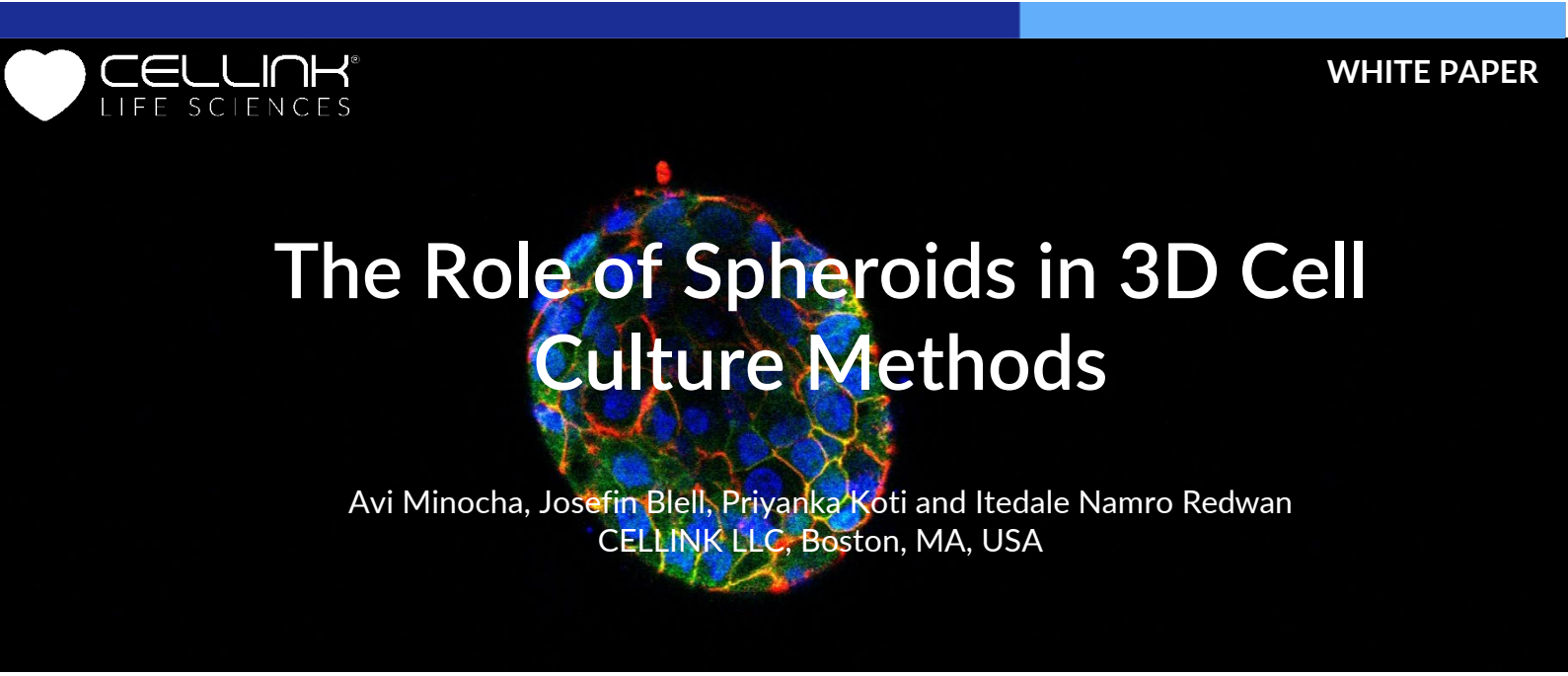

The Role of Spheroids in 3D Cell Culture Methods

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The 3D Revolution

In recent years, we have seen a paradigm shift in tissue engineering as scientists have transitioned to processes rooted in 3D cell culture. Numerous studies have explored what moving from 2D systems to 3D models entails, and it is well accepted that 3D models are the better option to mimic complex *in vivo* physiologies, demonstrate natural barriers and cell-cell junctions, and recapitulate tissue-specific environments that support 3D growth and the upregulation of oncoproteins.

Within the realm of 3D cell culture, spheroids have gained traction as an effective approach for modeling tissue. Spheroids, or cell clusters, are cells that under the right conditions, self-assemble into spherical models between 50 and 1,000 microns. These simplistic models create a basis for understanding complex processes, biochemical signaling and mechanosensing, and biophysics principles.

