Application Note

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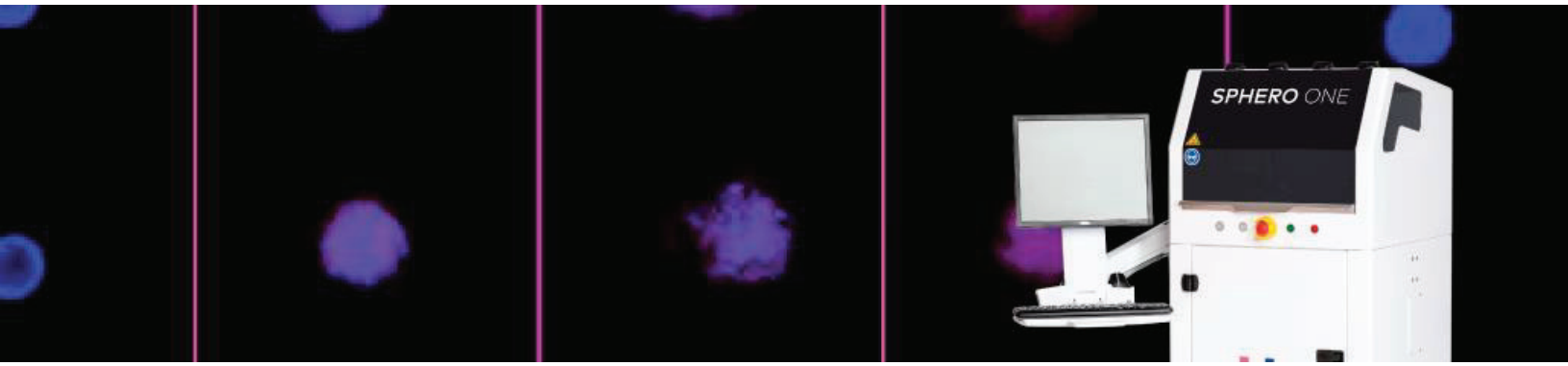

Preparation of HepaRG™ spheroid assay-ready plates using spheroONE

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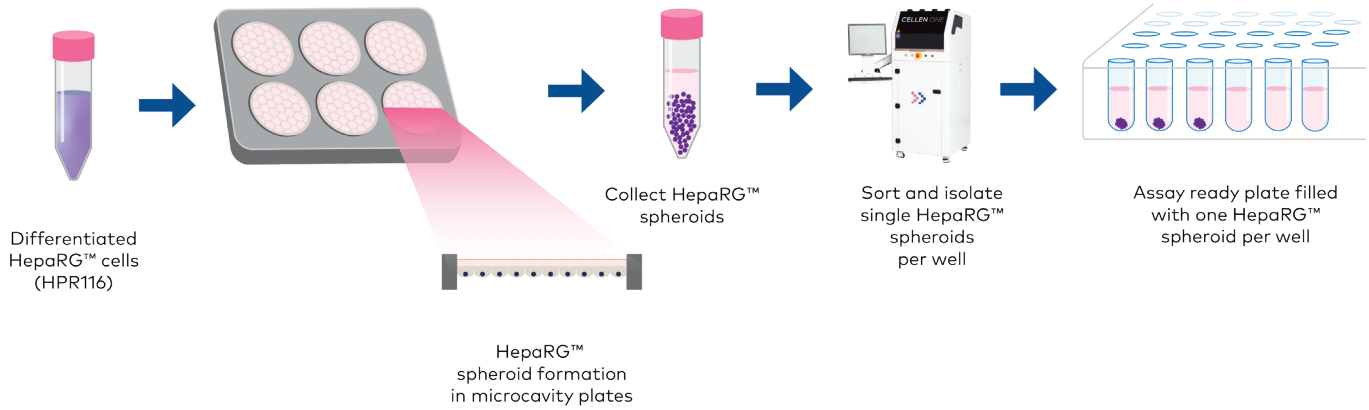
Abstract

Differentiated HepaRG™ cells are unique human bipotent cells capable of forming both biliary-like and hepatocyte-like cells. They express many detoxifying enzymes and are widely used in liver toxicity assays in early drug discovery. In this study, we demonstrated high throughput and efficient formation of highly functional HepaRG™ spheroids, their sorting and isolation using spheroONE, to obtain assay-ready plate containing homogeneous single HepaRG™ spheroids per well.



Introduction

Over recent years, the use of 3D cell models such as spheroid and organoids have significant progressed as they recapitulate features such as spatial architecture, diffusion barrier, differential gene expression which were lacking in traditional monolayer cell culture *[1]*.

