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Regenerative medicine: from new insights to new applications

ORGANTRANS

Controlled Organoids transplantation as enabler for regenerative medicine translation

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Upscaling from research to medical Sphericalplate 5D

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Executive Summary

For the creation of liver constructs in a standardised manner, spheroids of a predetermined size and composition will be used for bioprinting. To generate the spheroids, Sphericalplates 5D from Kugelmeier have been used which enable the formation of multicellular spheroids. An optimal cell number per microwell was optimised to be 200 cells per microwell. Spheroids retained some heterogeneity but on average the spheroids were relatively uniform in size with an average diameter of 124 µm [range 66 - 166 µm]. The spheroids created with the MP5D plates, 20,184 in total per plate, retain the expression of hepatic markers and are therefore excellent building blocks to continue to whole organ engineering within the ORGANTRANS project.

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1. Introduction

The current deliverable D2.2 is part of Work Package 2 (WP2). The overall aim of WP2 is to assure clinical grade spheroid production. One of the goals of WP2 is upscaling the spheroid production from the existing platform Sphericalplates, which harbour 753 microwells per well to Medicalplates, which harbour 20,184 microwells per well.

1.1. Spheroids

3-dimensional (3D) liver spheroids simply formed by gravity-induced aggregation and self-assembly and have been shown to sustain hepatic cellular phenotypes and viability in extended culture up to 4 to 5 weeks (Bell et al. 2016). Conventional 2D culture do not retain the hepatic cellular phenotype as important cell-cell interactions are lacking. The retention of physiologically relevant 3D architecture in liver spheroids will allow a more native tissue-like structure. Furthermore, transcriptomic and proteomic characterizations have shown that liver spheroids are very representative of native liver compared with other liver models in 2D culture format (Messner et al. 2018). Co-culture has been shown to be one of the strategies needed to increase the hepatic phenotype of stem cell derived hepatocytes (Chen et al. 2018). Therefore, we include supporting cell types in order to create multicellular spheroids. An added advantage is that liver spheroids are adaptable to a higher throughput workflow, vital for ORGANTRANS' success. These characteristics make liver spheroids an attractive model system for whole organ engineering.

1.2. Sphericalplates

The medical plate (MP5D) follows up on the Sphericalplate 5D (SP5D) which was conceived for research purposes. Like its predecessor, it comprises microwells shaped like inverted pyramids. Cells in suspension seeded into this geometry are induced to mutual adhesion and form spheroids. The SP5D design originally entailed circular housing compartments which partially distorted a low percentage of the 753 microwells adjacent to compartment walls, causing spheroid size deviation. To address this issue, a new engineered platform is designed to allow the production of 3'364 microwells in rectangular compartments. Every well is preserved in shape and distance to compartment walls, allowing the generation of up to 20'184 spheroids per plate. Together with novel tooling production with much higher precision and reproducibility of microwells in nickel shims, large volume production of MP5D is maintained.

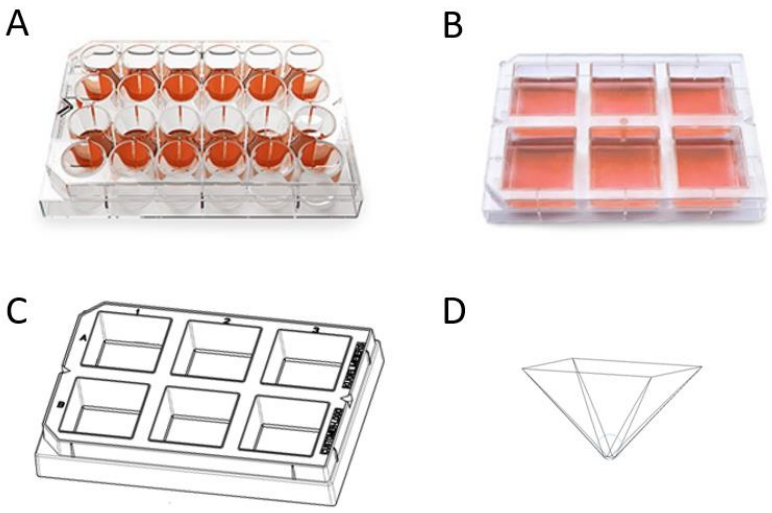

Figure 1. Kugelmeier microwell plates required for upscaling. Sphericalplate 5D with 753 microwells per well shown in (A). New medicalplate 5D (MP5D) with 3,364 microwells per well shown in (B). Schematic representation of MP5D showing a large volume well where the microwells are created using nickel shims (C). Schematic representation of a single microwell showing the inverted pyramid shape and rounded bottom for the creation of standardised spheroids.