A number of studies have demonstrated that spheroids are invaluable tools for developing drugs, fighting cancer and understanding complex cellular processes. For spheroids grown in biomimetic hydrogels or bioinks, the benefits are even greater. The versatility of embedded spheroids makes them uniquely suited for *in vivo*, *ex vivo* and *in vitro* studies. For instance, in drug discovery and cytotoxicity studies, multicellular cancer spheroids have demonstrated increased chemoresistance and weakened response to anticancer drugs, similar to *in vivo* tumor microenvironments. Second, in natural engineering and stem cell research, single-cell-derived spheroids have been used as *ex vivo* models that allow researchers to investigate cell fate, cell-cell interactions and protein expression in various stages of development. Lastly, in other applications like cardiovascular research, cardiac spheroids have been classified as relevant *in vitro* models for investigating myocardial infarction and heart function.

While there are a number of challenges that hinder spheroid culture, like high scalability and size control, recent advances in automated cell culture systems have made it possible to efficiently grow, feed and maintain spheroids in large quantities and at low costs.

Novel Workflows Solve Problems

Combining spheroids with bioinks and bioprinting has the potential for an effective workflow to generate 3D models. If consistent spheroids can be developed, adding them to a bioink to then be printed on a bioprinter gives researchers full control over the placement of these spheroids. Additionally, by selecting tunable biomaterials, the stiffness of these constructs can be better controlled. By controlling all these parameters, researchers can better replicate physiological conditions and produce models that yield more accurate results. CELLINK scientists suggest a workflow outlined in **Figure 1** that demonstrates how these elements can be combined for consistent and effective 3D cell culture.

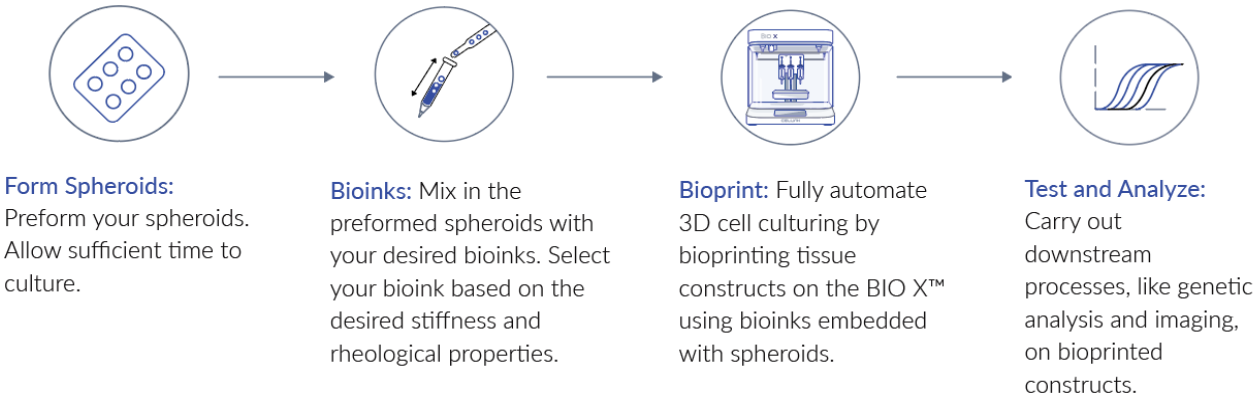


Figure 1. A proposed 4-step workflow for spheroid encapsulation and bioprinting.

Encapsulating spheroids in bioinks provides unique opportunities for discovery in bioprinting and 3D cell culture. With the proposed workflow, researchers could combine multiple fluorescently labeled cell-spheroid types into one cartridge, bioprint them into a 3D construct and observe how they interact with one another. For immuno-oncology applications, this could be highly relevant for growing mini tumors *ex vivo*. For instance, one could print T-cell and cancer spheroids and measure cell death over time. Alternatively, researchers could print cancer spheroids and later introduce targeted T cells to model invasive capabilities. Another example of this workflow could be forming single-cell spheroids with stem cells in ultra-low attachment plates, bioprinting them, and influencing their fate with various growth factors. A third example could dive into bioink degradability. By introducing proteases, one could slowly degrade bioinks and allow cancer spheroids to remodel and self-assemble into larger tumoroids. And last, one might try combining these methods with elements of perfusion in microfluidic devices. By bioprinting spheroids directly into microfluidic devices, like the Vaskit (CELLINK) or a similar device, one could study how spheroids behave under various culture conditions.