In the world of 3D cell models, the HepaRG™ human hepatic cell line has proved its worth for studying hepatotoxicity thanks to its metabolic characteristics making it a model of choice in anti-tumor drug screenings *[2],[3]*.

Drug screening must be carried out on spheroids selected on the basis of their size and morphology, which means that the spheroids must be sorted and isolated according to certain pre-selected criteria.

Cellenion has developed **spheroONE**, an innovative platform to sort, isolate and dispense large particles such as spheroids and organoids from bulk population of cellular aggregates.

Materials and methods

Spheroid bulk production

Medium preparation

The frozen HepaRG™ Pre-induction Medium Supplement with antibiotics (ADD610, Biopredic International) was placed in a water bath at 37°C until completely thawed. The HepaRG™ Pre-induction Medium, named MIL610, was reconstituted by adding the thawed ADD610 supplement to 100 mL of Basal Hepatic Cell Medium (MIL600, Biopredic International).

The frozen HepaRG™ Maintenance/Metabolism Medium Supplement with antibiotics (ADD620, Biopredic International) was placed in a water bath at 37°C until completely thawed. The HepaRG™ Maintenance/Metabolism Medium, named MIL620, was reconstituted by adding the thawed ADD620 supplement to 100 mL of Basal Hepatic Cell Medium (MIL600, Biopredic International).

Cell thawing

A vial containing 8 million ready-to-use differentiated HepaRG™ cells (HPR116080, Biopredic International) was thawed in a 50 mL sterile tube containing 9 mL of MIL610 prewarmed in a water bath at 37°C. The protocol of the HPR116 user guide provided by Biopredic International was then followed for cell thawing, cell viability measurement and counting.

Cell seeding and spheroids formation

7 wells of a microcavity Elplasia plate P24 (Corning, Ref: 4441, containing 554 microcavities per well) were pre-filled with 1 mL each of MIL610 prewarmed in a water bath at 37°C. In order to obtain on average 2000 HepaRG™ cells/spheroid, the cell suspension concentration was adjusted to $1.11x10^6$ cells/mL in MIL610. This cell suspension (1mL/well) was seeded into each media pre-filled wells and the plate was incubated for 4 days at 37°C with 5% CO_2 to allow spheroids formation.

Sample preparation

Once HepaRG™ spheroids were formed in each cavity, spheroids were transfer from the Elplasia plate into a 15 mL Falcon tube. Culture medium was removed, and spheroids were resuspended in 6 mL sterile PBS with Ca^{2+} et Mg^{2+} (Corning, Ref: 21-030-CM). The first half of the sample was loaded under sterile conditions into a 3 mL spheroONE sample reservoir and mounted into spheroONE to start sorting and isolation. Once completely processed, the remaining sample was loaded into the reservoir and mounted into spheroONE for a second round of spheroid sorting and isolation.

Spheroid sorting and isolating

spheroONE is an innovative device for sorting and isolation of large cellular aggregates. Using precision dispensing technology and advanced image-based sorting capabilities, spheroONE enables selection and isolation of spheroids, organoids and tumoroids. A Nano Dispense Capillaries (NDC, ca.Ø = 300 µm) was used throughout the following experiments.

Sample reservoir was put under pressure (150 mBar), and detection and isolation parameters were set to isolate spheroids with a diameter between 200 µm and 250 µm.

Four 384-well ULA plates (Corning, Ref: 4516) were pre-filled with 30 µL of MIL620. According to pre-selected parameters on the spheroONE, a single spheroid was isolated per well.

Spheroid morphology post isolation

Spheroid morphology was assessed 2 days after spheroONE isolation by microscopy (Axio observer Z1, 5x objective, Zeiss). Spheroids diameter was measured using Zen software tool (Zeiss).

Spheroids functionality

After 2 days of culture post spheroONE isolation, a series of viability and functionality tests were undertaken:

Spheroid viability using ATP

Spheroid viability was assessed using CellTiter-Glo® 3D Cell Viability Assay (Promega, Ref: G9682). The protocol was carried out according to manufacturer's protocol in a flat bottom 96 well plate on 10 randomly selected spheroids. A Microplate Reader (FLUOstar Omega Multimode, BMG LABTECH) was used to measure the luminescence.

Spheroid viability using Live-Dead stain

Spheroid viability was assessed using Hoechst 33342 (5 µg/mL) and Ethidium Bromide (EtBr) (1 µg/mL). Spheroids were incubated 5 min in the dark at 30°C, then washed 3 times with PBS. Stained spheroids were transferred into wells of a flat bottom 384 well plate and subsequently imaged and analysed with a fluorescence microscope (Axio observer Z1, Zeiss).

