Considerations for Three-Dimensional Cell Culture when using the Corning® Spheroid Microplate

Guidelines for Use

CORNING

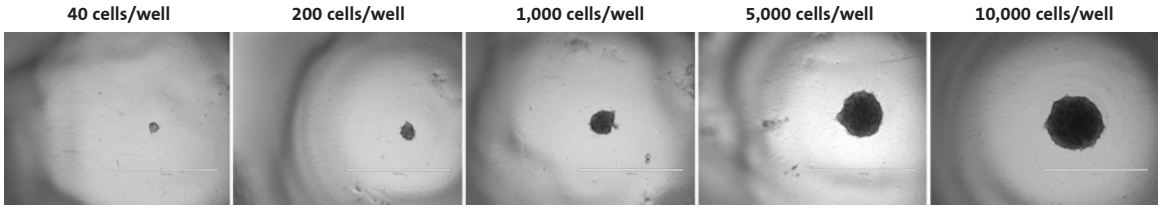
There has been a growing interest in three dimensional (3D) cell culture with more data showing drastic differences in cell behavior and response to drugs when cells are cultured in 3D compared to more traditional two dimensional (2D) models. With the added benefits of 3D systems there come additional challenges in terms of optimization, handling, and assaying. Here we discuss these obstacles and provide recommendations for dealing with them.

Spheroid Formation

Not all cells will readily form tight spheroids. However, there are some tips and techniques that can aid in spheroid formation. Specifically, optimizing the spheroid seeding density, media formulation, culture period, or adding an overlay of Corning Matrigel® matrix can aid in tighter spheroid formation.

Spheroid Size

Spheroid size is determined by the cell type, seeding density, and culture time. As the spheroid becomes larger, it becomes more difficult for nutrients and oxygen to reach the center of the spheroid, leading to a hypoxic core. Depending on the assay, this may or may not be desirable. Optimizing the seeding density will play a very large role in the ability of the spheroids to initially form, as well as determine how long the spheroids can be kept in culture.



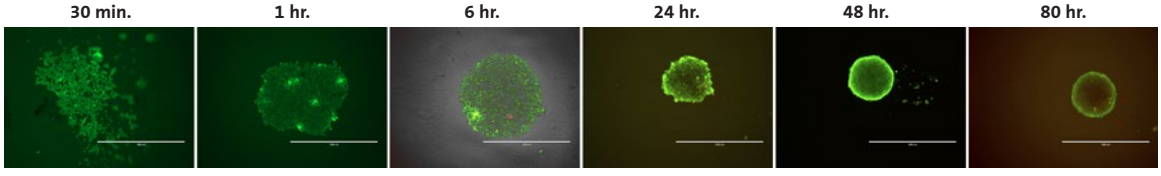
DU 145 spheroids after 72 hours of culture (4X objective). Scale bar = 1,000 µm.

Media Formulation

Media formulation may affect spheroid formation. This should be tested with each cell type. Supplements, such as methylcellulose, can increase the viscosity of the medium and have been shown to aid in spheroid formation[1].

Time to Form Spheroid

Some cells will readily form spheroids within hours, while other cells will require several days. Monitoring spheroid formation over time will help in determining the ideal culture period for each application.



Fluorescently labeled HT-29 spheroid formation over a 3-day period (4X objective)

Spheroid Handling

Media/Buffer Exchanges

Corning® Spheroid microplates are an automation friendly solution, which makes media and buffer exchanges easy to accomplish without the risk of disturbing the spheroid. If exchanges are done manually, we recommend careful pipetting to remove most of the medium in the well, ensuring pipet tips do not scratch the bottom or sides of the wells to avoid damaging the Ultra-Low Attachment surface coating, and leaving behind at least 10 to 20 µL residual volume to avoid disrupting the spheroids. It is also possible to do half medium changes more frequently instead of full exchanges.

Spheroid Transfer

Spheroids can be removed from the spheroid microplates using Axygen® 1 - 200 µL wide bore tips (Corning Cat. No. TF-205-WB-R-S) or a Corning 5 mL Stripette™. This can be helpful for spheroid embedding protocols, neurogenesis assays or any other application in which the spheroids need to be removed from the spheroid microplate.

Centrifugation

Cells seeded in the spheroid microplate can be centrifuged briefly at 300 x g to aid in spheroid formation, or for centering spheroids when an extracellular matrix (ECM), such as Corning Matrigel® matrix, is required.