Step 1. Forming Spheroids

Developments in microtechnologies have introduced a number of ways in which spheroids can be formed. Among these methods are hanging droplets, micromolds like ULA plates, microfluidic devices, magnetic levitation and pellet culture. Here, we provide a closer look at some of these methods, their challenges and benefits.

Hanging Droplets

One of the most common and simple techniques for spheroid formation is the hanging droplet method. This method involves forming spheroids in small volumes of inverted cell culture medium. An advantage of this method is that the size of the spheroid can be easily controlled by the droplet's volume or the density of cell suspension. It is also an inexpensive way to form spheroids on a small scale. This method does have its drawbacks, however, often being inconsistent and prone to human error.

Ultra-Low Attachment Plates

Ultra-low attachment, or ULA, well plates allow cells to settle and aggregate without sticking to the walls of the plate. ULA plates prompt self-assembly within cell suspensions and allow cells to form a spheroid within 1-4 days. Often, a biomaterial with non-adherent properties is used to coat well plates for this method. This technique offers ease in manipulating spheroid size, by allowing researchers to control the volume of suspension or the number of seeded cells. This process of dispensing cells can also be automated. While ULA plates provide a relatively quick and consistent method to develop spheroids, many have also proved to be challenging when it comes to changing cell medium or manually maneuvering spheroids without having them deteriorate. For these reasons, ULA spheroid cultures are often used for short-term studies. There are, however, existing well plates like the Sphericalplate 5D (SP5D) from Kugelmeiers that remove these drawbacks and allow for easy long-term culture and spheroid harvesting.

Microfluidics

Microfluidic devices mirror the scale of *in vivo* structures. They also integrate 3D culture, perfusion and real-time analysis on highly cell-dense channels. Devices like lab-on-a-chip use biomaterials that are more permeable to oxygen and nutrients, thus decreasing the risks of hypoxia. While microfluidic systems have great potential within 3D cell culture, they are often tedious and costly to scale-up.

Magnetic Levitation

The magnetic levitation method (MLM) involves coating cells with magnetic nanoparticles and passing them through a magnetic field. Within a well plate, the magnetic field pulls coated cells to the air-liquid interface, allowing them to assemble into spheroids. While an effective strategy, the MLM must also account for any changes that the nanoparticles or magnetic field bring to cell health or behavior, as well as the difficulty of extracting formed spheroids after turning off the magnetic field.

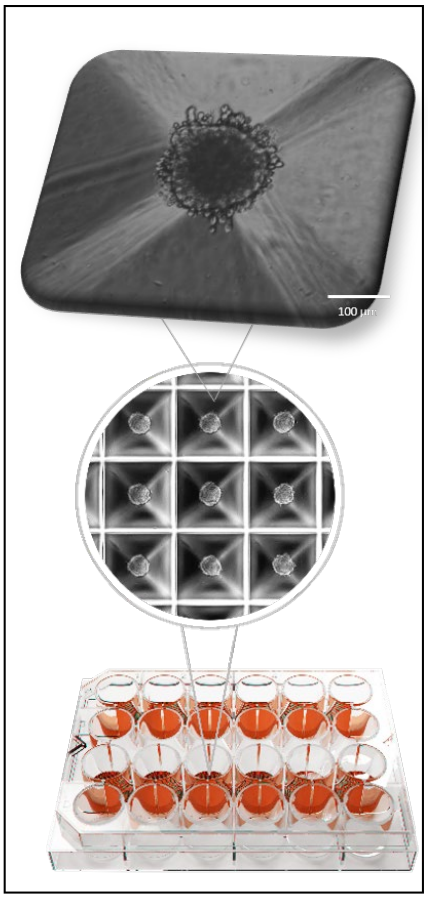
Step 2. Choosing a Bioink

Choosing the right bioink for spheroid culture can pose a number of challenges. Bioinks often require intensive synthesis and preparation, extensive gelation times and cell-specific protocols. Still, they are relevant for supporting long-term spheroid culture, and luckily, there are existing portfolios of bioinks and fine-tuned protocols that are suited for most cell types and applications. Readily sourced or commercially available bioinks like Collagen I and GelMA can make 3D spheroid culture easy and dependable.