Albumin secretion

To obtain sufficient volume for this assay, for each measurement, 10 µL supernatant from 10 randomly selected wells of the spheroid containing 384-well ULA plates were pooled together. Albumin secretion was then measured using a commercial Human Albumin ELISA Kit (Bethyl Laboratories, Ref: E88-129) according to manufacturer's protocol. A Microplate Reader (FLUOstar Omega Multimode, BMG LABTECH) was used to measure absorbance. A total of 3 measurements were undertaken.

Results and discussion

Bulk Spheroid preparation

Following 4 days of culture post seeding in P24 Elplasia plates, differentiated HepaRG™ cells formed tightly packed HepaRG™ spheroids. Using a microscope and its associated software, it was determined that the HepaRG™ spheroids formed were heterogeneous in size with diameters ranging from about 150 to 350 µm.

Spheroid isolation

After HepaRG™ spheroids formation, these were successfully pooled and processed using spheroONE to obtain individually sorted spheroid of diameter 200 to 250 µm in every well of four U-bottom ULA 384 well plates. After spheroid isolation, the plates were incubated at 37°C with 5% CO_2. After 2 days of culture, the plates were imaged and a single image for each well was recorded (**Figure 1**).

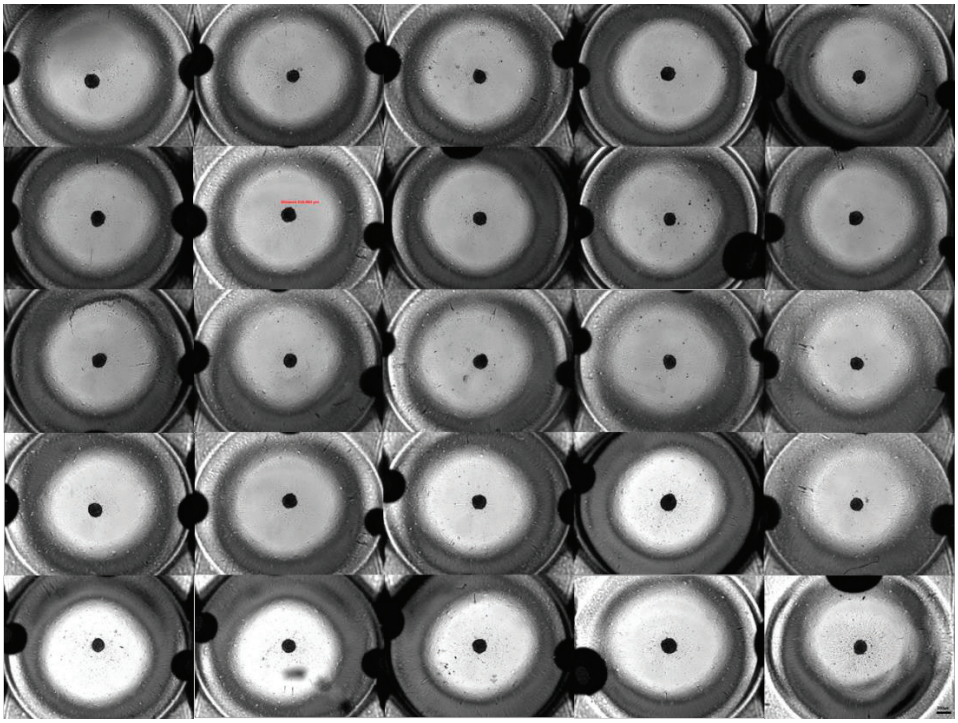


Figure 1: 5x5 tile of microscopy images of differentiated HepaRG™ spheroids in U-bottom ULA 384 well plate. 5x Objective. Spheroids diameter: 200-250µm. Scale = 200µm.

From the images acquired, the diameters of 100 spheroids were measured using a segmentation tool (Zen Software, Zeiss). From these measurements, a box plot (Figure 2) was generated to show the average spheroid diameter and their size distribution.