References:

- C.C. Bell, D.F. Hendriks, S.M. Moro, et al. Characterization of primary human hepatocyte spheroids as a model system for drug-induced liver injury, liver function and disease. Sci Rep, 6 (2016), p. 25187
- S. Messner, I. Agarkova, W. Moritz, J.M. Kelm. Transcriptomic, proteomic, and functional long-term characterization of multicellular three-dimensional human liver microtissues. Appl In Vitro Toxicol, 4 (2018), pp. 1-12
- C. Chen C, A. Soto-Gutierrez, P.M. Baptista, B. Spee. Biotechnology Challenges to In Vitro Maturation of Hepatic Stem Cells. Gastroenterology. 2018 Apr;154(5):1258-1272.

2. Results and Discussion

In order to standardise the multicellular spheroids formation, we first optimised the optimal cell number of cells within a single microwell of the Sphericalplates (SP5D). Thereafter we tested the formation of spheroids within the Medicalplates (MP5D) which contain which contain 20,184 microwells per well compared to the 9,000 microwells in the SP5D plates.

2.1. Optimal cell number for the generation of multicellular spheroids using SP5D Sphericalplates

For the optimisation of multicellular spheroid formation in microwell plates we used the existing Sphericalplates (which have the same geometry compared to the Medicalplates) and compared the addition of 200, 400 and 600 cells per microwell. The optimised ratio described in deliverable 1.3, 6:4:2 of respectively liver organoid cells, mesenchymal stem cells (MSCs) and human umbilical cord endothelial cells (HUVECs), was used throughout these experiments. In addition, we compared spheroid formation to commercially available microwell plates from Corning (Elplasia). Results indicated that 200 cells was the optimal amount of cells within the microwells creating spheroids of 124 µm [range 66 - 166 µm] in diameter. No large differences were observed between Elplasia and SP5D plates.

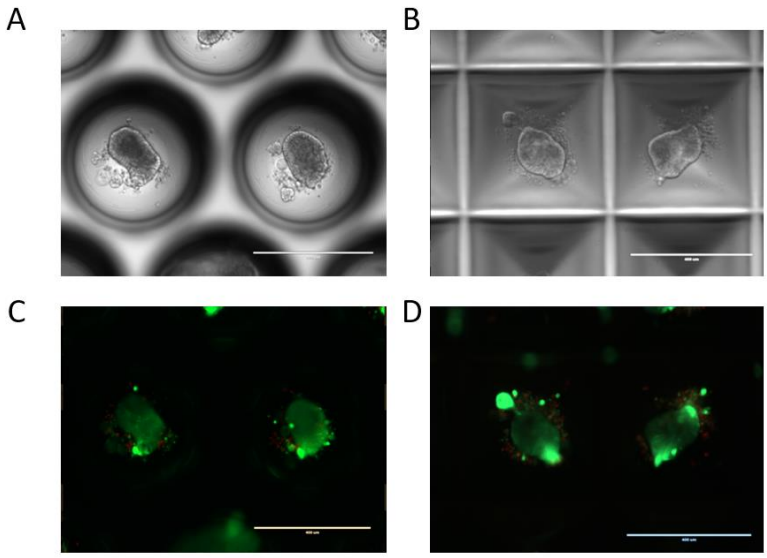

Figure 2. Results on the optimisation of the optimal amount of cells within Sphericalplates (SP5D) compared to Corning Elplasia plates. Spheroid formation at day 6 in Elplasia (A) and SP5D (B) plates. Live/dead staining in Elplasia (C) and SP5D (D) plates. Green indicates live cells, red indicates dead cells.

2.2. Upscaling of spheroid formation using Kugelmeier 5D Medicalplates (MP5D)

Although the Corning Elplasia plates have roughly 500 microwells per well and Sphericalplates (SP5D) have 753 microwells, for upscaling towards printed constructs this is too limited. Therefore, we have employed Kugelmeier 5D Medicalplates (MP5D) which contain roughly 3,364 microwells per well. Results indicate that with a similar amount of cells per microwell (200), spheroids can be created with in larger numbers and easily flushed out of the wells for further analysis (Figure 3 and Figure 4).