Collagen I (Coll I) is found in over 30% of the body's total protein, making it an excellent biomaterial for tissue engineering and 3D printing applications. It forms fibrous networks in the body, enhancing the structural integrity of the extracellular matrices (ECM) while promoting cell adhesion, growth, biological signaling and tissue

morphogenesis. Similarly, GelMA provides extraordinary versatility across applications, owing to its unique biological properties and tunability that enable excellent attachment and proliferation of various cell types.

Step 3. Automating Spheroid Culture with Bioprinting



There are several commercially available ULA plates that allow for the mass formation of spheroids, including Kugelmeiers' Sphericalplate 5D (SP5D). The SP5D is a 24-well plate with 750 microwells per well in 12 functionalised wells, allowing the formation of 9000 spheroids per plate. A nanocoating facilitates cell aggregation in microwells. It allows for the formation of uniform and size-controlled spheroids for scalable applications that require hundreds of spheroids in small volumes of cell culture medium or ECM. The SP5D (**Figure 2**) offers different microwell groupings, allowing users to control spheroid size and refresh culture media without disruption.

Today's bioprinters are thoughtfully designed to provide a platform in which cell viability is the highest priority. High-end printers like CELLINK's BIO X come equipped with temperature control features that allow spheroids to remain at homeostatic conditions during printing processes and enable researchers to use a wide range of low-viscosity and temperature-sensitive biomaterials like Collagen I, GelMA, ColMA or alginate in their studies. Bioprinters with multiple methods of extrusion add an additional advantage to this workflow as they give researchers improved flexibility when it comes to printing spheroids. For instance, the droplet printhead, electromagnetic printhead, pneumatic printhead and syringe-pump printhead are all suited for printing low-viscosity, spheroid-laden bioinks.

Figure 2. An example of a tightly formed A549 spheroid sitting inside a microcavity of a Sphericalplate 5D.

Step 4. Quantifying Impact

Several methods of analysis can be implemented during or after spheroid culture, including viability checks, immunocytochemistry, live-cell imaging and rheological measurements. The latter methods can be used to assess spheroid growth, health and function, as well as spheroid-spheroid communication.

To better explain the proposed workflow with SP5D plates, bioprinting and analysis, CELLINK scientists bioprinted HepG2 and A549 spheroids in Coll I or GelMA. Both cancer cell lines were cultured for 4 days on an SP5D until spheroid formation. On Day

4, spheroids were harvested and carefully mixed into low-viscous Coll I (CELLINK) or GelMA (CELLINK) bioinks. Collagen, which plays a crucial role in tumor progression and growth, was selected to mimic the tumor microenvironment for HepG2 cells. GelMA, which is highly tunable for various cellular applications, was selected for encapsulating A549 spheroids. Using two different bioinks allowed researchers to observe how spheroids interact and grow within inks of varying stiffness and properties. Spheroid-laden bioinks were printed using a BIO X and cultured at 37°C for 7 days.

After culture, constructs were removed from the incubator, washed with PBS and stained with a LIVE/DEAD kit according to CELLINK protocols. As shown in **Figure 3**, not only are spheroids viable by Day 7, but they also show extensive growth within Coll I bioink. Another fascinating aspect of spheroids is their natural tendency to develop hypoxic cores. After 7 days of culture, the presence of a hypoxic core is apparent in most HepG2 spheroids.

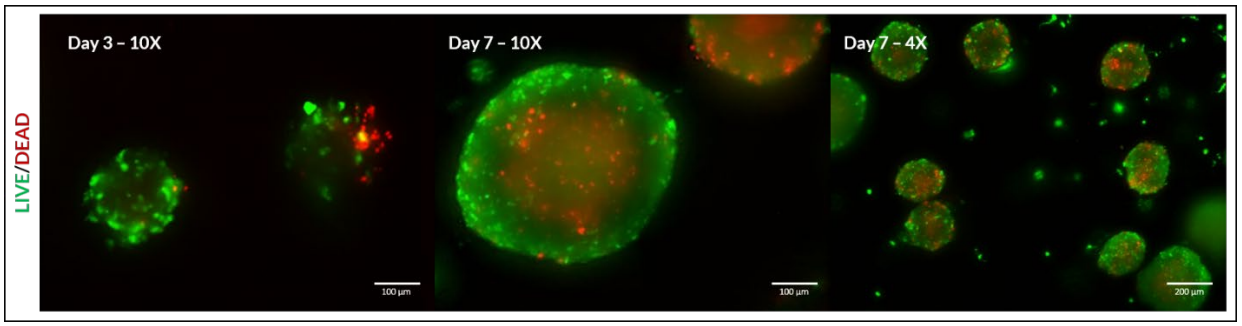


Figure 3. Viability assay for HepG2 cells cultured in Collagen I for 7 days. Live (green) and dead (red).

