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U N I V E R S I T Y**

FACULTY OF MEDICINE

Development of microfluidic systems for the formation of brain components

Doctoral thesis

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Annotation

Organoids, as 3D cell culture models, are *in vitro* multicellular structures designed to emulate tissue or organ-like properties that better replicate the cellular environment *in vivo*, providing opportunities to produce more relevant research results. Cerebral organoids offer a unique platform to investigate neurodevelopment and neurological diseases. However, current methods for generating cerebral organoids suffer from limitations such as time-consuming protocols and high heterogeneity among organoids. Due to the unique properties of the microfluidic method, we can address these challenges, and we present an automated microfluidic device for the effective formation and differentiation of cerebral organoids. Here, we focused on fabrication and optimization for initial cell formation and cultivation. By using *in silico* simulations, we supported the effectiveness of the microfluidic chip in replicating cell culture microenvironments. We then used this newly developed system for the cultivation and differentiation of cerebral organoids and utilized numerous analytical methods to prove that our novel microfluidic device could be successfully utilized to generate high-quality cerebral organoids. Finally, as proof of the versatility of our system, we used our functional microfluidic system to culture and differentiate retinal organoids. Lastly, in collaboration with OTH Regensburg, we worked on the development of the microfluidic system for single-cell sorting. Taken together, these results highlight the potential of custom-designed microfluidic technology in improving the efficiency of cerebral organoid culture as well as its utility in other cell-based experimental workflows.

Abstract

Introduction

Microfluidics enables precise control of microscale environments, essential for cultivating 3D tissue structures like cerebral organoids derived from iPSCs, which mimic human brain functions. Unlike static methods, microfluidics offers dynamic control and single-cell analysis capabilities. This dissertation focuses on developing a microfluidic chip for organoid culture and droplet generation for single-cell separation.

Methods

A microfluidic chip for organoid culture was fabricated using stereolithography 3D printing and soft lithography, producing PDMS chips with flow channels and cultivation wells. Computational Fluid Dynamics simulations optimized nutrient diffusion and shear stress. Cerebral organoids from human pluripotent stem cells were cultivated for 50 days and analyzed with qPCR and immunohistochemistry. Retinal organoid cultivation was tested as proof of concept. A separate chip, created with laser ablation and wet etching, was designed for droplet-based single-cell separation.

Results

Compared to standard methods, the microfluidic system demonstrated enhanced aggregation and differentiation of cerebral organoids, confirmed through microscopy and molecular analyses. Retinal organoid cultivation validated the chip's versatility, while the droplet-generation chip showed potential for single-cell applications.

Conclusion

This study demonstrates the advantages of microfluidics in optimizing cerebral and retinal organoid cultivation and its utility in the droplet generation for single-cell analysis. All these results highlight the unique potential of microfluidics to be used for diverse biomedical applications.

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Glossary

SLA	– Stereolithography
3D	– Three-dimensional
PDMS	– Polydimethylsiloxane
CFD	– Computational fluid dynamics
qPCR	– Quantitative PCR
hESCs	– Human embryonic stem cells
iPSCs	– Induced pluripotent stem cells
WP	– well plate
Re	– Reynolds number
Ca	– Capillary number
UV	– Ultraviolet
CAD	– Computer-aided design
FDM	– Fused deposition modelling
ABS	– Acrylonitrile Butadiene Styrene
PLA	– Polylactic Acid
PETG	– Polyethylene Terephthalate Glycol
DLP	– Digital Light Processing
IPA	– Isopropyl alcohol
2PP	– Two photon polymerization
EBL	– Electron beam lithography
NIL	– Nano imprinting lithography
ESCs	– Embryonic stem cells
2D	– Two-dimensional
ECM	– Extra cellular matrix
FDM	– Finite difference method
FEM	– Finite Element Method

FVM	– Finite Volume Method
EB	– Embryoid body
CO	– Cerebral organoid
PBS	– Phosphate Buffered Saline
BSA	– Bovine Serum Albumin
EDTA	– Ethylenediaminetetraacetic acid
NIM	– Neuro induction medium
L _G	– Length of contour perimeter
L _F	– Length of outer convex contour
RNA	– Ribonucleic acid
GFP	– Green fluorescent protein
DSP	– Double Side Polished
HF	– Hydrogen fluoride
TMAH	– Tetramethylammonium hydroxide
ROCKi	– Rho-kinase inhibitors
DNA	– Deoxyribonucleic acid
mPEG	– Methoxy polyethylene glycol
IHC	– Immunohistochemistry
HMDS	– Hexamethyldisilazane
APTES	– (3-Aminopropyl)triethoxysilane

1 Introduction

1.1 Microfluidics

Microfluidics has emerged as a game-changer field in life sciences, showing promise in research and industry through its capacity to miniaturize and enhance fluid handling¹. Originally seen as a groundbreaking technology, microfluidics is now viewed as a maturing field². With devices operating at nano or micro scales, the field has advanced significantly, enabling faster reaction times, novel functionalities, and the study of phenomena previously inaccessible at larger scales³.