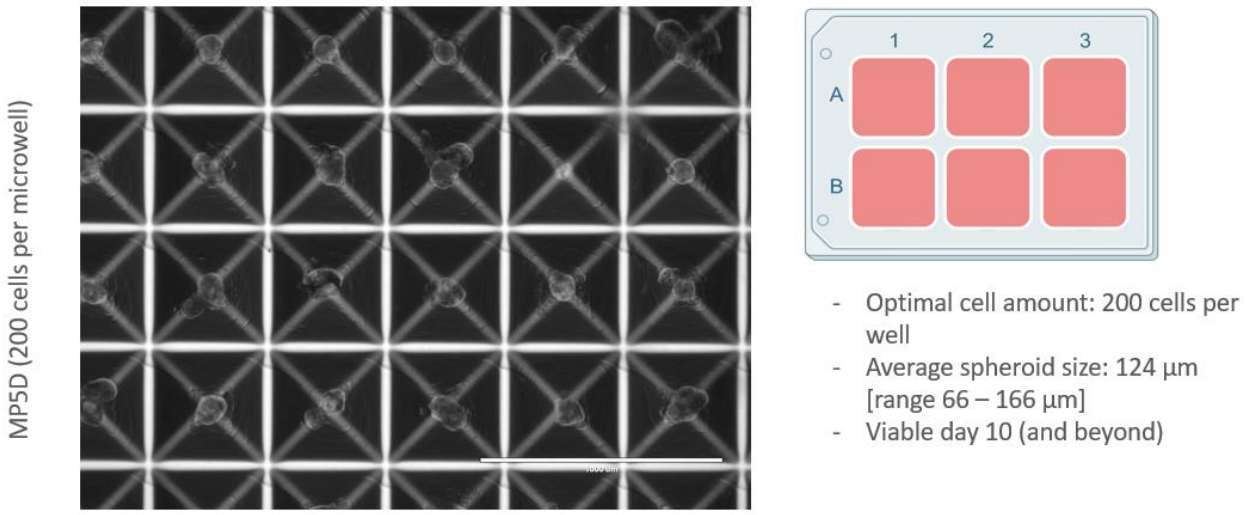

Figure 3. Tricellular spheroid formation in 5D medicalplate (MP5D). Formation of spheroids (left) from liver organoids combined with mesenchymal stem cells (MSCs) and HUVECs (macrovascular endothelium) in a ratio of 5:4:1 on day 6. Schematic overview (right) of Kugelmeier 5D medicalplate (MP5D) with 20,184 microwells.

In order to observe if spheroids can be harvested from the medical plates (MP5D) we have flushed out the spheroids with an excess of culture media and analyze if spheroids remained intact. Results indicated that the spheroids remain intact with the procedure (Figure 4). If can be observed that a degree of heterogeneity remains in the spheroid size and density. For the selection of spheroids in the future, we will need to determine what the difference is in terms of functionality, in order to be implemented in the selection in task 3.2 and 3.3.

