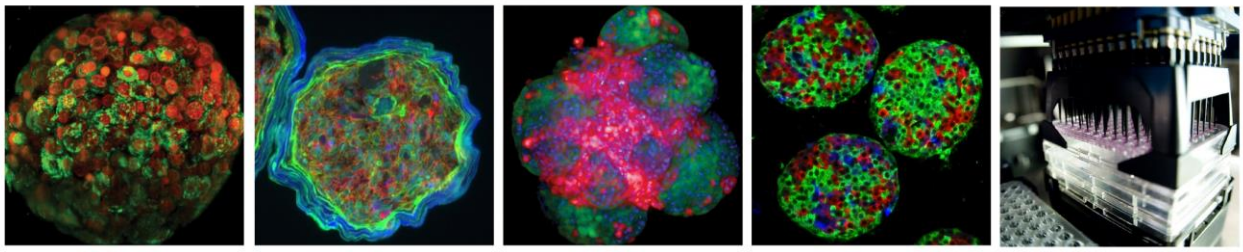


InSphero White Paper

Perfecting cellular self-assembly: Prerequisites for industrial-scale production of 3D microtissues for drug discovery and development

How enrichment of self-assembly-competent cells using the 3D Select™ Process enables mass production of robust, standardized microtissues.

Introduction

It is critical to remove compromised cell populations from the source material to produce a tissue of consistent quality and viability.

With the adoption of tissue-engineered 3D cell culture models for substance testing, novel challenges have arisen which demand industry-changing innovation for robust and reproducible bio-manufacturing. Whereas in the past mammalian cells have been grown on an industrial scale primarily for production of peptides and proteins, bio-manufacturing technologies must be developed to mass produce organotypic tissue models for routine use. In the case of regenerative medicine, typically only a small number of larger, high-complexity tissue constructs were required for the purpose of replacing defective tissue *in vivo*. This meant production times could be long, with only limited scalability required[1]. In contrast, the number of tissues needed for drug testing campaigns throughout drug discovery and developmental stages introduces a different framework, where 500,000 or more small tissues may be needed for a drug testing campaign[2]. Reproducible and robust production of several thousands of highly functional 3D cell culture models in a relatively short timeframe presents a daunting challenge, one that requires novel technologies and processes, particularly when primary and stem cell sources, or complex co-culture models are being employed. The cellular self-assembly approach has proven most amenable to mass production of organotypic 3D models, as the absence of scaffold materials lends itself to simple integration with automation and liquid handling instrumentation[3].

The key to any robust tissue production process is the high quality and consistent cell viability of the primary material used. For proliferating cell line-based tissue models this is not a pressing issue. However, if non-proliferating primary cells or cells differentiated from stem cells are being used, the quality of the source material, including not only cells but also culture medium, is of upmost importance. The medium must be formulated to provide an optimal environment during tissue formation, and for complex tissues, must be suitable for more than one cell type.

One inherent constraint of either isolating fresh cells from a respective tissue, or thawing cryopreserved cells, is that the cell population contains a certain percentage of dead cells, as well as cells that are compromised such that they will no longer be capable of forming a tissue by self-assembly. This can introduce donor- and production-run-specific variability in self-assembly efficiency, making it critical to remove such compromised cell populations from the source material to produce a tissue of consistent quality and viability.

Here we present the 3D Select™ technology, which ensures that only cells capable of forming a microtissue are used in combination with optimized medium. We compare microtissues produced using the 3D Select™ Process and more conventional 3D cell culture methods, and show that InSphero 3D Select™ Technology produces more uniform, higher quality tissues, and ensures less dependency on the donor material.

The concept: Enrichment of cells competent for cellular self-assembly

Industrial microtissue production methods should be amenable to a wide variety of cell sources, especially for primary and stem cells. Ideally, when establishing an *in vitro* assay to be used for long-term, multisite studies, the production method should not need to be altered or re-engineered. The primary difference between the two methods compared in this study is a unique pre-selection step in the InSphero 3D Select™ Process (Figure 1). This step serves to eliminate not only dead and apoptotic cells but also cells that are not capable of being integrated into the tissue.