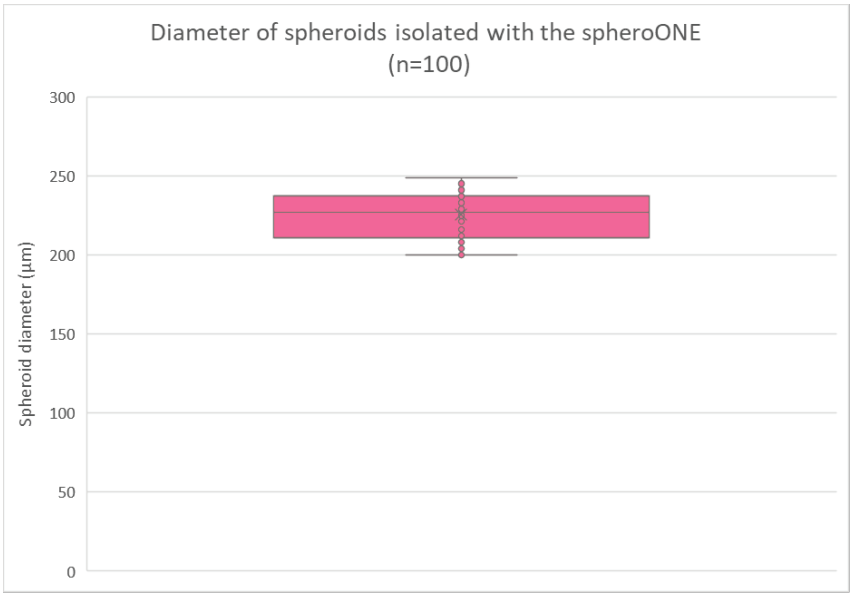


Figure 2: Boxplot of HepaRG™ spheroids diameter following 2 days of culture post spheroONE isolation.

These microscopy images and diameter measurements confirmed that all spheroONE isolated HepaRG™ spheroids had a diameter ranging from 200 to 249µm with an average value of 226 µm.

Spheroids morphology

Additional visual inspection of those microscopy images highlighted that spheroONE did not compromised spheroid integrity with all isolated spheroids being tightly packed and uniform (Figure 3).

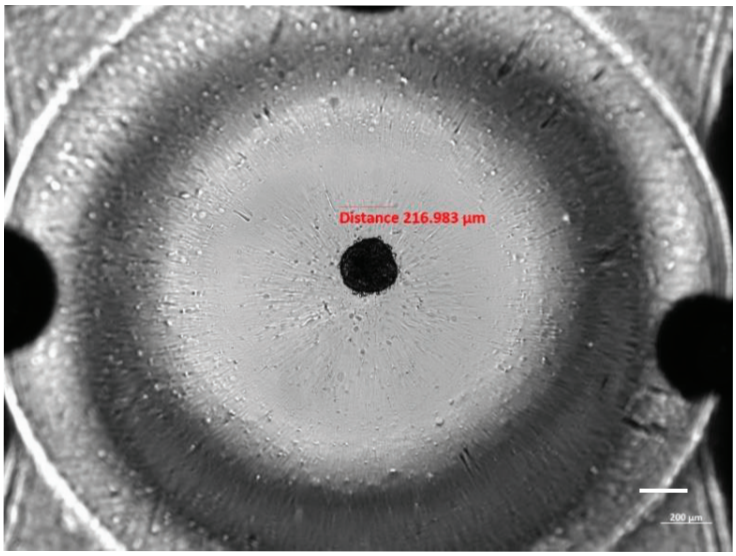


Figure 3: Microscopy image of differentiated HepaRG™ spheroid in U-bottom ULA 384 well plate following 2 days of culture post spheroONE isolation. Objective x5. Scale 200µm.

Spheroids functionality

Spheroids viability using ATP

From the luminescence readouts, it was possible to calculate the amount of ATP produced by each spheroid using a standard curve. The results showed good reproducibility with each spheroid producing between 962 and 1123 nM of ATP with an average value of 1032 +/-23 nM. This value was in line with Biopredic International internal QC data (558 to 1156 nM of ATP for spheroids composed of 2000 HepaRG™ cells cultured in MIL620 in ULA 96 well plate (Corning Costar®, Ref: 7007) demonstrating that the formed HepaRG™ spheroids showed good viability and energy status.

Spheroids viability using Live-Dead stain

Additionally, a few isolated spheroids were stained using a “Live-Dead” stain, transferred in flat bottom 384 well plate, and subsequently imaged under fluorescent microscopy in order to identify if dead cells were present in those spheroids.

