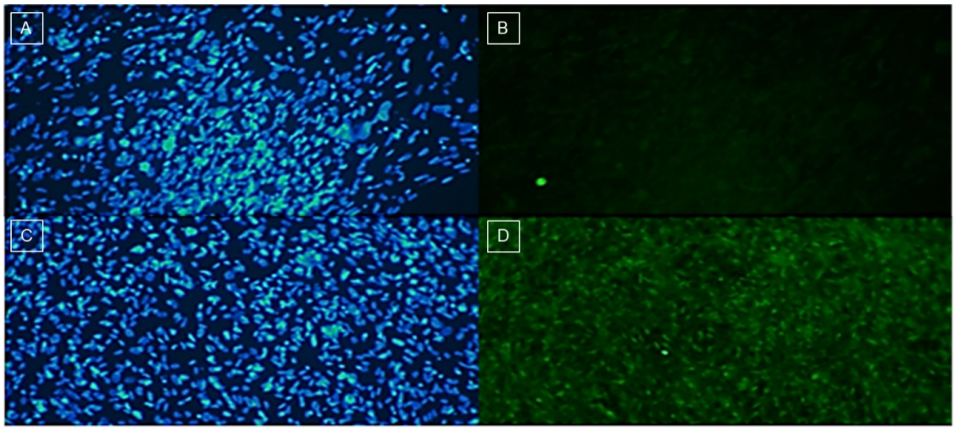


Figure 7. Antibody staining confirms reporter expression in stem cells. Confirmed edited iPSCs were expanded and plated for ICC enhancement of the reporter as reporter was not expressing in native state. (A) parental stained DAPI, (B) ICC enhanced negative parental, (C) edited stained DAPI, and (D) ICC enhanced positive reporter.

