Unveiling Drug Responses in Liver Spheroids: Multiplexing 3D Cell-Based Assays and Imaging in a Microwell Platform

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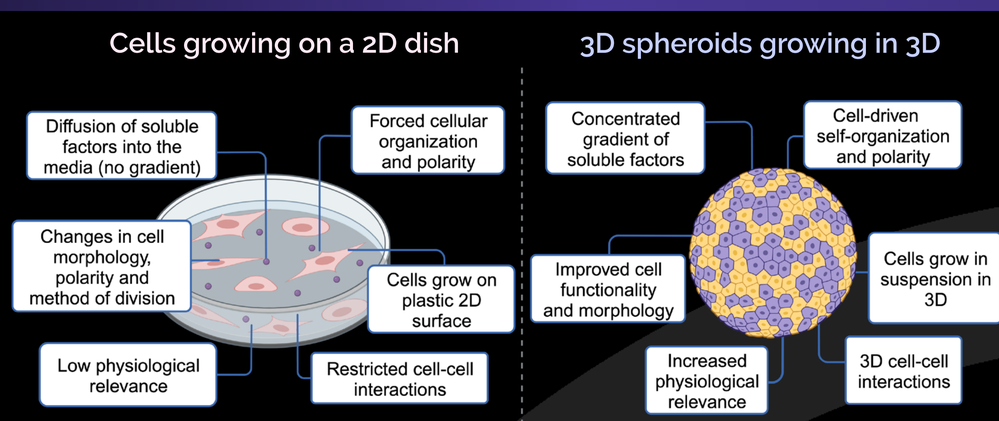
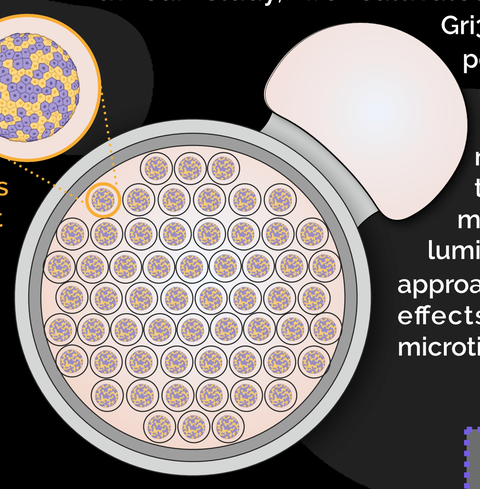
INTRODUCTION



Yet most 3D cultures are still not ready for screening. Using conventional plates such as ultra-low attachment (ULA) U-bottom poses challenges in:

1. Reproducibility
2. Scalability
3. Imaging
4. Automation
5. Handling
6. Loss of microtissues
7. Assay development

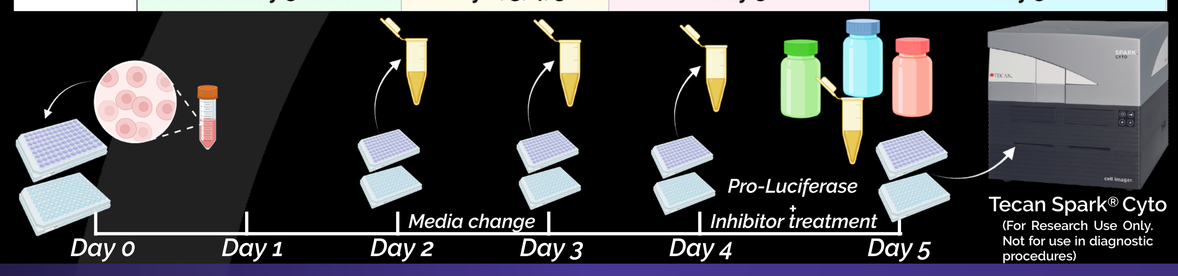
To tackle these hurdles, we introduce Gri3D®: an innovative hydrogel microwell 96WP system designed for uniform cell seeding, efficient aggregation, and the generation of individual microtissues in suspension-like conditions. Importantly, the resulting microtissues are strategically positioned within the same focal plane, enabling simultaneous high-resolution imaging. In our study, we cultivated hepatocyte spheroids simultaneously in Gri3D® and ULA 96WP to compare ease of use, performance, sensitivity, and scalability across multiple assays. We concentrated our efforts on hepatotoxicity. Using Spark Cyto imaging multi-mode plate reader, we concurrently assessed the viability and enzymatic activities of the 3D microtissues, combining cell imaging and luminesce readouts. This innovative 3D multiplexing approach enabled us to glean crucial insights into the effects of various drugs on CYP activity and microtissue viability, all within a single experiment.

