TECHNICAL NOTE:

GENERATION OF REGULAR 3D SPHEROIDS WITH SPHERICALPLATE 5D® FOR THE SUBSEQUENT CELLVIEWER ANALYSIS

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INTRODUCTION

The 3D multicellular spheroids offer an exciting model to investigate *in vivo*-like functionality where cells are grown in conditions that are not drastically different to those seen in real tissue, as in contrast to classical 2D cell culturing. Nevertheless its wide applications are currently limited due to lack of reliable and high throughput methods for generating well-defined and complex co-culture 3D structures. Spheroids are typically formed using pellet culture, liquid overlay, hanging drop, spinning flask and magnetic levitation methods. However, these conventional methods have limitations, such as a lack of reproducibility and a wide distribution of spheroid shape and sizes, leading to not comparable results. Heterogeneous-sized spheroids show different properties: a chemical gradient formation occurs from 200 µm diameter, a necrotic core and a proliferative rim appear from 400 µm to 600 µm, incomplete drug penetration is detected in 650-800 µm diameter spheroids. Sphericalplate 5D® (Kugelmeiers AG) represents an interesting technological solution to these issues. It consists of 12 functionalized wells with 750 round-bottomed microwells per well along with high-end ultra-low attachment nanocoating, which leads to perfect cell aggregation. Sphericalplate 5D® is perfectly compatible and integrated in CELLviewer workflow, since it generates a large, homogeneous spheroids population (about 9000 spheroids per plate), that is suitable for CELLviewer 3D culture and time-lapse analysis, thus adding reproducibility and reliability to tests results.

MATERIALS AND METHODS

- MCF-7 cell line (ATCC® HTB-22™)
- DMEM with 1 g/L glucose, sodium pyruvate and L-glutamine (Corning® Life Sciences)
- FBS 10% (Gibco™, Life Technologies, Thermo Fisher Scientific)
- L-glutamine 2 mM (Sigma-Aldrich, Merck)
- Penicillin-Streptomycin solution (Sigma-Aldrich, Merck)
- Dulbecco's Phosphate Buffered Saline with $MgCl_2$ and $CaCl_2$ (Sigma-Aldrich, Merck)
- Percoll® solution, pH 8.5-9.5 (25°C), cell culture tested (Sigma-Aldrich, Merck)
- Sphericalplate 5D® 24-well cell culture plate (Kugelmeiers AG)
- MitoGreen (PromoKine, PromoCell)
- CELLviewer imaging system and disposable cartridge
- CELLviewer 50 mL DOCK
- ImageJ software (US National Institutes of Health)

Note: One functionalized well of the Sphericalplate 5D® contains 750 microwells. Depending on the medium used, some air bubbles can remain within the microwells. If so, they usually release either by light tapping of the Sphericalplate 5D® or by centrifugation at 1000 x g for 1 min. The plate allows a wide range of different sizes of standardized clusters depends on the cell type. On average, for a cluster to reach 100 µm diameter, 150-600 cells per microwell are needed. For fast-growing cells, it is recommended to seed fewer cells, i.e. 40 cells per microwell. To create very large clusters it is feasible to load a larger quantity of cells per well, i.e. 2'000 cells per microwell.

