Establishment of perfect 3D spheroids for cancer research – your guideline

Introduction

In cancer research, great importance is attached to cell line-based model systems to understand the emergence and growth of tumors in humans or find new therapeutic drugs. So far, traditional 2D *in vitro* culture systems are commonly used as model systems. These systems enable a well-controlled and homogeneous cell environment and promote microscopic analysis. However, they incompletely recapitulate the natural microenvironment of cells, and a lot of results from 2D drug screenings fail when transferred to animal models or humans. Therefore, 3D cell culture models have become increasingly popular. [1] Tumor cells grown in suspension aggregate into dense 3D spheroids consisting of cells and deposited extracellular matrix (ECM). These constructs allow physical cell-cell and cell-ECM communication in each dimension and the activation of typical signaling pathways of solid tumors. [2] Their dense 3D network acts as a physical barrier for drugs and includes a high number of quiescent cells with an increased survival rate and cancer-specific gene expression. Due to these hallmarks, spheroids can reproduce the microenvironment of tumors *in vivo* and have the ability to overcome the limitations of 2D systems. [2, 3] Furthermore, cancer cells in 3D spheroids reveal a greater resistance against chemotherapeutic drugs than in 2D systems; thus, spheroids are well suited for drug testing systems. [3]

Hence, the development of complex 3D cultures for cancer research applications can have a significant impact on generating more meaningful results with a greater transferability to patients. In addition, the value of spheroid cultures is increasing rapidly due to novel microfabricated platforms amenable to high-throughput screening (HTS). However, different parameters should be considered for the assembly of a cancer-relevant model using 3D spheroids.

Cell spheroids are formed when certain conditions of their *in vitro* culture system are fulfilled and can be adjusted by different parameters: i) spheroid morphology, ii) spheroid number, iii) spheroid formation over time, iv) spheroid size, and v) cell line and coculture. The following note summarizes a guideline for the 3D spheroid culture with attention to the previously listed parameters.

Spheroid morphology and the number of spheroids per well have a significant impact on readout

Spheroids are commonly cultured in specially treated well plates or other cell culture consumables, which can be coated by the customer himself (e.g., using BIOFLOAT™ FLEX coating solution).

These culture systems are characterized by a hydrophilic surface, preventing protein and cell attachment, and forcing cells into spontaneous aggregation. Different spheroid culture systems may influence the spheroid morphology even when using the same cell line (Figure 1). In current standard spheroid culture systems, cells form spheroids with cell satellites and irregular shapes (Figure 2). In contrast, BIOFLOAT™ coated plates allow the formation of one round cell spheroids per well, which is an important criterion for generating a reproducible, reliable cell and high-throughput analysis.

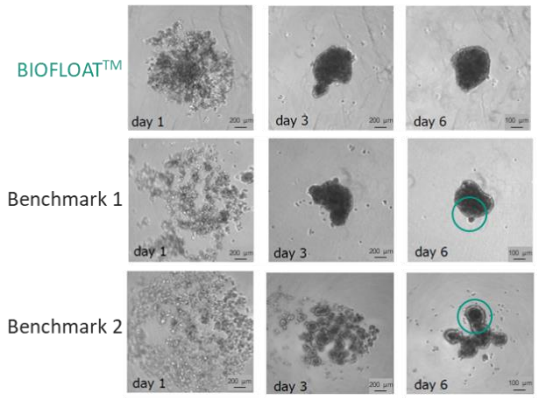

Figure 1: Microscopic images of hepatocyte spheroids in a BIOFLOAT™ 96-well plate compared to two benchmark products on days 1, 3, and 4. Scale bar: 200 µm.

High-throughput screening of spheroids requires an even distribution of one (symmetrical) spheroid per well for the specific readout by automated systems. Irregular shapes or generation of multiple spheroids per well negatively influences the quality of the results and may lead to poor reproducibility. The usage of spheroid culture plates can lead to multiple spheroid formation (Figure 2; benchmark). In the majority of BIOFLOAT™ wells, only one spheroid per well is generated. Therefore, BIOFLOAT™ is a suitable product for high-throughput screening with valuable readout.

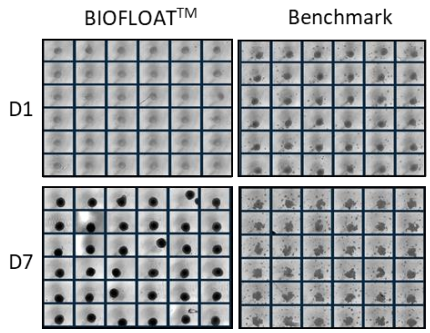

Figure 2: Overview images of 96-well plates seeded with 3T3 cells after days 1 and 7 in BIOFLOAT™ plates (left) and a benchmark product (right).

Adjusting the size and density of spheroids

At the beginning of each experimental setup, the size and density of the spheroids must be determined to set the right time point of analysis. This can be affected by the cell type model, but also, the cell seeding number can play a role. For example, cell lines from the same origin (human adenocarcinoma H3122, H228, and H1975) with equal initial cell numbers (2000 cells/well) form spheroids with different morphologies after four days of culture (Figure 3). This highlights the effect of the cell line type on spheroid size and density.