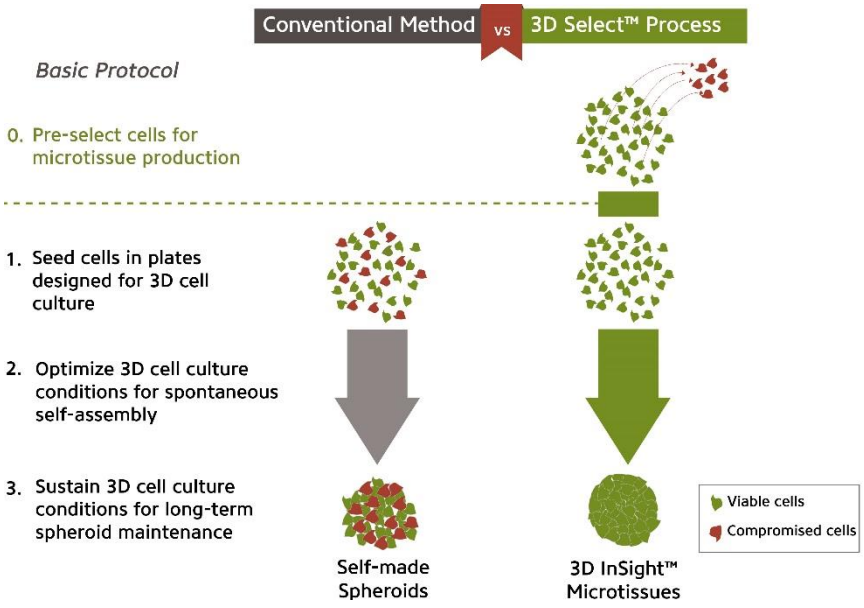


Figure 1. Comparison of Conventional Method versus 3D Select™ Process. The InSphero 3D Select™ Process[1] ensures elimination of compromised cells during re-assembly, resulting in standardized, viable microtissues with minimal evidence of necrosis.

[1] Patent pending

Both microtissue production approaches apply a similar method for seeding cells in plates designed to create a culture condition appropriate for spontaneous self-assembly into 3D microtissues (e.g., hanging drop or ultra-low attachment (ULA) coated wells) [4,5,6]. Pre-selection enriches for the cell populations capable of forming a tissue. In this way, the 3D Select™ Process ensures production of uniform, healthy, and viable microtissues with minimal evidence of necrosis, which can compromise functional activity.

Results and discussion

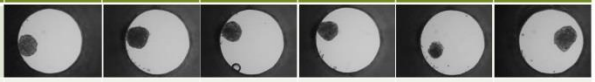
Under appropriate cell culture conditions, dispersed cells spontaneously self-assemble and form compact, dense spheroidal-shaped microtissues within 4-5 days that can be cultured in appropriate maintenance media for up to 5 weeks, depending on the cell types. However, the success of microtissue formation across different donor lots can vary significantly due to inherent differences in the quality of cells from different batches, a variable that is normalized by the 3D Select™ Process.

Self-assembly efficiency

To evaluate and compare spheroid formation efficiency by the two methods, cryopreserved primary hepatocytes from six different donors were used, and assessed for microtissue formation using bright-field imaging to capture spheroid formation after five days in culture (Figure 2). The 3D Select™ Process resulted in more reliable microtissue formation efficiency across the six donors (Figure 2).

The 3D Select™ Process is robust and ensures spheroid formation with high ATP content. Using the conventional method, hepatocytes from three of six donors failed to form spheroids and ATP content was notably compromised.

A InSphero 3D Select™ Process

Donor	1	2	3	4	5	6
Day 5						
Microtissue Formation	Yes	Yes	Yes	Yes	Yes	Yes
ATP Content (pmol/microtissue)	25.4 ± 1.1	30.6 ± 5.2	19.1 ± 0.9	24.8 ± 4.3	11.6 ± 0.7	26.5 ± 1.9

B Conventional method

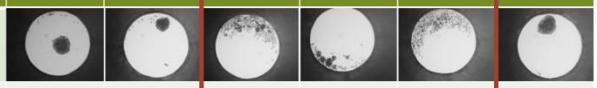
Donor	1	2	3	4	5	6
Day 5						
Microtissue Formation	Yes	Yes	No	No	No	Yes
ATP Content (pmol/microtissue)	16.6 ± 2.2	15.2 ± 3.7	1.7 ± 0.6	6.0 ± 2.5	1.7 ± 0.4	19.0 ± 3.1
Δ from 3D Select™	-34.7%	-50.3%	-91.1%	-75.9%	-85.1%	-28.2%

Figure 2. The 3D Select™ Process ensures consistent microtissue formation. Cryopreserved human hepatocytes from six different donors were either produced using **(A)** the 3D Select™ Process, or **(B)** conventional seeding with no preselection. The 3D Select™ Process enables generation of liver microtissues from all six donors, whereas the conventional method failed to produce tissues from three donors and showed marked decrease in ATP content.