This area of fluid mechanics focuses on the flow of fluids through micro-sized channels and the creation of devices that can handle extremely small fluid volumes, typically in the range of 10^{-15} to 10^{-6} liters⁴. Microfluidics involves the design and production of microsystems, such as microreactors and micro-channels^{5,6}, and typically consist of valves, chambers, and pumps, which together enable precise control over fluid movement and reactions⁷. This interdisciplinary field connects multiple areas, including physics, chemistry, biology, and engineering. Through microfluidic technology, biological and clinical tests can be performed in a highly efficient and parallelized manner⁸. Compared to traditional equipment, microfluidic devices offer significant advantages: they consume less power and time, reduce costs of manufacturing and handling, and provide greater flexibility and precision in liquid manipulation. They also minimize sample and reagent use, support high-throughput screening, enable rapid sample processing, and achieve high accuracy and resolution⁹.

1.2 Physical principles for microfluidic in biology

To work with microfluidics, it is essential to first understand the physical phenomena that occur at the microscale. Microfluidics enables the manipulation and analysis of fluids in structures at the micrometre scale. At the microscale, different forces become dominant over those experienced in everyday life¹⁰. Due to scaling effects, simply shrinking existing large devices and expecting them to function effectively at the microscale is often counterproductive¹¹. New designs must be created to take advantage of forces that work on the microscale. The parameters that become dominant in microfluidics include fluid flow, diffusion, fluid resistance and surface effects.

1.2.1 Fluid flow

The flow of the fluid ion microscale differs from that on a macroscale and it plays an important role in the design and fabrication of microfluidic devices¹². The flow of fluids along solid surfaces is called internal flow, and the type of flow is characterized by the Reynolds number (Re). It is the ratio of inertial forces to viscous forces:

$$Re = \frac{\rho v D_h}{\mu} \quad (1)$$

Where ρ is the fluid density, v is the characteristic velocity of the fluid, μ is the fluid viscosity, and D_h is the hydraulic diameter. Laminar flow is the movement of fluid particles in regular, orderly paths that run parallel to the walls of a tube or channel. Laminar flow is characterized by $Re < 2000$, low viscosity, high momentum diffusion and small momentum convection. Pressure and velocity are almost independent of time in laminar flow. Turbulent flow is the random motion of the fluid particle in a particular one-way direction. Turbulent flow is characterized by $Re > 4000$, high momentum diffusion, low momentum convection, and a rapid variation of pressure and velocity in space and time. Transition flow is characterized by the transition between the laminar and turbulent flow with Re between 2000 and 4000. Flow is almost laminar in microfluidic channels due to the small dimensions of microfluidic channels^{1,13}.

1.2.2 Diffusion

Diffusion is the movement of molecules driven by a concentration gradient. As diffusion times are short, concentration gradients can be created in miniaturized devices¹⁴. In one dimension, movement due to diffusion can be governed by the Einstein–Smoluchowski relation:

$$x = \sqrt{2Dt} \quad (2)$$

Where x is the distance a particle moves in a time t , and D is the diffusion coefficient of the particle. In microfluidics, diffusion is important for mixing and for the transport of nutrients and signaling molecules in biological applications.

1.2.3 Fluidic resistance

Fluid resistance in microchannels is determined by a set of equations with well-known solutions¹⁵. The flow rate within a microchannel is given by:

$$Q = \Delta P / R \quad (3)$$

Where Q is the flow rate, P is the pressure drop across the channel, and R is the channel resistance. The most common channel geometry, because of its presence in blood transport, is the circular tube. The resistance of a circular geometry can be calculated using the formula:

$$R = \frac{8\mu L}{\pi r^4} \quad (4)$$

Where μ is fluid viscosity, L is the channel length, and r is the channel radius. The resistance of a rectangular microchannel with a high aspect ratio can be found by:

$$R = \frac{12\mu L}{wh^3} \quad (5)$$

Where w is channel width and h is channel height¹.

1.2.4 Surface effect tension and capillarity

The surface area-to-volume ratio and surface tension forces play a key role in fluid flow within microchannels. Large surface area to volume ratio aids in capillary electrophoresis in microchannels by removing excessive heat¹⁶. Surface tension results from cohesion between liquid molecules at the liquid/gas interface. The surface free energy of a liquid measures the amount of tension present on its surface. The distance that water will travel through a capillary is directly related to the water's surface free energy and inversely related to the radius of the capillary. When microchannels with dimensions in the micron range are used, the distances liquids can travel due to capillary forces alone become significant. The manipulation of surface tension can enhance fluid transport and mixing in microfluidic devices.

