Platform Design, Culture Optimization and Assay Development in Liver Spheroids

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1. Abstract

Innovative in vitro liver models are essential for drug testing and the study of complex liver disease. In this project, 3D liver spheroids were generated from primary hepatocytes using molded agarose cones in 384-well plates, allowing long-term maintenance of liver-specific functions. By varying spheroid size and incorporating up to 10% non-parenchymal cells (NPCs), spheroid morphology and functionality, including albumin and urea production, were significantly improved. In contrast, 2D cultures on collagen lost function within one week. The inclusion of NPCs also promoted structural complexity, with features resembling vascular and bile canaliculi. Optimized RNA extraction and imaging protocols enabled detailed molecular characterization. These findings highlight the potential of 3D liver spheroids as a reliable platform for predictive toxicity testing and disease modeling, with future improvement focusing on culture standardization the addition of further cell types.

2. Introduction

The liver performs blood filtration, detoxification, bile production, and plasma protein synthesis. Hepatocytes show functional zonation: periportal cells produce proteins, while pericentral cells handle detoxification. [1] Together with non-parenchymal cells, they support regeneration after injury. 3D spheroids better mimic native liver tissue than 2D cultures, forming proliferative, quiescent, and necrotic zones. Spheroids are a classic 3D model. [2] In this project, 3D-printed molds were used to create agarose cones for uniform spheroid formation. Challenges with plastic molds led to the development of stainless-steel molds for smoother structures. Future work will include RNA analysis, immunofluorescence, and optimized embedding for cryosectioning to study spheroid behavior over time.

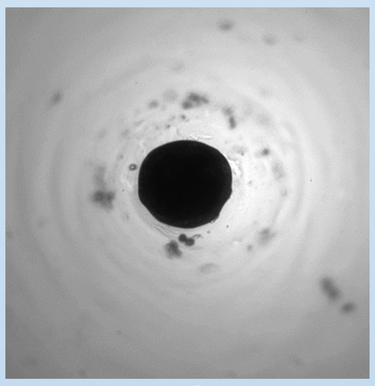

Figure 1: EVOS image of mouse hepatocytes cultured as spheroid (in agarose wells) (Stettler, 2025a)

3. Aims and Leading Questions

Aim 3: Develop assay protocols for RNA analysis and spheroid embedding to determine if spheroids are more pericentral or periportal in nature

Leading questions:

1. Is there a difference in the bulkRNA profile of the spheroids over time with respect to freshly isolated cells, and do these profiles align more closely with periportal or pericentral hepatocytes?

2. Does immunofluorescent labelling of embedded spheroids support these findings?

4. Methods and Material

3D liver spheroids were generated from primary mouse hepatocytes to better model liver-specific processes. Spheroids were formed in agarose wells, with 2D collagen cultures and media-only wells as controls. Different ratios of hepatocytes and non-parenchymal cells (NPCs, 10–50%) were tested to study their effect on liver function. For characterization, high-quality RNA was extracted and analyzed using Oxford Nanopore sequencing, enabling detailed insights into gene expression relevant for drug development and toxicology. Histological evaluation was performed with Hematoxylin & Eosin staining, providing structural information. In addition, Imaging Mass Cytometry (IMC) was applied, combining antibody-based detection with mass spectrometry to study cell populations and tissue organization in spheroids. Together, RNA sequencing, histology, and IMC form a comprehensive toolkit to characterize liver spheroids at both molecular and structural levels.

5. Results

H&E staining showed that the spheroids formed compact, round structures with preserved cell morphology. Some nuclei were lost at the edges, even after additional fixation steps. RNA extraction trials demonstrated acceptable A260/A280 values, while A260/A230 purity was initially reduced but improved after additional wash steps, though not yet within the reference range. Over time, most samples showed sufficient purity. RNA yield was slightly below the 1000 ng threshold required for nanopore sequencing and could be improved by pooling more spheroids. Imaging Mass Cytometry (IMC) is currently being optimized for OCT-embedded spheroids. While only DNA and HNFα could be reliably detected in spheroids, control liver tissue confirmed antibody specificity.

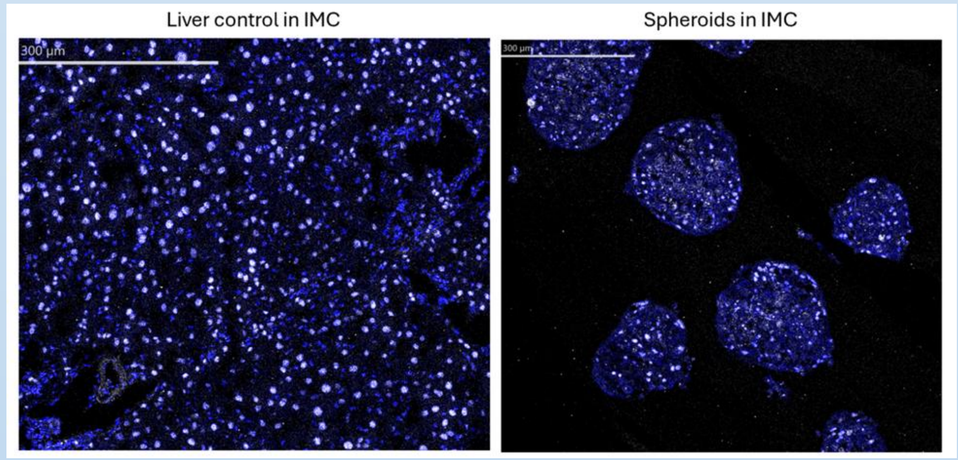

Figure 2: IMC of liver control and spheroids. On the left side of the figure the liver control is illustrated marked with DNA (blue colored) and HNFα (white colored). On the right side the spheroids are illustrated which are labeled with DNA (blue colored) and HNFα (white colored) (Stettler, 2025b)

6. Discussion

RNA extraction from spheroids proved more challenging than from free hepatocytes, resulting in lower yields and reduced A260/A230 purity, likely due to agarose contamination or incomplete lysis. Strategies such as additional wash steps, enzymatic agarose digestion, and collecting more spheroids are being explored to improve RNA quality before transcriptomic analyses can be performed. Imaging Mass Cytometry (IMC) on OCT-embedded spheroids yielded limited marker detection, likely caused by embedding-related epitope masking and mechanical stress during cryosectioning. H&E staining confirmed overall spheroid morphology and structural integrity, although occasional loss of nuclei (‘nuclear ghosting’) was observed, probably due to suboptimal fixation or freezing. These results highlight that technical optimizations in RNA extraction and embedding are crucial for reliable molecular and structural characterization of liver spheroids, enabling future studies on liver zonation and function.

References

[1] Thompson, W. L., & Takebe, T. (2021). Human liver model systems in a dish. *Develop Growth Differ. 63,* 47-58. https://10.1111/dgd.12708

[2] Park, S. Y., & Hong, H. J., Lee, H. J. (2022). Fabrication of Cell Spheroids for 3D cell culture and Biomedical Applications. *BioChip Journal, 17,* 24-43. https://doi.org/10.1007/s13206-022-00086-9

Figures

Fig. 1 Stettler, S. (2025a). *EVOS image of mouse hepatocytes cultured as spheroids (in agarose).* medi.

Fig. 2 Stettler, S. (2025b). *IMC of liver control and spheroids. On the left side of the fiugre the liver control is illustrated marked with DNA (blue colored) and HNFα (white colored). On the right side the spheroids are illustrated which are labeled with DNA (blue colored) and HNFα (white colored). m*edi.