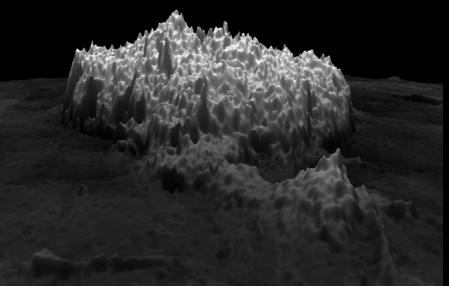


Biopixlar® Application Note

Microfluidic Spheroid Bioprinting

Dr. Andreas Svanström, Lead Application Scientist, Fluicell

INTRODUCTION

The discovery of novel and effective drugs is hampered by a low likelihood of acceptance (LOA), where more than 90% of all clinical drug candidates fail.[1] The selection of drug candidates is based on in vitro and in vivo experiments that largely fail to recapitulate the bona fide physiological effects in humans, calling for better predictive human surrogate models for drug discovery.

An important step in providing an alternative to 2D culture and animal models is the advancement of 3D models, where different cell types are combined in a 3D environment to mimic a tissues' extracellular matrix, biochemical and mechanical cues as well as cell: cell interactions seen in vivo.[2] More importantly, producing and combining multiple human tissue surrogate models that together demonstrate a biological system, eventually replacing animal models, is in line with the 3R principle (Refine, Reduce and Replace the use of animals).

The non-alcoholic fatty liver disease (NAFLD) is a chronic disease that affects 20-50% of the population[3], recognized by an abnormal accumulation of fat, which may eventually lead to liver failure and the need for liver transplantation[4]. Current models to target NAFLD include liver spheroids made by using ultralow attachment plates and the seeding of cells[5,6], applicable for high-throughput screening. However, the models are simple in nature by the lack of in vivo cell, microenvironment and structural heterogeneity of the liver[7].

Here, we present a novel application method to produce liver spheroids by using the Biopixlar bioprinting platform (Fluicell), carefully positioning cells in solution onto a substrate followed by gel encapsulation and culture (Figure 1). The method illustrates the potential to recreate the liver heterogeneity, with cell: cell contact prior to gel embedding, and tissue model maturation.

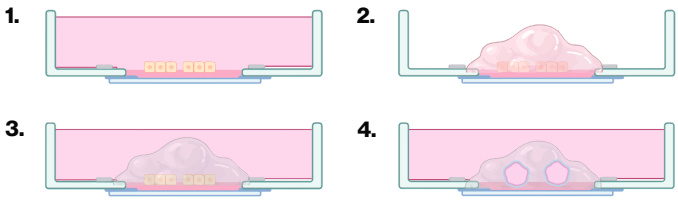


Figure 1. Illustration of the methodology. Cells are harvested from a standard 2D culture and printed in a pattern of choice, embedded with gel and studied over time. Imaged made using Biorender.com

Spheroid bioprinting - step by step guide

1. Bioprint suspended cells onto a cell culture dish containing PBS.
2. Replace the PBS with Matrigel.
3. Induce Matrigel gelification at 37° C for 20 min and add cell culture media.
4. Maturate the spheroid model for 4-7 days.

SPHEROID FORMATION

Cells were reproducibly printed onto a substrate in squared patterns, aligned with preprinted sites of cell attachment agent, in similar cell number (Figure 2A and 2C), consistent with a previously study using the same microfluidic based bioprinting technology[8]. Of note, cells did not attach to the substrate without the use of a cell attachment agent. The printed cells expanded in 3 dimensions into spheroid structures within 1 week time, showing a similar area given by perimeter measurements (Figure 2B and 2C). Until 4 days post printing and gel embedding, the expanding spheroids were efficiently measured using phase microcopy (Figure 3A), measuring a height of 43 µm. Spheroids cultured for longer time were imaged using 2-photon microscopy, with a z-stack of 152 µm (2 µm increments) (Figure 3B).

CONCLUSION AND DISCUSSION

The method of printing cells onto substrates whilst kept in solution (e.g., PBS or media) using Fluicell's microfluidic based bioprinter offers a novel way to produce spheroids with a defined starting point, here illustrated with cell number, architecture, and cell types. This provides a methodology to create advanced in vivo-like tissue models, represented by spheroid or organoids, that may be dispensed into plates for larger throughput drug toxicity assays or combined to form complex biological systems to study systemic drug effects. For imaging, phase microscopy is an excellent option to monitor the initial 3D growth (In this study, <40 µm height) whilst the models are in culture, whereas 2-photon microscopy is preferable for endpoint analysis, providing a high resolution and z-stacking on ticker tissue models (In this study, ≤ 150 µm height).