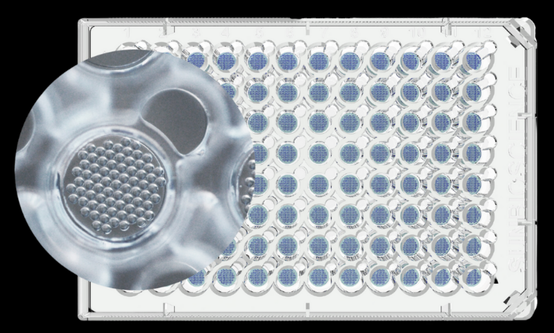


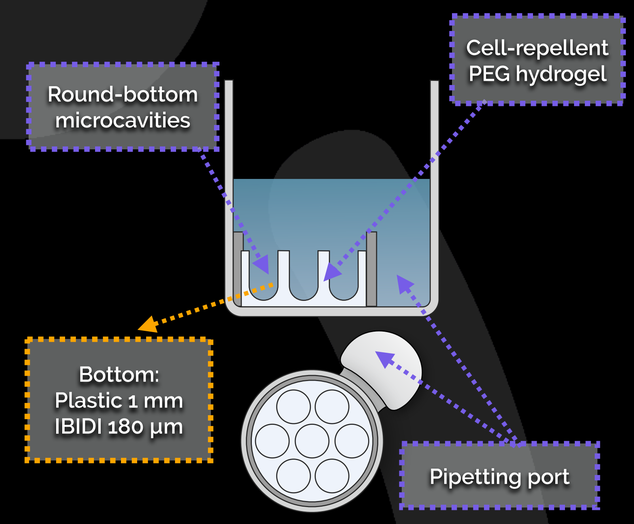
METHODS

Upcyte® hepatocyte spheroids are generated in parallel in Gri3D® 96WP 600 µm microwells (SUN bioscience) and in standard ULA 96WP plates (Corning). Spheroids form from a single-cell suspension and are cultured for up to 5 days. The microtissues are exposed to inhibitors of liver enzyme activity for 24 hours, and both CYP (cytochrome P450) activity and microtissue viability are assessed using commercially available kits (Promega). Both plates are evaluated on a Spark® Cyto (Tecan, For Research Use Only). All the experiments were carried out in Doppl's Laboratory (Lausanne, Switzerland).

ASSAY	CellTiter-Glo® 3D	LDH-Glo™	P450-Glo™ CYP3A4	P450-Glo™ CYP1A2
Readout	Luminescence Day 5	Luminescence Day 2, 3, 4, 5	Luminescence Day 5	Luminescence Day 5







RESULTS

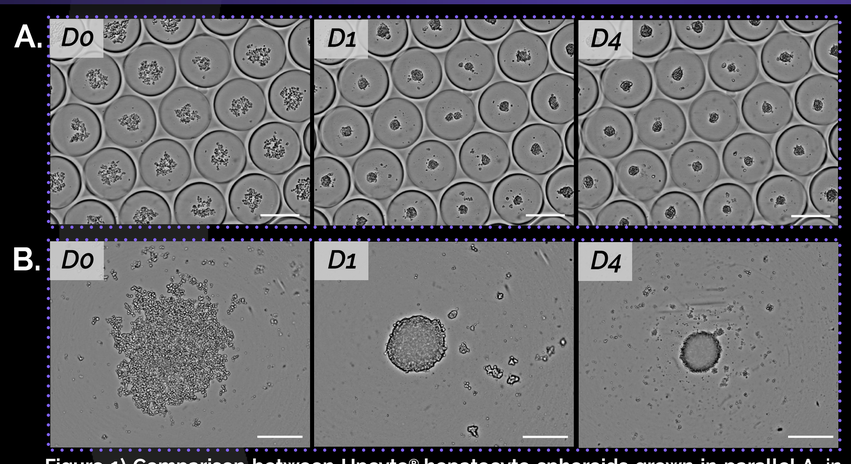
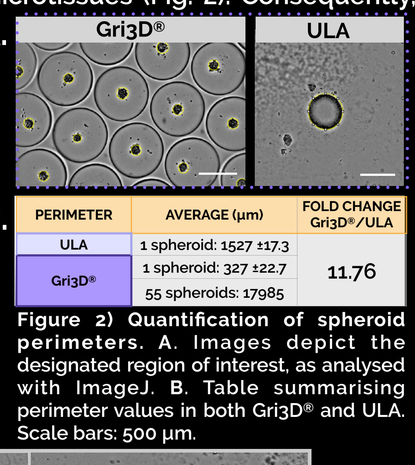


Figure 1) Comparison between Upcyte® hepatocyte spheroids grown in parallel A. in Gri3D® 96WP 600 µm microwells or **B.** in ULA 96WP plates. Both sets of spheroids originate from the same single-cell suspension and are cultured for up to 5 days with a seeding density of 7'500 cells per well. Images captured with a Tecan Spark® Cyto - 4x magnification. Scale bars: 500 µm.