Microtissue health and viability

To compare viability of liver microtissues produced with and without the cell selection step, we assessed total microtissue ATP content using the Promega CellTiter-Glo® assay[7] on each well. A higher ATP content was observed over all donors using the 3D Select™ Process. Even in instances where the conventional method did form spheroids, there were obvious differences in the viability of microtissues from the same donors (Figure 2B). For example, although microtissues produced from donors 1 and 6 formed successfully using both methods, the ATP content in those created using the conventional method was approximately 30% less.

Organotypic morphology

Microtissues generated using the conventional method generally contained hepatocytes that had lost distinct cellular boundaries and the cuboidal shape typical of hepatocytes.

To further compare and characterize the health and organotypic nature of microtissues that formed successfully, we used Hematoxylin and eosin (H&E) staining to assess their morphological integrity and cellular organization using representative microtissues produced from donor 1 and 6. Liver microtissues produced using the 3D Select™ Process (Figure 3A) displayed a contiguous, dense tissue defined by clear cell boundaries, cuboidal-shaped hepatocytes, and well-defined, healthy nuclei. Hepatocyte cytoplasm was generally well structured with minimal eosinophilic staining, demonstrating the superior morphology and healthy liver-like tissue structure resulting from the 3D Select™ Process. In stark contrast to this morphology, microtissues generated using the conventional method (Figure 3B) generally contained hepatocytes that had lost distinct cellular boundaries and the cuboidal shape typical of hepatocytes. Large areas of necrotic cells were evident by eosinophilic staining of cytoplasm, which was largely unstructured, and with cells frequently absent of defined nuclei, or displaying pyknotic nuclei. In addition, the size of the resulting microtissues was significantly smaller, indicating that many cells were not able to self-assemble into a tissue structure.

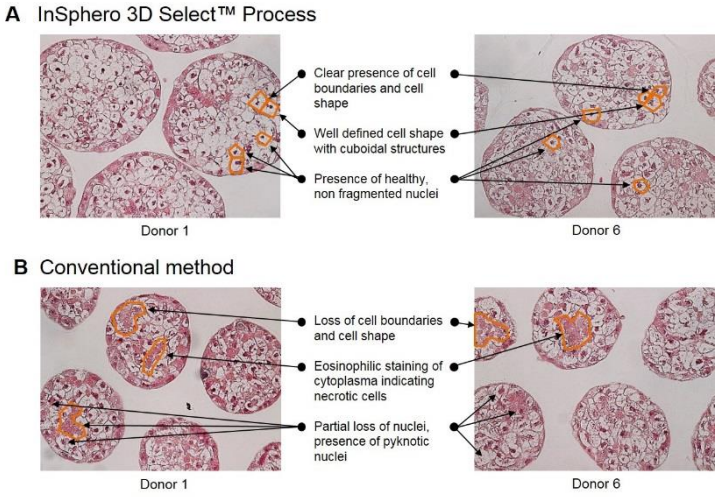


Figure 3. Structural and morphological characteristics of microtissues for donors 1 and 6 produced by the **(A)** InSphero 3D Select™ Process and **(B)** conventional method. Microtissues made using the 3D Select™ Process displayed organized, cuboidal hepatocyte structure with healthy nuclei, free of eosinophilic staining cytoplasm. In contrast, conventional spheroids showed less defined cellular boundaries, disorganized/pyknotic nuclei, and large zones of necrotic cells.

