Establishment of clonal Organoid cultures after CRISPR-Cas9 genome editing using the Yamaha CELL HANDLER™

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Introduction

Stem cells are cells that can divide to give rise to different types of daughter cells, while retaining their 'stemness'. Organoids are three-dimensional structures grown from these stem cells, that can be cultured and expanded long-term in the laboratory[1]. Since the development of the first culture methods of adult intestinal stem cell-derived Organoids[2], these methods have been adopted to allow Organoid generation from a wide variety of different tissues, both healthy and diseased.

Although Organoid Technology does not require special equipment and can therefore be set-up in almost every tissue culture facility, Organoid culture and manipulation can be time-consuming depending on the intended application. Previously, we described gene editing methods such as CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas9 modified for use in Organoids[3–5]. Clonal Organoid lines can be robustly generated using the published methods however, it is challenging and time-consuming.

The first step in methods to generate genetically modified cellular models results in a mixed, polyclonal, population of cells. Depending on the applications, it is required to develop the model into a clonal line by deriving Organoids from single modified stem cells. In particular, when generating lines with CRISPR-Cas9, the generation of a clonal line is a key benefit of the CRISPR Technology. As with all methods, the efficiency of genetic modification is <100%, therefore selection of the modified cells, for example marked by GFP (Green Fluorescence Protein) expression, is required. Secondly, CRISPR-based genome editing will result in a mixture of genotypes, depending on how the induced DNA damage is repaired in each individual stem cell. Therefore, to obtain genetically 'pure' cultures after CRISPR-based genetic engineering, clonal expansion and subsequent sequencing is required. For this, individual Organoids (derived from individual stem cells) are picked and transferred to individual wells. These individual Organoids are subsequently passaged and expanded as clonal Organoid cultures. Depending on the number of clonal cultures desired, this can be a laborious process. Technical advances including automated systems that can recognize Organoids and transfer them to a different culture plate, can help to decrease time required to select lines and improve accuracy.

The Yamaha CELL HANDLER™ is an image-based cell selection system, that can be used to identify, select, and pick up Organoids to transfer them to individual wells. Selection of Organoids is based on (fluorescent) phenotype and can be automated based on morphological features.

Here, we report on the feasibility of the Yamaha CELL HANDLER™ to select CRISPR/Cas9 modified Organoids based on GFP expression which greatly improves the throughput of clonal Organoid selection and expansion.

Methods

Organoids were disrupted into single cells and electroporated to introduce pCas9-GFP construct (Addgene No. 48138)[5] encoding the Cas9 nuclease plus an sgRNA (5'-TAACCGGCCCTGGGAATATA-3') targeting the *AAVS1* locus. Organoids were reseeded and left to recover for two days before the CELL HANDLER™ was used to select GFP+ Organoids and transfer them to individual wells. Organoids were transferred to a 384-well plate (one Organoid per well) and left to grow for a maximum of 25 days. DNA was isolated using QuickExtract™ DNA isolation kit (Lucigen, Cat No. QE0905T). PCR using primers 5'-GGTTCTGGCAAGGAGAGAGA-3' and 5'-CACCTCTCCATCCTCTTGCT-3' was performed to assess mutations in the target region of the gene of interest (**Figure 1**).

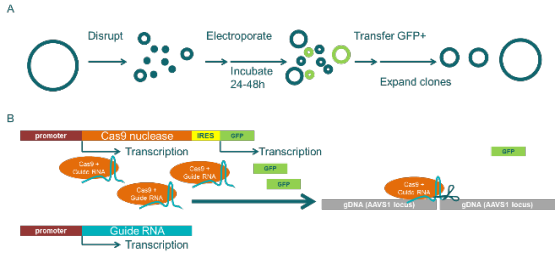

Figure 1. Overview of the experimental procedure. A: Schematic overview of the handling of Organoids to obtain CRISPR/Cas9 modified clones. B: Graphical representation of transient expression construct used to achieve targeted modification of the AAVS1 Locus.

Results

We set out to explore if the Yamaha CELL HANDLER™ can identify Organoids as objects based on size, shape and fluorescence and if manipulation of Organoid by the machine affects cell viability/outgrowth efficiency. As such, this study explored the feasibility of the use of the CELL HANDLER™ in Organoid manipulation.

CELL HANDLER™ can specifically select electroporated Organoids based on GFP expression and transfer them to 384-well plates.
To assess it, a bulk Organoid culture 48 hours after electroporation with a construct transiently expressing CRISPR-Cas9, GFP and a guide targeting the non-essential *AAVS1* locus, was used as input. ~20 % of the culture was GFP+ (determined by FACS analysis 48h after electroporation). We selected 40 GFP+ Organoids and transferred these to separate wells of a 384-well plate. Organoid transfer took 2 minutes (**Figure 2 and 3**).