Upcyte® hepatocyte cells aggregate and form compact spheroids over a 5-day culture period. We seed 7'500 cells per well, resulting in a single spheroid in each ULA well, and 55 microtissues of ~136 cells in each Gri3D® well (Fig. 1). In Gri3D®, the total surface area exposed to media and reagents is more than ten-fold greater due to the presence of multiple smaller microtissues (Fig. 2). Consequently, we observe a higher ATP concentration measured from an equivalent total cell number (Fig. 3). Upon exposure to CYP enzyme inhibitors Ketoconazole (CYP3A4) and α-Napthoflavone (CYP1A2), the viability of microtissues remains unchanged, evidenced by the constant LDH release (Fig. 4). Notably, spheroids in Gri3D® show much higher CYP3A4 basal activity compared to those in ULA. When treated with the well-known inhibitor, Ketoconazole, this activity can be halved in Gri3D®. However, such a reduction remains undetectable in ULA. As for CYP1A2, its basal enzymatic levels are virtually non-detectable; thus, the inhibitory effect of α-Napthoflavone is not discernible (Fig. 5).



PERIMETER	AVERAGE (µm)	FOLD CHANGE Gri3D®/ULA
ULA	1 spheroid: 1527 ±17.3	
Gri3D®	1 spheroid: 327 ±22.7	11.76
	55 spheroids: 17985	

Figure 2) Quantification of spheroid perimeters. A. Images depict the designated region of interest, as analysed with ImageJ. **B.** Table summarising perimeter values in both Gri3D® and ULA. Scale bars: 500 µm.

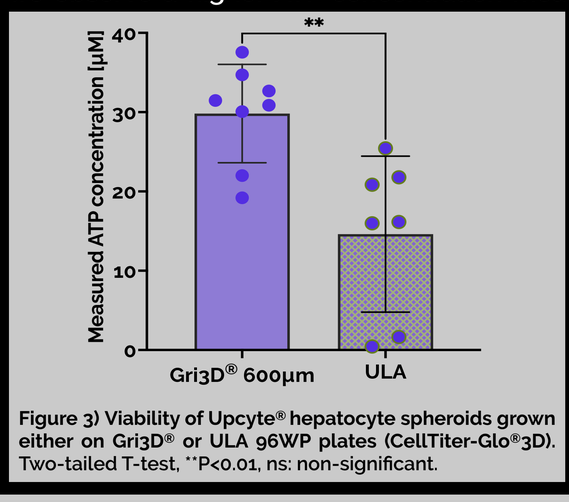


Figure 3) Viability of Upcyte® hepatocyte spheroids grown either on Gri3D® or ULA 96WP plates (CellTiter-Glo®3D). Two-tailed T-test, **P<0.01, ns: non-significant.