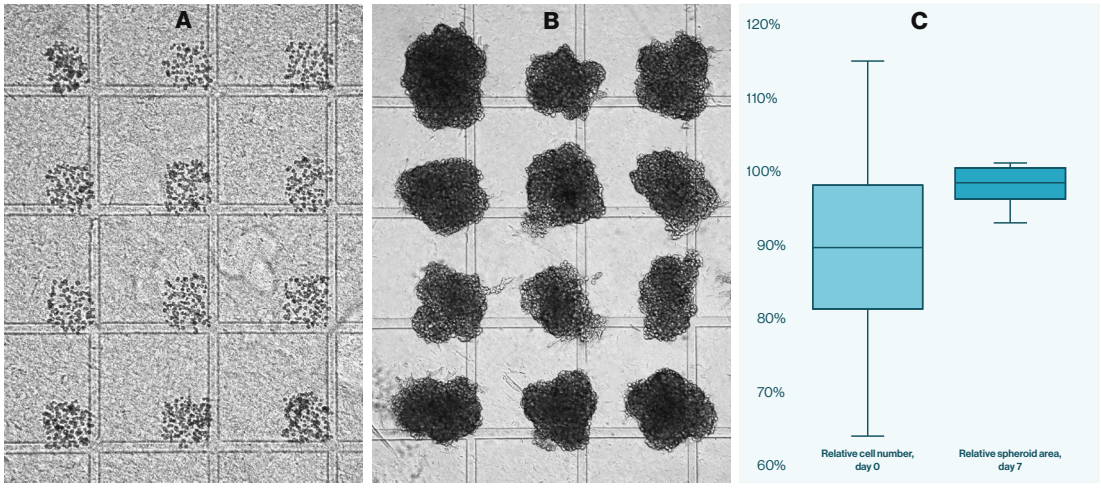


Figure 2. A) HepG2 cells printed in 4 by 3 squares on a grided dish (500 × 500 µm² grid size) and embedded in Matrigel. **B)** Following gel embedment, cells were cultured for 7 days to form spheroids. **C)** Relative number of cells per printed square, measured directly after printing and relative spheroid area, measured after seven days.

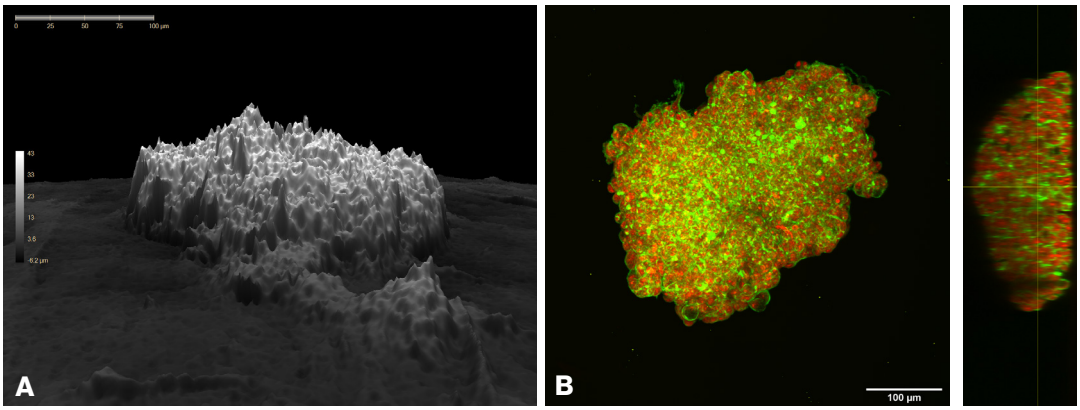


Figure 3. A) HepG2 cells cultured for 4 days post printing and gel embedding, imaged using phase microscopy. **B)** HepG2 and 3T3-J2 cells cultured for 7 days post printing and gel embedding, imaged using 2-photon microscopy. Spheroids cytoskeleton and nuclei stained using Actin-Phalloidin (AF488, green) and propidium iodide (red), respectively. Image shows maximum intensity projection. (https://www.p-inst.com).

METHODOLOGY

Cell culture

HEPG2 (Sigma) and 3T3-J2 (AddexBio) cells were maintained according to producers' recommendation and cultured in cell culture treated flasks (Falcon).

Surface

A flat proprietary mesh-like substrate was prepared and partially coated with Matrigel (Corning). The coating was made by adding 10 µl of a 1:1 ratio of Matrigel: Cell media of RPMI (Gibco) supplemented with 10% FBS (Gibco) to the substrate, covering the solution with a 13 mm PTFE membrane (Advantec), incubating for 5 min at room temperature following 5 min at 37° C, 5% CO2, with a closed lid. Prior to printing, the lid was removed, the membrane was slightly dried in air prior to pealing the membrane off the substrate surface and adding 3 ml of PBS (Cytiva).