MCF-7 cells were grown at 37 °C and 5% CO_2 in DMEM medium, supplemented with 2 mM L-glutamine, 10% FBS, 100 units/mL penicillin and 100 mg/mL streptomycin. Under a laminar flow sterile hood, the Sphericalplate 5D® plate is rinsed first with 1 mL of sterile PBS followed by 1 mL of complete medium. Every well is preloaded with 0,5 mL of complete medium, then 0,5 mL of cell suspension at a concentration of $3*10^5$ cells/mL is pipetted in every well for a total volume of 1 mL per well.
The plate is then incubated at 37 °C and 5% CO_2 for 24 hours to promote spontaneous cell aggregation and uniform-sized 3D spheroids formation. The 3D Spheroids are withdrawn from 4 wells and trasferred in a 50 mL centrifuge tube. Sample is centrifuged for 5 minutes at 800 rpm and the surnatant is discarded by gently aspirating. The pellet is resuspended in DMEM medium supplemented with Percoll®, to improve sample stable focusing during long-term imaging in CELLviewer cartridge (please refer to TN_MKT_03 Spheroid vianility assessment in CELLviewer conditioned culture medium). Basicly, 3000 spheroids are resuspended in 20 mL of Percoll® supplemented DMEM medium to achieve a working concentration of 150 spheroids/mL. Consequently, MitoGreen 400nm solution is added to the culture medium and the sample is then pipetted inside a 50 mL Falcon tube closed with a CELLviewer 50 mL DOCK. MitoGreen probe uptake is thereby quantitatively analyzed within CELLviewer over the experiment lifetime. CELLviewer digitally isolates a single 3D spheroid and focuses the sample with a fluidic feedback mechanism. CELLviewer software, CELLcontrol, manages the imaging setup to automatically acquire sample images in Bright-field channel and GFP channel at 0,5 fps with 20X magnification. ImageJ software is used for image analysis using Measure function to calculate 3D spheroids diameter. All image acquisitions from FITC channel are stacked together and the same square shaped ROI (including spheroid borders) is applied to all the images. Adjust Brightness/Contrast function is used to homogeneously remove fluorescence background to all the stack images. Max Grey Value function is used to quantitatively assess fluorescence signal increase over the experiment lifetime.

RESULTS

$1,5*10^5$ MCF-7 cells are seeded in 1 mL of complete medium for every well to let them aggregate. Homogenous-sized spheroid population shows mean diameter of 73 ± 4 µm at 24 hours of incubation and mean diameter of 73 ± 5 µm after 48 hours (Fig. 1). Spheroids diameter remains constant within 24 and 48 hours.

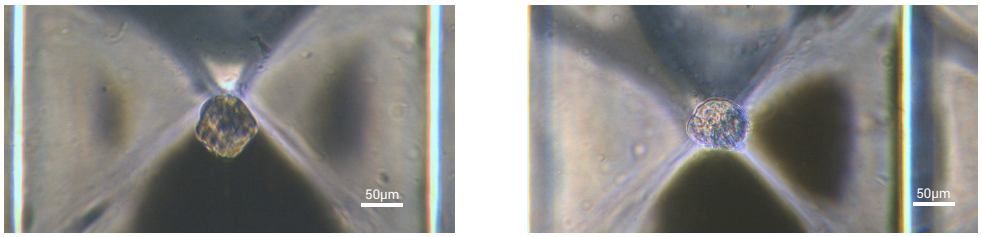

Figure 1. Bright-field analysis of MCF-7 spheroids grown in Sphericalplate 5D®. Left: Bright-field acquisition at 24 hours. Right: Bright-field acquisition at 48 hours. Image acquisition with Leica Microsystems inverted Epi-fluorescence microscope DMLB Fluo MS15062. **Scale bar: 50 µm.**

Single 3D spheroids, composed of different cell lines (depending on customers' requirements) are digitally isolated in CELLviewer cartridge and a fluidic feedback mechanism focuses the sample in the analysis chamber for time-lapse, long-term culture and time-lapse live imaging. As shown in Figure 2 below, 100 µm diameter MCF-7 spheroid is cultured for 4 hours in CELLviewer. A bright green fluorescent signal assesses spheroid viability, since MitoGreen conceptually overlaps mitochondrial regions of all viable cells composing a 3D spheroids. Fluorescence signals gradually increases throughout the experiment time course, reaching a maximum signal intensity within the first 1 hour and a half from the experiment start, due to MitoGreen probe progressive uptake by multicellular 3D spheroids.