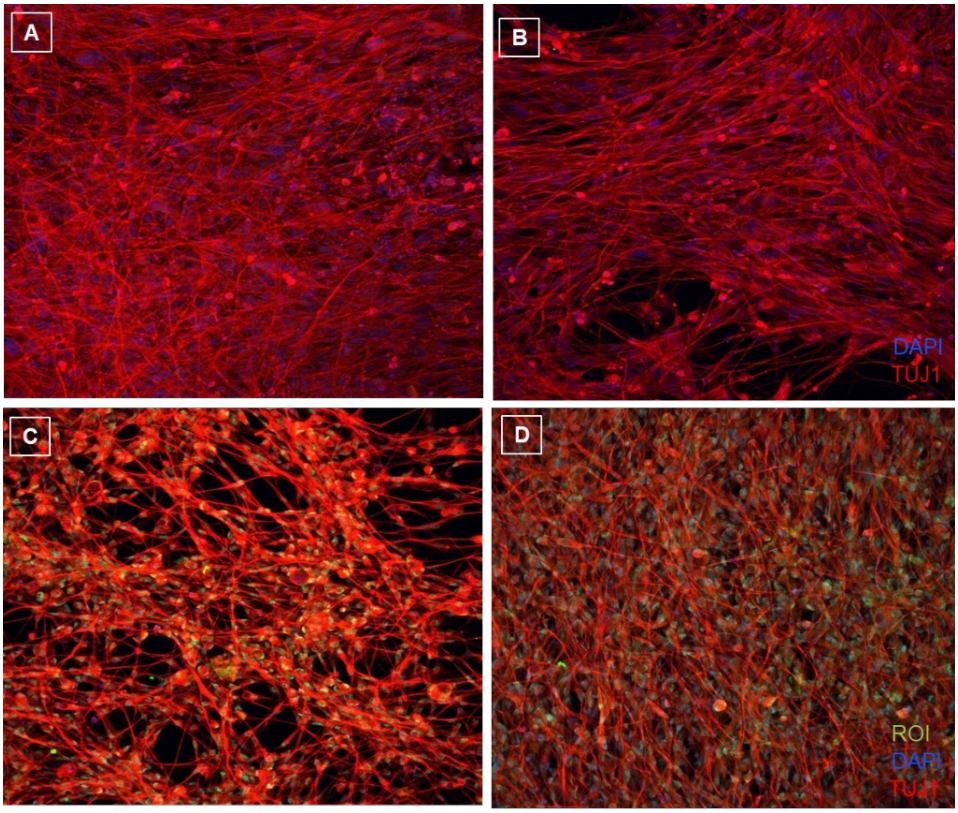


Figure 8. 11-week-old edited forebrain neurons respond to drug treatment. ICC enhanced neurons for reporter of interest (ROI) (green), TUJ1 (red), and DAPI (blue). (A, B) Parental 11-week-old forebrain neurons stained with TUJ1 and DAPI. (C, D) Edited 11-week-old forebrain neurons stained with TUJ1, DAPI, and ROI. Differentiation followed STEMDiff Forebrain Neuronal Kit and replating neurons for imaging followed the JOVE Protocol (Calabrese, 2019).

Lastly, to determine whether the expression of the reporter gene could be visualized in a more mature neuronal model, one clone was differentiated into 3D cerebral organoids and treated with compound to induce GOI expression. Whole organoids were cleared and/or sectioned, stained for TUJ1, DAPI, and the GOI. As shown in Figure 9, the cerebral organoids were positive for both the reporter gene and TUJ1, confirming successful development of the desired reporter line.