Removal of Spheroids from Corning Matrigel® Matrix

For applications that require the spheroid to be embedded in Corning Matrigel matrix there are several options to recover the spheroid from the matrix. One option is to reduce the temperature of the microplate in order to liquefy the Matrigel matrix so that the spheroid can be removed. Another option, depending on the concentration of the Matrigel matrix, is to add a cold buffer or medium to the well in order to dilute/liquefy the Matrigel matrix. This may need to be repeated in order to free the spheroid. Additionally, Corning Cell Recovery solution (Corning Cat. No. 354253) may be used, which depolymerizes the Matrigel matrix and can be used to recover the spheroids.

Single Cell Recovery

For assays requiring single cell suspensions, spheroids can be dissociated by incubating with reagents such as Accutase®, 5 mM EDTA, 1X Trypsin/EDTA, or 1X, 5X, or 10X TrypLE™.

Spheroid Assays

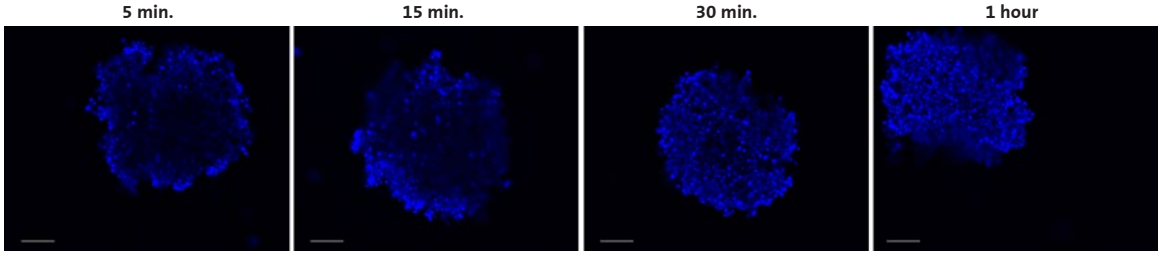
The black sidewalls and clear bottom of the spheroid microplate make it an ideal option for numerous fluorescent, luminescent, and colorimetric assays that can be conducted directly in the spheroid microplate. Depending on the size of the spheroid it may become difficult for some assay reagents to penetrate fully. Therefore, optimization of each assay is recommended using appropriate positive and negative controls.

Homogenous Assays

There are several commercially available 3D-specific reagents that have been optimized for use with spheroids. We recommend CellTiter-Glo® 3D cell viability assay (Promega Cat. No. G9683) for enumerating total ATP content of spheroids.

Fluorescent Imaging

Staining a 3D structure may require protocol optimization compared to the 2D equivalent. In general, the larger and tighter the spheroid, the longer and more complex it will be for complete staining to occur. If cell permeabilization is required, reagent choice, and length of incubation time may need to be considered. We have had success using a variety of stains including primary and secondary conjugated antibodies with cells cultured in the spheroid microplate. You can also pre-label cells prior to seeding in the spheroid microplate, or use fluorescent protein expressing cells in order to ensure that all cells are labeled as needed for the application.



HT29 cells cultured in 3D in the Corning spheroid microplate for 24 hours, exposed to 1 µg/mL Hoechst stain for various amounts of time (10X objective). Scale bar = 100 µm.