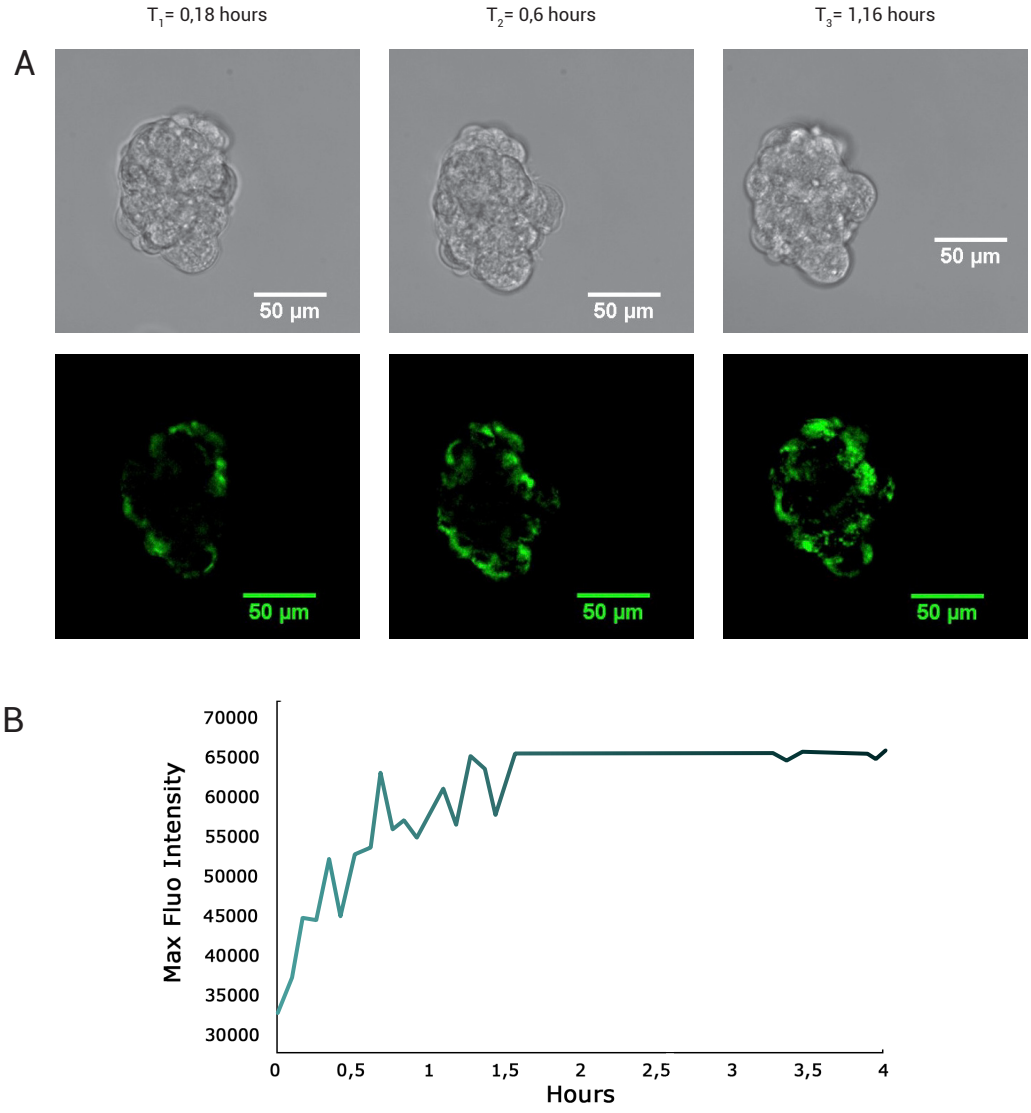

Figure 2. Analysis of MitoGreen labelled MCF-7 spheroid. **A** *CELLviewer acquisitions in Bright-field channel (top) and in GFP-channel (below) at 3 different timepoints.* **B** *Scatter Plot of Mitogreen Max Fluorescence Intensity (Grey Values) over the course of experiment.* **Scale bar: 50 µm.**