Immunocytochemistry was performed using various antibodies, including E-cadherin, ZO-1, albumin and β-catenin. Results show high expression of albumin and E-cadherin for HepG2 spheroids in Coll I, as well as an apparent difference in expression for ZO-1 and β-catenin between 2D and 3D GelMA spheroids (**Figure 4B**). Furthermore, the results suggest that improved 3D models with localized gene expression better resemble *in vivo* conditions than conventional 2D models.

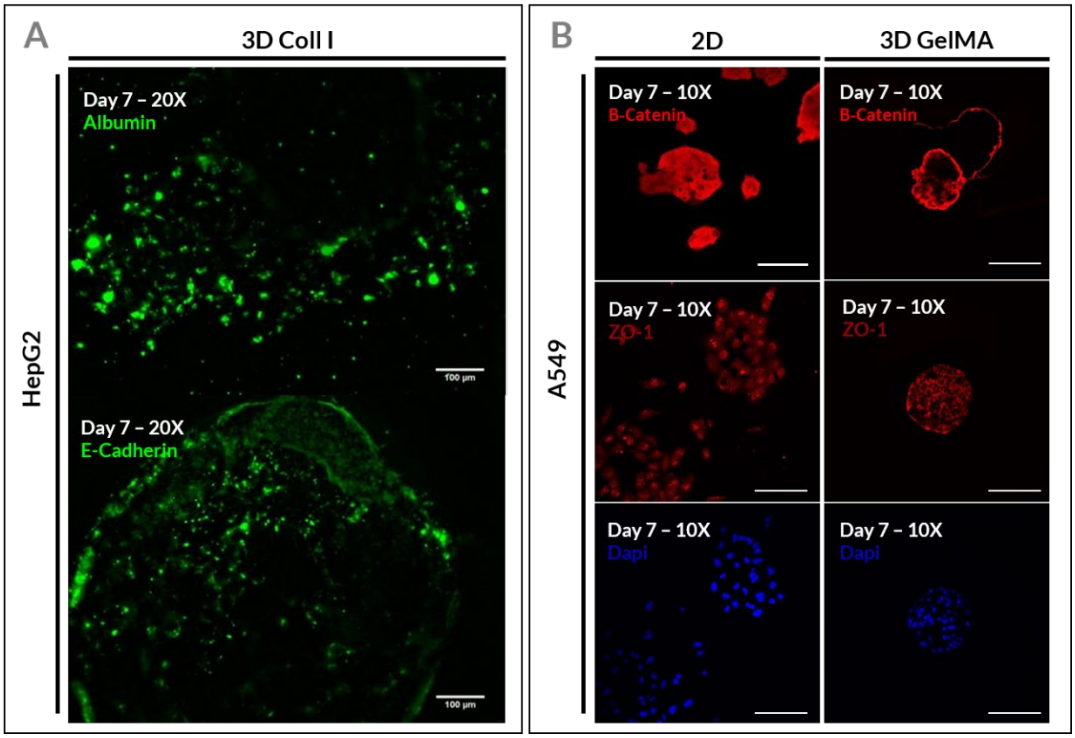


Figure 4. Various gene expression patterns in 2D and 3D spheroids. All images were captured using the same exposure time. Scale bar = 100 µm.

An Exciting Future for 3D Cell Culture

3D cell culture continues to impact the world of cell biology and tissue engineering. Implementing workflows that leverage innovative technologies, as seen in the SP5D, and bioprinters will help propagate 3D cell culturing practices and improve *in vivo* and *ex vivo* tissue models. Such models widen the scope of bioprinting applications and provide a unique glimpse into spheroid-spheroid and spheroid-matrix interactions. With improved models, researchers will continue to develop better solutions for the challenges that face us in the life sciences today.

In the quest for improved methods to recapitulate *in vivo* environments, we will continue to see the increasing role of bioprinting. Automating such workflows will remain a critical step to effectively scale models, and bioprinting will undoubtedly be the method of choice to make this happen.

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