InSphero 3D InSight™ Microtissues produced using 3D Select™ Technology are a superior in vitro *model for drug discovery and development.*

Uniformity and reproducibility

The 3D Select™ Process serves as the foundation for reproducible production of 3D microtissues, as it ensures only cells which have the capacity for cellular self-assembly are used. To further assess the consistency and uniformity of microtissues produced using 3D Select™ Technology, we evaluated microtissue size measured across 84 production runs, spanning 7 different hepatocyte donor lots and different co-culture conditions (Figure 4A). Although highly diverse primary donor material was used for these production runs, the overall size deviation across all production runs was only 10% (265 ± 28.6 µm, Figure 4B). Microtissue size deviation within each run across different plates was less than 5%. These data show that the 3D Select™ Process ensures the high reproducibility required for industrial production of 3D liver microtissues.

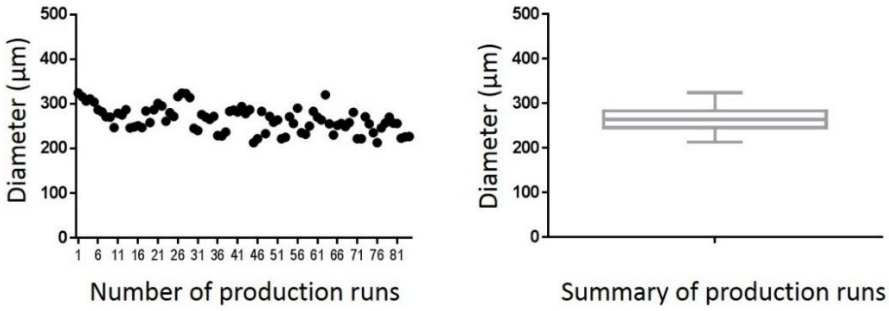


Figure 4. 3D InSight™ Human Liver Microtissue Size. **(A)** Plot of the individual diameter (µm) of 3D InSight™ Human Liver Microtissues across 84 production runs shows uniformity of microtissue size. **(B)** Box and whisker plot summarizing the diameter microtissues across 84 production runs. The average diameter was 265 µm ± 28.55 µm.

A comparison summarizing key differences between the two methods, using liver microtissues as an example (Table 1) further shows that InSphero 3D InSight™ Microtissues produced using 3D Select™ Technology are a superior *in vitro* model for drug discovery and development.

Table 1. Liver microtissues produced using conventional method versus InSphero 3D Select™ Process

Spheroid Production	Conventional Method	3D Select™ Process
Self-assembly-driven re-aggregation	Yes	Yes
Donor dependency	High	Low
Robustness in tissue formation	Low	High
Organotypic liver structure	Yes	Yes
Presence of necrotic areas	Common	Rare
In vitro culture lifespan	Up to 5 weeks	Up to 5 weeks
ATP Content (cell viability measure)	Decreased	High

Conclusions

Implementation and application of reproducible tissue-based assays requires the provision of consistent tissue quality in a scalable manner. The 3D Select™ Technology filters cells that are not capable of being functionally integrated into an engineered microtissue. Even though the conventional self-assembly production process leads to microtissue formation (with ~50% success rate), production robustness and reproducibility improves significantly when compromised cells are removed before seeding. The InSphero 3D Select™ Process is currently being applied to produce a broad suite of assay-ready microtissue models for liver, pancreatic islets, various tumor types, skin, and brain using cells from multiple species, including human, rat, dog, and monkey.

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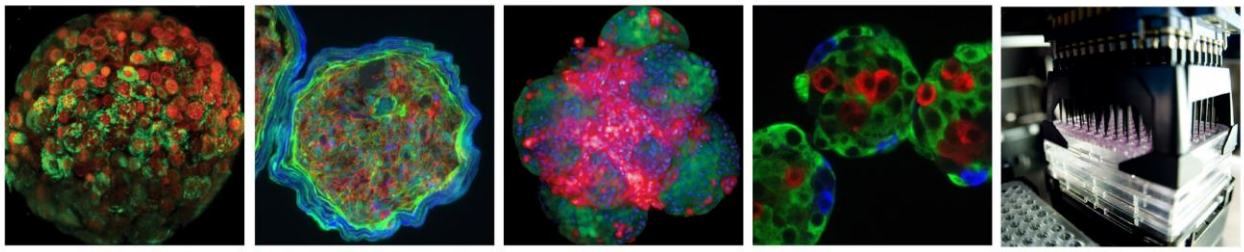

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