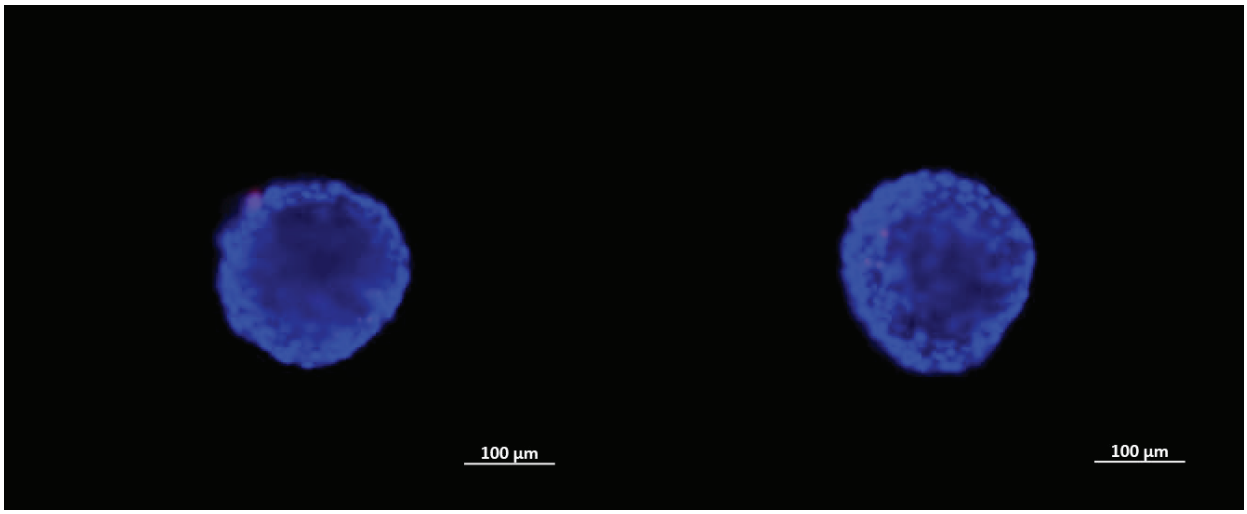


Figure 4: Microscopy images of differentiated HepaRG™ spheroids in flat bottom 384 well plate, 2 days of culture post spheroONE isolation after Live-Dead staining. Objective x10. Scale 100µm. Blue staining = viable cells. Red staining = dead cells.

Almost all cells were alive in the spheroids (mostly blue with no red) and only very few dead cells (red) were seen in those spheroids after spheroONE isolation (Figure 4).

Both these assays confirmed that HepaRG™ spheroids were neither damaged nor their cell viability affected following sorting and isolation using spheroONE.

Albumin secretion

From the absorbance measurements and using a standard curve, it was possible to calculate the secreted albumin concentration for each set of 10 wells. The results from the 3 measurements showed that spheroids secreted between 49 and 62 ng/mL of albumin with an average value of 55 +/- 4 ng/mL. This value was in line with previously published values demonstrating that the formed HepaRG™ spheroids showed good functionality.

Both ATP viability and albumin dosages corresponded to standard values obtained during quality control assessment of HepaRG™ 3D cell models undertaken at Biopredic International which confirm the proper functionality of HepaRG™ spheroids isolated using spheroONE.

These experiments confirmed successful preparation of highly homogeneous U-bottom ULA 384 well plates filled with tightly packed, viable and functional single HepaRG™ spheroid per well thanks to spheroONE sorting and isolation capabilities.

Conclusions

In this study, we demonstrated how large numbers of functional HepaRG™ hepatic spheroids were easily prepared by self-assembly using ready-to-use differentiated HepaRG™ cells (HPR116 from Biopredic International) seeded in ultra-low adhesion microstructured Elplasia® microplates. These bulk quantities of HepaRG™ spheroids were subsequently size-sorted and isolated using spheroONE to obtain highly homogeneous ready-to-use assay plates containing one spheroid per well. Functional and viability assays demonstrated that HepaRG™ spheroids in ready-to-use assay plates were comparable in performance to spheroids made using more labor-intensive manual methods. Overall, combining HPR116 cells and spheroONE for high throughput and automated preparation of functional HepaRG™ spheroid loaded assay ready plates is highly amenable to a range of 3D hepatic models screening applications that are widely implemented at different stages of Drug Discovery.

Acknowledgement

Differentiated HepaRG™ cells and culture reagents were provided by Biopredic International to Cellenion.

Product Code

Differentiated HepaRG™ cells: HPR116080 (Biopredic International, France).
spheroONE system: F00CS (Cellenion, France).

References

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[3] Andersson, T. B., Kanebratt, K. P., & Kenna, J. G. (2012). The HepaRG cell line: A unique *in vitro* tool for understanding drug metabolism and toxicology in human. *Expert Opinion on Drug Metabolism & Toxicology*, *8*(7), 909-920.

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