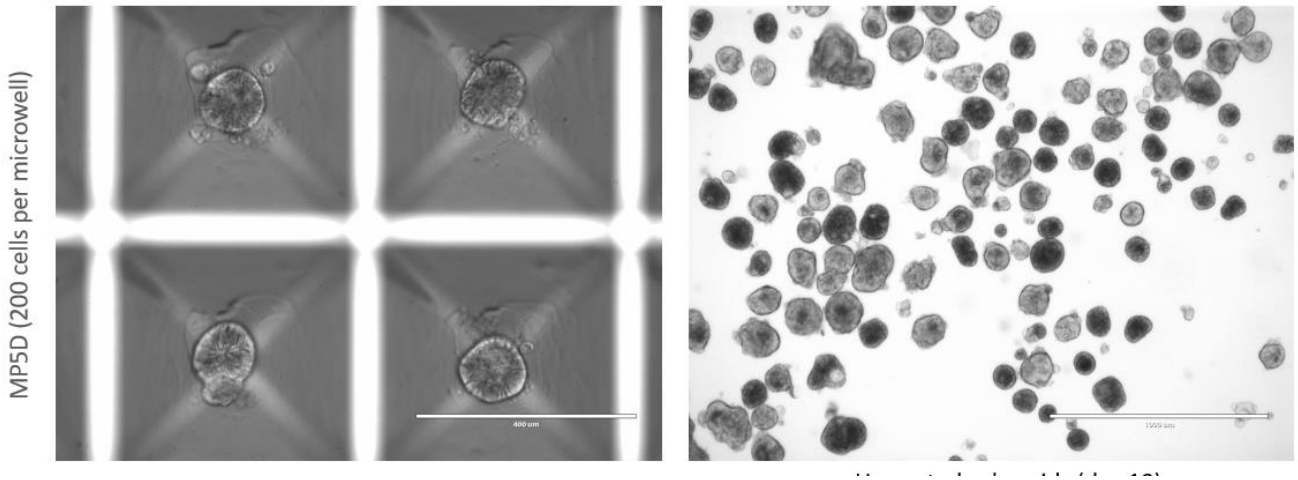

Figure 4. Multicellular spheroid formation in 5D medicalplate (MP5D) and harvesting. Formation of spheroids (left) from liver organoids combined with mesenchymal stromal cells (MSCs) and HUVECs (macrovascular endothelium) in a ratio of 6:4:2 in MP5D plates. Flushed out spheroids on day 10 (right).

An initial characterisation of the spheroids indicates that the cells are positive for hepatic marker keratin 18 and supporting cell type markers can be seen in the multicellular spheroids indicating that they retain their hepatic phenotype in MP5D plates (Figure 5) very similar as described in deliverable 1.3.

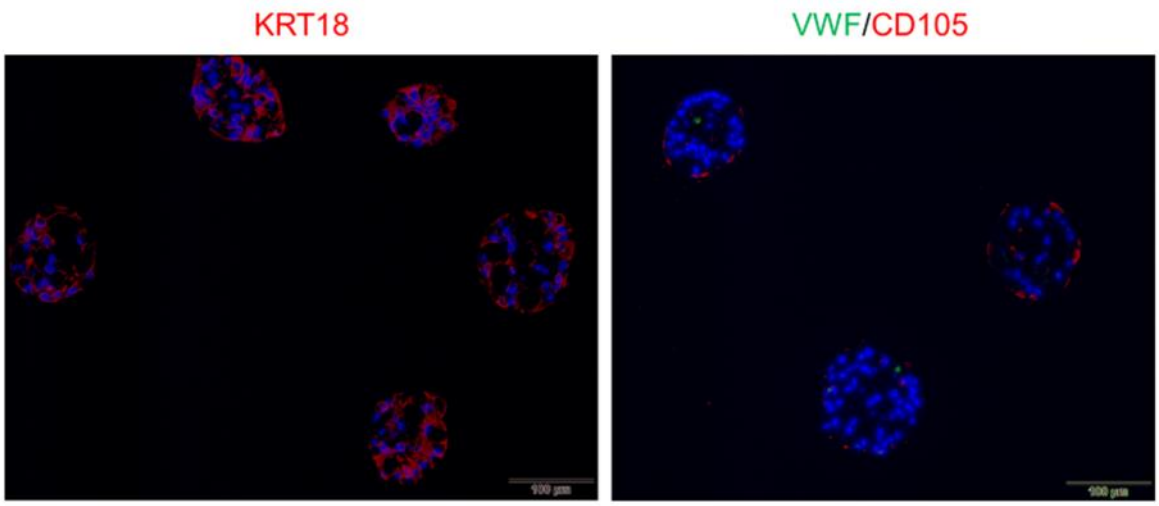

Figure 5. Multicellular spheroid formation in 5D medicalplate (MP5D) and stainings. Immunofluorescent staining showing hepatic marker Keratin 18 and endothelial marker Von Willebrand Factor (VWF) as well as endothelial and mesenchymal stem cell marker Endoglin (CD105).

3. Conclusions

We have optimised the optimal amount of cells per microwell to be 200 cells to create a multicellular spheroids. The upscaling of SP5D plates was successful which allows a 4.5-fold increase in the amount of spheroids per well, from 753 to 3,364 microwells. The yield for an entire plate of SP5D plates is over two-fold more spheroids. Results indicated that the spheroids formed well in the microwells and they could be harvested by flushing them out with a pipette. Spheroids retained some heterogeneity but on average the spheroids were relatively uniform in size with an average diameter of 124 µm [range 66 - 166 µm]. The spheroids created with the MP5D plates, 20,184 in total per plate, retain the expression of hepatic markers and are therefore excellent building blocks to continue to whole organ engineering within the ORGANTRANS project.