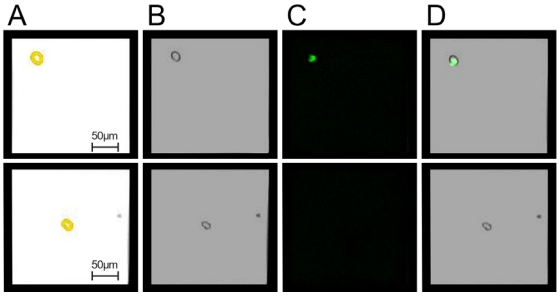

Figure 2. Selection of Organoids based on GFP expression. Microgrid on top contains GFP positive Organoid, microgrid below contains GFP negative Organoid. A: Brightfield image with recognized Organoids highlighted in yellow, B: Brightfield image of Organoids recognized in A, C: Green fluorescent image of Organoid, D: Merged image of Brightfield and Green fluorescent images.

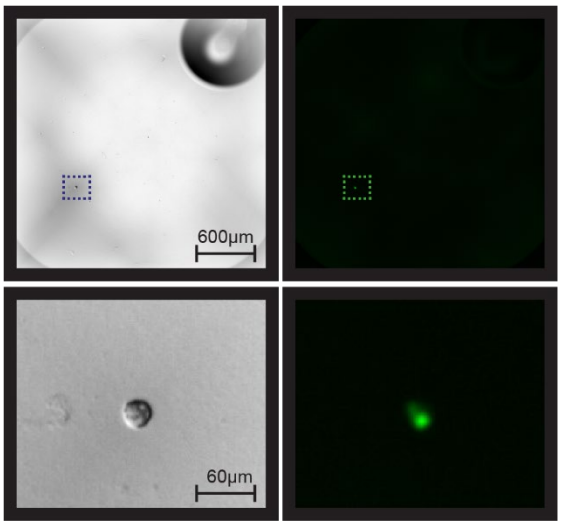

Figure 3. Image of a GFP positive organoid after being transferred by the Cell Handler.
Top is the full image covering >90% of the well, below is an enlargement (10x) of the indicated area. On the left are Brightfield images and to the right are GFP images

Organoids remain viable and proliferate after transfer to a 384-well plate.

Organoids were monitored for a maximum of 25 days after being transferred to a 384-well plate by the CELL HANDLER™. Whereas 96% (3% CI_{95}) of Organoids were viable after transfer under normal conditions, not all transfected Organoids remained viable after transfer. However, >50% of the transfected Organoids increased in size sufficiently to perform normal DNA isolation and PCR techniques within 25 days, indicating normal proliferation (**Figure 4**).

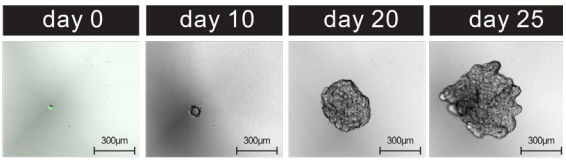

Figure 4. Expansion of a clonal Organoid in a 384-well plate, after picking by the CELL HANDLER™. Transient GFP expression was only visible at day 0 (48h after electroporation). The same Organoid was imaged 10, 20 and 25 days after transfer.

Table 1. Summary of organoid transfer efficiency, outgrowth and successful modification of target site

Selected organoids for transfer	
Total selected	60
Transfer efficiency	86%
Transferred organoids	
GFP positive	80%
Expanded	57%
Analysed	33%
Analysed organoids	
Mutant	63%

These results indicate that the handling by the CELL HANDLER™ does not prevent Organoid outgrowth after transfer.
DNA was isolated from the single clones which were able to proliferate. Sequencing of the CRISPR/CAS9 targeted AAVS1 locus revealed that 63 % of clonally expanded Organoids were found to contain mutations at the targeted cleavage site, suggesting successful targeting (**Table 1**).

Conclusion

Here, we show that the Yamaha CELL HANDLER™, originally designed to select individual cells, can be used to identify, select and transfer colon Organoids based on morphology and fluorescence. Organoids can be transferred with a speed of 1280 Organoids/hour. Organoids remained viable and proliferative after manipulation by the CELL HANDLER™.
Generation of CRISPR modified organoid lines is tedious work at the best of times. Attempting to generate organoid lines without use of antibiotic resistance or growth factor selection can make it practically impossible in difficult to transfect organoids lines. With the CELL HANDLER™ we now can efficiently recognize and specifically transfer and expand transiently Cas9-GFP transfected organoids in a manner that is suited to selection of rare events. This enables the generation of CRISPR clones without any requirement of growth selection, even for experiments with low transfection and or Cas9 modification rates such as homologous recombination-based sequence changes. In addition, as transfected organoids can be specifically transferred, it would be possible to determine whether specific modifications are lethal as one would expect approximately half of these organoids to be viable and two thirds of those organoids to be modified (can vary depending on guide sequence). Deviation from these numbers would be a strong indicator that the modification is non-viable.

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