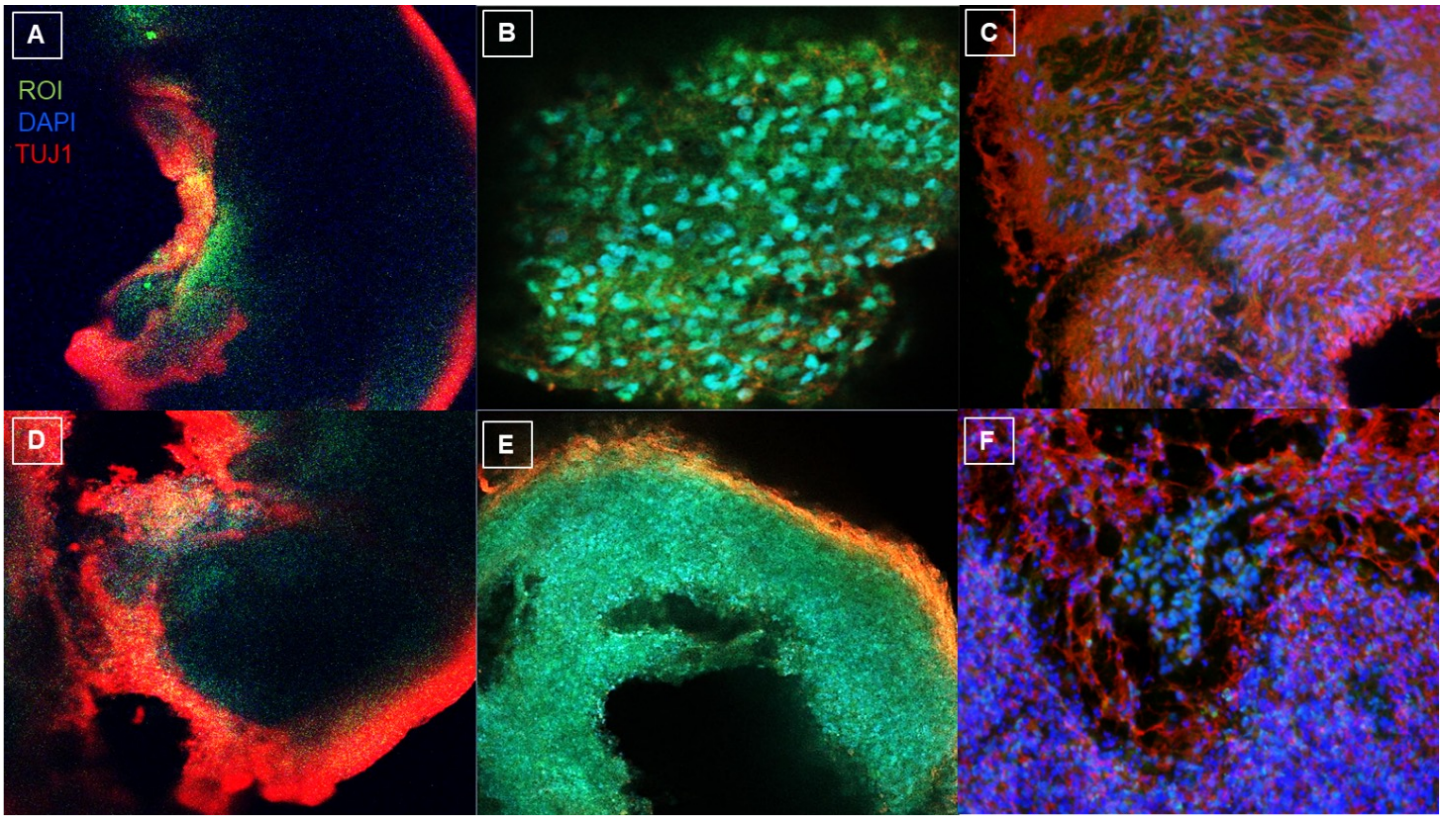


Figure 9. 11-week-old reporter iPSCs differentiate successfully into cerebral organoids. ICC enhanced organoids for reporter of interest (ROI) (green), TUJ1 (red), and DAPI (blue). (A, D) 11-week-old ICC enhanced whole cerebral organoid 10X images. (B, E) 11-week-old ICC enhanced whole cerebral organoid 20X images. (C, F) 11-week-old ICC enhanced sectioned cerebral organoid 20X images. All of them have been stained for TUJ1 and DAPI and differentiated using the STEMDiff Cerebral Organoid Kit.

Discussion

CRISPR/Cas Technology allows researchers to study diseases, investigate treatment options, and even cure disease through reversing mutations. In vitro studies of genetic diseases improve when cell colonies are monoclonal and contain a homogenous population of a desired edit. However, CRISPR/Cas9 editing often results in heterogeneous populations containing positively edited cells, cells containing an edit that is only partially integrated into the target sequence, and unedited cells. Identifying those cells positive for the edit of interest involves techniques such as limiting dilution or FACS to single sort cells which then expand into monoclonal colonies that can be assessed for the edit. However, many cell types cannot survive the pressure and fluidics during sorting or the stress of growing alone in a well without extracellular growth factors and cytokines to promote viability. For the researchers in this case study, this problem became apparent as they were unable to derive a monoclonal edited reporter iPSC line that survived any of the traditional methods.

CellRaft Technology and the CellRaft AIR System provide a gentle solution to single cell cloning. In this workflow, viability was the key to success using the CellRaft Array. The Array allowed the stressed cells from electroporation to be rescued and recovered, improving the viability of even fragile edited cells. While most cloning methods put a single cell in a well, the CellRaft Array allowed for thousands of potentially edited cells to be screened within individual microwells spatially segregated from one another, compared to hundreds in limiting dilution. With increased screening capacity, improved viability, and the ability to isolate established colonies of cells into collection plates, rather than single cells, the difficulties of the editing process were overcome with CellRaft Technology.

Conclusions

In this Clone Challenge, collaborating scientists sent parental iPS cells to Cell Microsystems, with the expressed goal of developing a critical reporter cell line for their research. At CMS, our team generated edited monoclonal iPS clones in only 5 weeks. With the help of the collaborators, the downstream validation to confirm successful editing took a total of 7 weeks. Further downstream characterization confirmed that the edited clones were capable of being differentiated into mature forebrain neurons and cerebral organoids that expressed the reporter of interest. These data demonstrate that CellRaft Technology was able to easily and quickly generate a fully validated, edited patient-derived iPS clone in only 3 months, which enabled the researcher to embark on their critical drug discovery studies after over a year of struggling with other methods and a lack of progress.

References

1. Calabrese, B., Powers, R. M., Slepian, A. J., Halpain, S. Post-differentiation Replating of Human Pluripotent Stem Cell-derived Neurons for High-content Screening of Neuritogenesis and Synapse Maturation. J. Vis. Exp. (150), e59305, doi:10.3791/59305 (2019).
2. Lloreda, C. L. (2021, April 7). New maps link thousands of genetic variants to disease genes. Broad Institute. https://www.broadinstitute.org/news/new-maps-link-thousands-genetic-variants-disease-genes
3. Redman, M., King, A., Watson, C., & King, D. (2016, August 1). What is CRISPR/cas9?. ADC Education & Practice Edition. https://ep.bmj.com/content/101/4/213