Cell preparation

A sub confluent flask of cells was washed in PBS (Cytiva), detached using Accutase (Gibco) and washed in media by centrifuging for 3 min at 300 G. Cells were resuspended in PBS, syringed 3× (total volume) using a 25 G needle (BD) and filtered using a 40 µm cell strainer (Fisher scientific). The single cell suspension was washed once in PBS by centrifuged at 300 G for 3 min and counted. One million cells were aliquoted, centrifuged at 300 G for 3 min and resuspended in PBS with a final concentration of 15 mg/ml of PEG (Fisher Scientific) and 106 cells/ml in 100 µl.

Cell printing

Printheads of 30 µm and 50 µm were prepared according to manufacturer's recommendations (Fluicell). The 30 µm printhead was loaded with a cell attachment agent, poly-l-lysine (Sigma), of 0.5 mg/ml and printed onto a Matrigel-coated cell culture dish containing PBS (Cytiva) using the Biopixlar bioprinting system (Fluicell). The printing was executed using an automated printing protocol made with the Biopixlar software (Fluicell) at standard pressure settings, fine-tuned to make a flow zone of 50 µm. Following the printing of the cell attachment agent, cells were manually printed in an area of 150 × 150 μm^2, and the PBS (Cytiva) was replaced with Matrigel (Corning). The gel was allowed to set for 20 min at 37° C, 5% CO_2 prior to adding 1.5 ml of cell medium to the dish. The printed cells were cultured for 4–7 days.

Analysis

Images were taken using bright field microscopy (Zeiss) and the number of cells printed on each site, as well as the area of the spheroids following culture, were counted and measured (ImageJ) respectively. Values were normalized by average value. 3D models were imaged by phase microscopy (PHI) on day 4 whilst in culture. For 2-photon microscopy (Prospective instruments) on day 7, samples were prepared by fixating the spheroids in 4% PFA (Sigma), washing in PBS (Cytiva) and staining with actin-phalloidin-conjugated (Actin cytoskeletal staining) and propidium iodide-conjugated (Nucleus staining) fluorophores.

REFERENCES

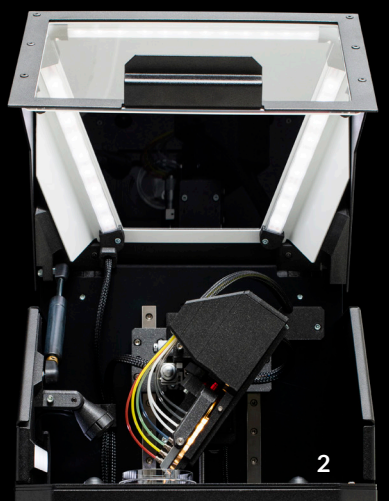
1. Biomedtracker® and Pharmapremia® (2020).
2. Moysidou, C-M. et al. Advances in Engineering Human Tissue Models. *Front. Bioeng. Biotechnol.* **8**, 620962 (2021).
3. K. Riazi, H. et al. The prevalence and incidence of NAFLD worldwide: a systematic review and meta-analysis. *Lancet Gastroenterol. Hepatol.* **7**, 851–861 (2022).
4. Pouwels, S. et al. Non-alcoholic fatty liver disease (NAFLD): a review of pathophysiology, clinical management and effects of weight loss. *BMC Endocr. Disord.* **22**, 63 (2022).
5. Kozyra, M. et al. Human hepatic 3D spheroids as a model for steatosis and insulin resistance. *Sci Rep* **8**, 14297 (2018).
6. Lasli, S. et al. A Human Liver-on-a-Chip Platform for Modeling Nonalcoholic Fatty Liver Disease. *Adv. Biosyst.* **3**, 1900104 (2019).
7. Trefts E, et al. The liver. *Curr. Biol.* **27**, 21 (2017).
8. Jeffries, G.D.M., et al. 3D micro-organisation printing of mammalian cells to generate biological tissues. *Sci. Rep.* **10**, 19529 (2020).

ABOUT BIOPIXLAR®

Biopixlar is Fluicell's family of high precision 3D bioprinting platforms. The Biopixlar platforms uses Fluicell's innovative open volume microfluidic technology and is capable of creating tissues, 3D cell cultures and cell arrays with single-cell precision. Biopixlar desposits cells directly in solution without any bioink, which ensures high cell viability and efficient intercellular communication. Biopixlar is available in two verions: as the modular Biopixlar platform and as the more compact Biopixlar AER.

ABOUT FLUICELL®

Fluicell is a Swedish life science company, specializing in high precsion research tools for biological and pharmaceutical research, in vitro disease models and cell-based regenerative medicine research and development. Fluicell provides innovative research instruments for single-cell biology and 3D bioprinting, based on proprietary microfluidic technology.



fluicell®

https://fluicell.com/
info@fluicell.com

Flöjelbergsgatan 8C,
431 37 Mölndal, Sweden