4. Degree of Progress

After the creation of a uniform media (task 2.3 and deliverable 2.3) and optimal cell ratio (task 1.3 and deliverable 1.3), the task 2.1: Standardized and high-throughput production of hepatic spheroids in Sphericalplate 5D (KUG, UU), was successful (D2.2) and 100% completed. The optimal cell number for the microwells was optimized to 200 cells per microwell. The upscaling from the 5D Sphericalplates (753 microwells per well) towards 5D medicalplates (3,364 microwells per well) was successful. In a next step, task 2.3: Genomic and proteomic profiling of standardized spheroids (KUG, UU) we will further analyze multicellular spheroids by, for instance, genome-wide transcriptome analysis, which will allow a global view of cellular state and internal communications. Moreover, this transcriptome analysis can also be used in risk assessment for the putative tumorigenesis potential of the cell product. This analysis will yield in an SOP with the aim to standardize analysis protocols for later clinical release criteria after which WP2 is concluded.

5. Dissemination Level

The deliverable is public, the protocols will be part of a future publication of the generation of hepatic spheroids and its intended use for bioprinting and transplantation into mice.

Protocols are provided as 12Addendum 1. Hepatic Multicellular Spheroid formation using Elplasia vs. SP5D plates and optimization of cell number per well..

6. Addendum 1. Hepatic Multicellular Spheroid formation using Elplasia vs. SP5D plates and optimization of cell number per well.

Preparation

- Pre-warm spheroid plate overnight
    - Add 500 µL of complete medium to each functionalized well
- Incubate two 6-well plates with 500 µL 1xTryple containing DNase (0.5 mg/mL) in each well
- Label and pre-cool 15-mL conical tubes
- Turn on the centrifuge (4°C)
- Pre-warm complete medium in 37°C water bath
- Prepare 1xTryple + DNase (0.5 mg/mL)

Methods

Intrahepatic Cholangiocyte Organoids (ICOs):

1. Discard the old culture media.
2. Add 1 mL cold PBS/HBSS and break up the basement matrix by gently scraping and pipetting up and down.
3. Transfer the organoid suspension to a 15-mL conical tube (3 wells of a 12-well per tube) and further disrupt the basement membrane by pipetting up and down with a P200 (20x).
4. Add cold PBS/HBSS (10-13 mL) to dilute the old basement matrix. Invert the tube 3-5 times and centrifuge at 400g for 3’ at 4°C.
5. Discard the supernatant. Add 500 µl cold PBS/HBSS and break the organoids mechanically by pipetting up and down with a P200 (20x). Add cold PBS/HBSS (10-13 mL).
6. Invert the tube 3-5 times and centrifuge at 300g for 3’ at 4°C.
7. Discard supernatant and resuspend cells in 0.5-1 mL 1xTryple containing DNase (0.5 mg/mL) using a P1000.
8. Transfer cell suspensions to a well of 6-well plate containing 500 µL 1xTryple + DNase (0.5 mg/mL) and incubate at 37°C.
9. Every 5 minutes mix the cell suspensions by pipetting up and down with a P200.
10. When >90% cells are single cell transfer to new 15 mL conical tube.
11. Invert the tube 3-5 times and centrifuge at 200g for 4’ at 4°C.
12. Remove the supernatant and resuspend the cell pellets in 1 mL complete medium.
13. Take 10 µL for cell counts and put cells on ice.

HUVECs & MSCs:

1. Discard the old culture media.
2. Wash the cells with 10 mL PBS.
3. Discard PBS and add 2.5 mL 1xTrypLE + DNase (0.5 mg/mL)
4. Incubate cells for 5 min at 37°C
5. Every 5 minutes check to see if cells are detached.
6. When >90% cells are detached and 10 mL cold AD+ and transfer cells to 15-mL conical tube.
7. Centrifuge at 300g for 3’ at 4°C.
8. Remove supernatant and resuspend pellets in 1 mL complete medium.
9. Take 10 µL aliquot for cell counts and put cells on ice.