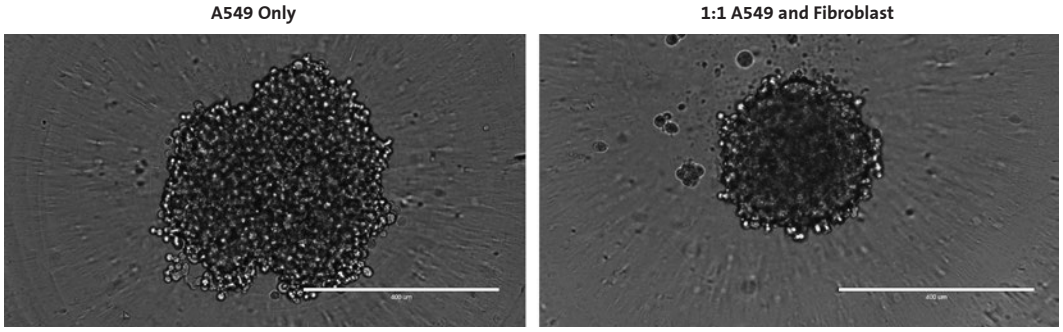
Advanced Applications

Extracellular Matrices Embedding

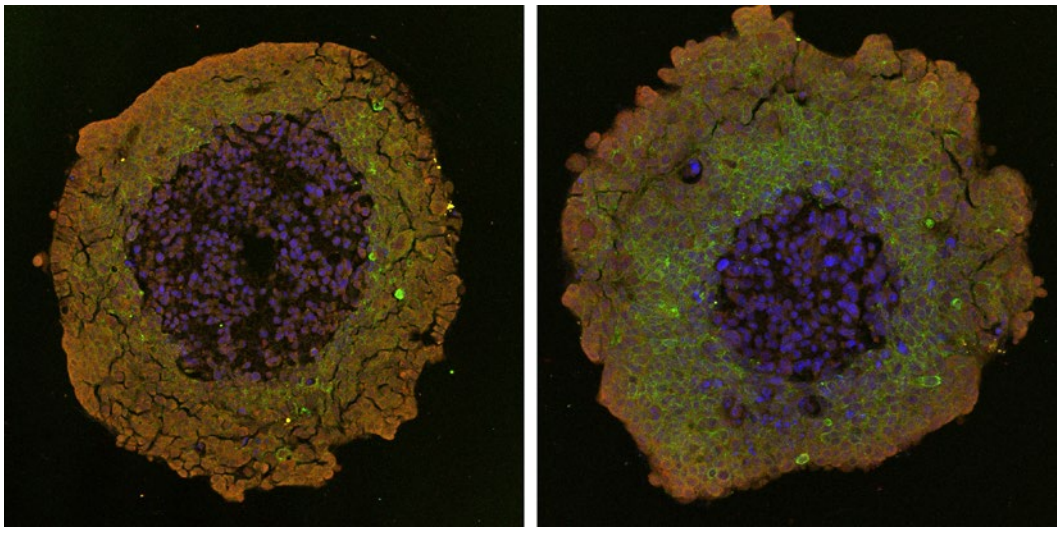
Some applications require the addition of an ECM such as Corning® Matrigel® matrix or Collagen. The concentration should be optimized for the specific application. Organoid cultures typically call for undiluted Matrigel matrix in the concentration range of 8 to 10 mg/mL, while other applications call for a more dilute concentration[2]. The ECM can be added during spheroid formation, or as an overlay to an already formed spheroid. For example, endothelial cells can be maintained in a spheroid formation by utilizing a Matrigel matrix overlay 24 hours after cell seeding. Depending on the length of culture and frequency of media changes, the ECM may need to be replenished.

Co-culture

Multiple cell types can be cultured together in a variety of ways to study cell-cell interactions, create more *in-vivo* like models, or add structure to a cell line that does not easily form a tight spheroid. The cells can be seeded at the same time or added at different time points depending on the application.
Depending on the cell types used, media formulations may require optimization to provide sufficient nutrients and growth factors to specialized cell types included in the co-culture environment. For example, when working with specialized cell types such as fibroblasts and endothelial cells, it is recommended to begin with the media formulation suggested for the specialized cell type. Seeding densities will also require optimization as cell proliferations rates may vary.



48-hour images of mono- and co-culture A549 and primary human lung fibroblasts at a final cell seeding density of 2,000 cells/well (10X objective). Of note, the multicellular spheroids become denser and tighter upon co-culture with fibroblasts compared to mono-culture conditions of A549 tumor cells alone.



HT29 adenocarcinoma cells were seeded at 500 cells/well alone and with Human Umbilical Vein Endothelial Cells (HUVEC) at a 1:2 ratio, and were overlaid with 2.2 mg/mL Corning Matrigel matrix 24 hours after cell seeding. Cells were cultured for 9 days and were paraffin-embedded, sectioned, and stained for E cadherin (green), DAPI nuclei (blue), and hypoxia marker HIF-1a (red). The hypoxic core appears smaller in the co-culture spheroid than in the HT-29 mono-culture spheroid.

Additional Resources

- Corning Spheroid Microplates User Guide (CLS-AN-235)
- Spheroid Processing and Embedding for Histology (CLS-AN-431)
- A Novel Three Dimensional Immune Oncology Model for High Throughput Testing of Tumoricidal Capability (CLS-AN-425)
- Neurosphere Formation, Differentiation, and Migration of Human Neural Stem Cells Cultured in Corning Spheroid Microplates (CLS-AN-334)
- Co-culturing and Assaying Spheroids in the Corning Spheroid Microplate (CLS-AN-390)
- CAR-T Cell Screening in Tumor Spheroids using Corning Spheroid Microplates (CLS-AN-447)
- 3D Hepatotoxicity Screening Using Corning HepatoCells, Spheroid Microplates, and SCREENWELL Hepatotoxicity Library (CLS-DL-AN-380)

References

1. Leung BM, Lesher-Perez SC, Matsuoka T, Moraes C, and Takayama S. Media additives to promote spheroid circularity and compactness in hanging drop platform. Biomater Sci. 2015; 3:336-344.
2. McCracken KW, Howell JC, Wells JM, et al. Generating human intestinal tissue from pluripotent stem cells in vitro. Nat Protoc 2011; 6:1920-1928.

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