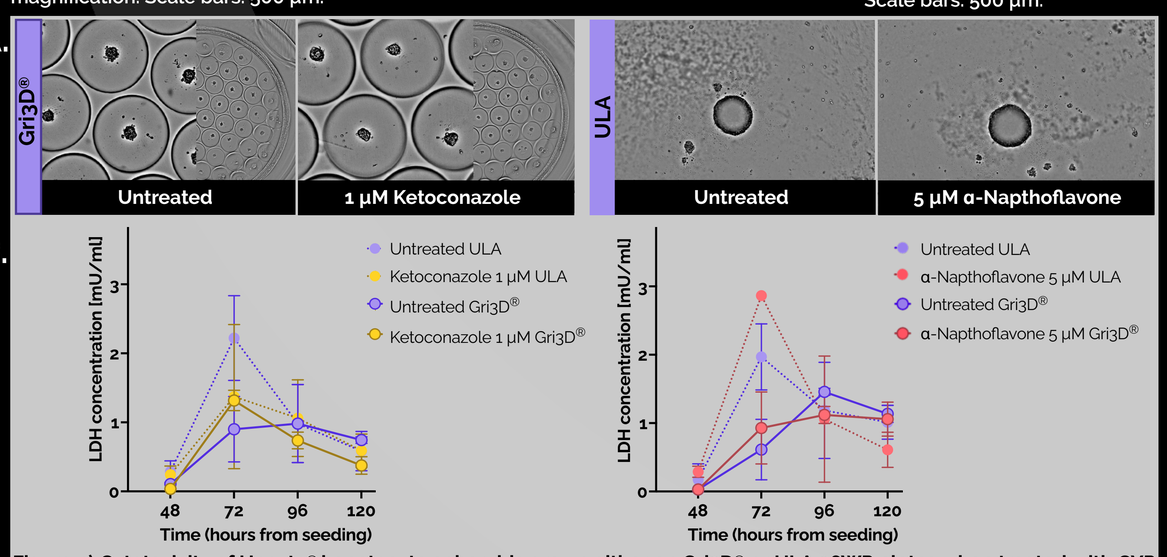


Figure 4) Cytotoxicity of Upcyte® hepatocyte spheroids grown either on Gri3D® or ULA 96WP plates when treated with CYP inhibitors. A. Brightfield images of spheroids, both treated and untreated, reveal no discernible difference. **B.** LDH release over time of Upcyte® hepatocyte spheroids grown on either Gri3D® or ULA 96WP plates (LDH-Glo™).

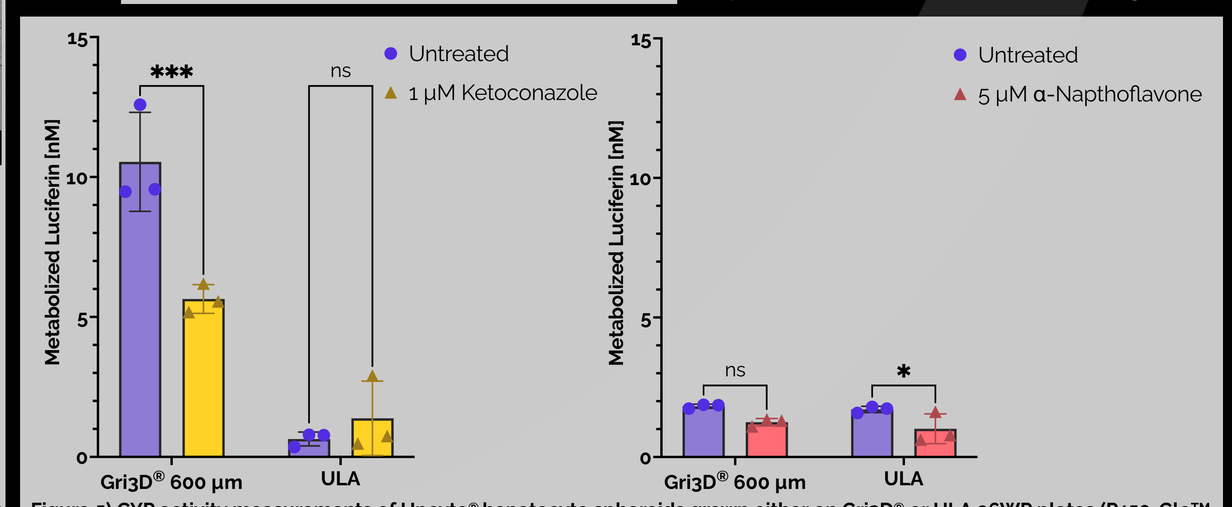


Figure 5) CYP activity measurements of Upcyte® hepatocyte spheroids grown either on Gri3D® or ULA 96WP plates (P450-Glo™ CYP3A4 and CYP1A2). Left: CYP3A4 activity inhibition upon treatment with Ketoconazole. **Right:** CYP1A2 activity inhibition after treatment with α-Napthoflavone. Two-way ANOVA with Fisher's multiple comparisons, *P<0.05, P***<0.001, ns: non-significant.

CONCLUSIONS

- Utilizing a 96-well plate format, Gri3D® 600 µm microwells facilitates the creation of over 50 microtissues per well, allowing for scalability, uniformity, and robustness.
- Both spheroid viability and enzymatic activity detection are improved in Gri3D®, which positions the platform as an optimal choice for advanced 3D-based drug evaluations.
- The synergistic use of Gri3D® technology with a compatible multimode plate reader, together with multiplexed cell-based assays, enriches our understanding of liver spheroid responses to drug exposure.
- Our pioneering approach offers a promising solution to address long-standing challenges in large-scale 3D cultures and compound evaluation on physiologically relevant models.

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