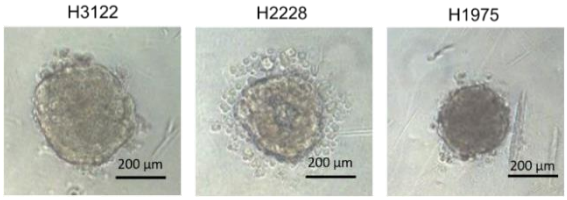

Figure 3: Microscopic pictures of cell spheroids of adenocarcinoma cell lines (H3122, H228, H1975) in 96-well plate cultivated on BIOFLOAT™ FLEX treated surfaces.

However, the spheroid size can also be manipulated by the initial cell number. HepG2 cells with an initial cell number of 6000 cells/well form large spheroids after 22 days (Figure 4 B). The seeding of HepG2 cells at cell numbers lower than 6000 cells/well results in smaller spheroids but with minor size variations when using 6000 cells/well (Figure 4 B). In contrast, seeding of either 6000 3T3 cells/well or 3000 3T3 cells/well show similar-sized spheroid area after 22 days (Figure 4 A), whereas seeding of initial cell numbers lower than 3000 cells/well results in smaller spheroids with minor variations in size. To conclude, the spheroid size depends on the initial cell number, and this effect can vary between different cell lines.

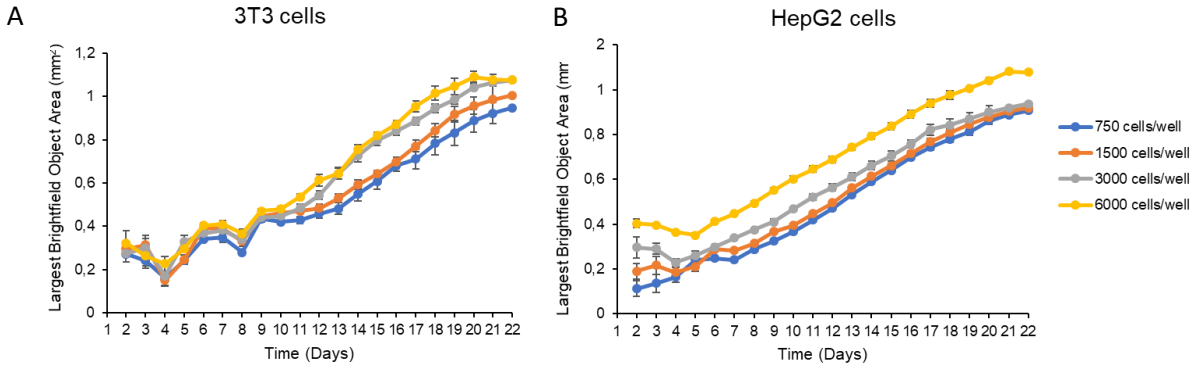

Figure 4: Effect of initial cell density on spheroid size over time. 3T3- or HepG2- cells were seeded at 750, 1500, 3000, and 6000 cells per well into BIOFLOAT™ 96-well plates. Spheroid area in mm² was measured over 21 days for 3T3 (left) and HepG2 (right) cell lines. Data are presented as mean ± standard deviation (SD) of 12 spheroids analyzed per condition.

Refine your spheroid model similar to *in vivo* conditions – using a coculture model

In vivo cancer cells grow in a complex environment consisting of different cell species acting as supporting components of various tasks. To recapitulate the cellular heterogeneity found in solid tumors, spheroids are often cultured in cocultures of different ratios of cancer to stromal cells to better mimic the *in vivo* conditions [2, 4]. Most often, one cell line fulfills supportive functions (feeder cells) for the other cell line. For example, osteoblasts in coculture with human umbilical vascular endothelium cells (HUVECs) in BIOFLOAT™ treated wells form a uniform formed spheroid with separated cell populations in a single spheroid (Figure 6).

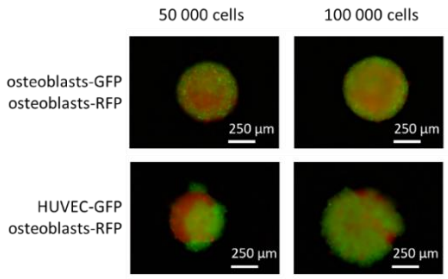

Figure 6: Fluorescence microscopic images of spheroids with osteoblasts (first row) and in coculture (second row) with HUVECs (HUVECs in green; osteoblasts in red). Scale bar: 250 µm.

Conclusion

3D cell culture methods confer a high degree of clinical and biological relevance to *in vitro* models. However, *in vivo* situation remodeling by *in vitro* 3D models and targeting application of the model require certain needs. Round and uniform spheroids can be easily generated by choosing BIOFLOAT™ for spheroid culture. Furthermore, the cell spheroids morphology can be tuned by changing different parameters, such as the initial seeding number or cell line model. In conclusion, 3D cell spheroids are well suited to the required needs and allow the coculture of different cells to build up complex and relevant models for fundamental cancer or pharmaceutical research.

References

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2. Costa, E.C., et al., *3D tumor spheroids: an overview on the tools and techniques used for their analysis.* Biotechnol Adv, 2016. **34**(8): p. 1427-1441.
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