Co-culture in Elplasia and SP5D:

1. Transfer ICO cells, HUVECs and MSCs in 6:4:2 ratio to appropriate 15-mL conical tubes:

Elplasia (600)	**ICO cells**	**HUVECs**	**MSCs**	**Total cells**
Cells per micro-well	300	200	100	*600*
Cells per well	166,200	110,800	55,400	*332,400*
Cells per 3 wells	**498,000**	**332,400**	**166,200**	***996,600***

Elplasia (400)	**ICO cells**	**HUVECs**	**MSCs**	**Total cells**
Cells per micro-well	200	134	66	*400*
Cells per well	110,800	74,236	36,564	*221,600*
Cells per 3 wells	**332,400**	**222,708**	**109,692**	***664,800***

Elplasia (200)	**ICO cells**	**HUVECs**	**MSCs**	**Total cells**
Cells per micro-well	100	67	33	*200*
Cells per well	55,400	37,118	18,282	*110,800*
Cells per 3 wells	**166,200**	**111,354**	**54,846**	***332,400***

SP5D (400)	**ICO cells**	**HUVECs**	**MSCs**	**Total cells**
Cells per micro-well	200	134	66	*400*
Cells per well	150,000	100,500	49,500	*300,000*
Cells per 6 wells	**900,000**	**603,000**	**297,000**	***1,800,000***

SP5D (200)	**ICO cells**	**HUVECs**	**MSCs**	**Total cells**
Cells per micro-well	100	67	33	*200*
Cells per well	75,000	50,250	24,750	*150,000*
Cells per 6 wells	**450,000**	**301,500**	**148,500**	***900,000***

ICOs in Elplasia and SP5D:

Elplasia (600)	**ICO cells**
Cells per micro-well	600
Cells per well	*332,400*
Cells per 3 wells	***997,200***

Elplasia (400)	**ICO cells**
Cells per micro-well	400
Cells per well	*221,600*
Cells per 3 wells	***664,800***

Elplasia (200)	**ICO cells**
Cells per micro-well	200
Cells per well	110,800
Cells per 3 wells	***332,400***

SP5D (400)	**ICO cells**

Cells per micro-well	400
Cells per well	300,000
Cells per 6 wells	***1,800,000***

SP5D (200)	**ICO cells**
Cells per micro-well	200
Cells per well	150,000
Cells per 6 wells	***900,000***

2. Add appropriate medium to the corresponding 15 mL conical tube to reach a total volume of 1.5 mL (for 3 wells) or 3 mL (for 6 wells).

3. Mix cell suspension with a P1000 (set at 500 µL) and pipette 500 µL into 3 (Elplasia) or 6 (SP5D) wells per condition, as depicted in *figure 1*.

4. Take pictures of each condition and place plate in incubator.
    - Record time and date
    - Record which well was used for pictures

5. Refresh half the medium after collecting day 4 samples, and every 2 days henceforth.

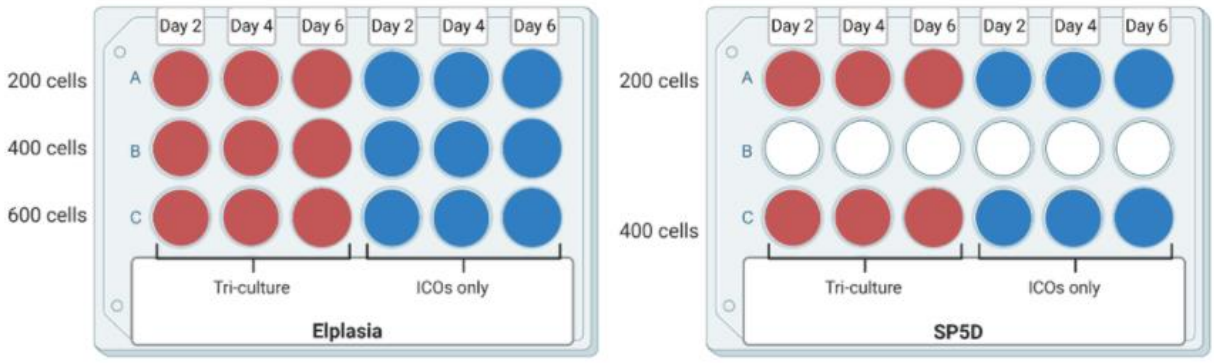

Figure 6. Schematic overview of the optimisation of the optimal amount of cells within Sphericalplates (SP5D) compared to Corning Elplasia plates.