For two-phase microfluidics, capillarity is an important term. Capillary number representing relative effect of the viscous forces to the surface tension forces:

$$Ca = \frac{\mu V}{\sigma} \quad (6)$$

Where μ is the dynamic viscosity of the liquid, V is the characteristic velocity and σ is the interfacial tension between two fluid phases. The capillary number is used whenever the forces resulting from fluid motion are to be compared to the forces resulting from surface tension. This is the case if a liquid is moved across a second fluid layer, e.g., a gas or an immiscible second liquid. A good visual example of these effects is droplets suspended in an inert liquid in droplet microfluidics. The viscous forces of the surrounding inert liquid may deform the droplets due to, e.g., local increases in pressure due to variations in the flow conditions. However, the interface tension forces between the two liquids are usually significantly higher, in which case the droplet may be locally deformed but not destroyed. In this case, $Ca \ll 1$ ¹⁷.

1.3 Fabrication techniques for microfluidics

Microfluidic fabrication techniques have evolved alongside the increasing complexity and miniaturization of features integrated into final devices. Consequently, efforts have been directed towards developing fast and reliable techniques.

1.3.1 Photolithography

The most used technique with general fabrication protocol is photolithography. The process involves several steps that use light to transfer a 2D pattern from a photomask onto a photosensitive material called a photoresist, which is applied to the substrate (Fig. 1). First, a thin layer of photoresist is spin-coated onto the silicon substrate to a uniform thickness. This viscous material is light-sensitive, which will define the pattern based on how it interacts with light exposure. There are two types of photoresists: the positive and the negative. When exposed to UV radiation, positive resist becomes soluble, while negative resist becomes cross-linked and hence insoluble. The photoresist-coated substrate is subsequently exposed to UV light through a patterned stencil known as photomask. The alignment of the photomask is critical for ensuring that the pattern is transferred accurately. Following exposure, the substrate undergoes development. The developer solution is used to dissolve the soluble photoresist, leaving patterned photoresist and exposed substrate. Finally, any remaining photoresist may be removed, or additional processing steps, such as etching (negative patterns) or dissolving (positive patterns), can be applied to create three-dimensional structures on the substrate based on the developed pattern. This leaves a microstructure of perfectly patterned silicon once all of the photoresist has been removed. This structure may then be utilized directly or as a mold to fabricate devices^{18–20}.

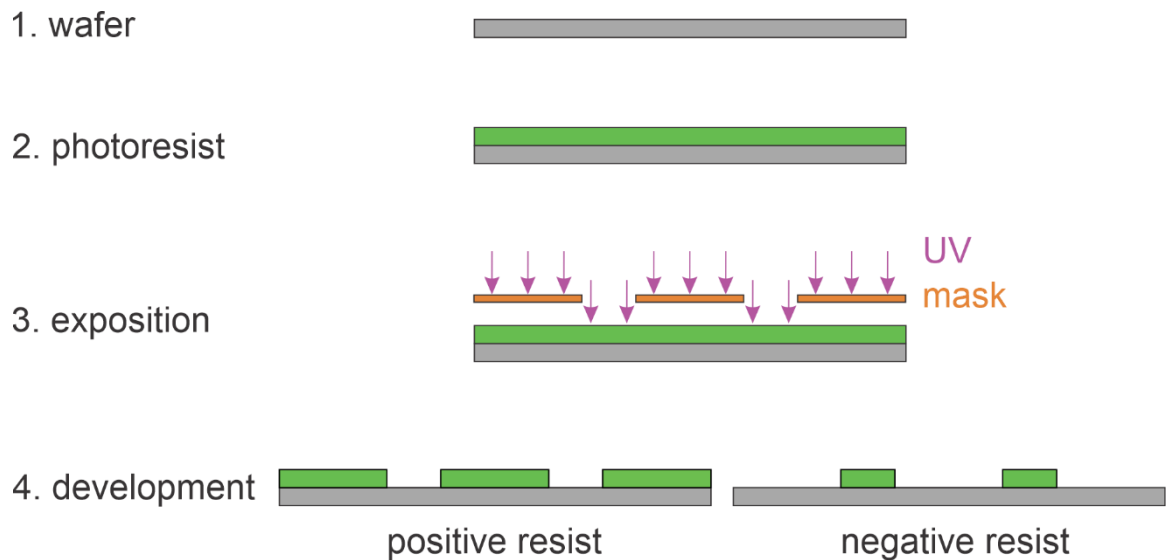


Fig. 1: Photolithography process

1.3.2 Etching

This fabrication technique is widely used in microfabrication and microelectronics to selectively remove material from a substrate, forming intricate patterns or structures. This process is essential in creating micro- and nanoscale features on surfaces, and it often follows lithographic patterning steps to define specific areas for removal. Etching can be classified into two main types: wet etching and dry etching, each with distinct mechanisms and applications. Wet etching involves immersing the substrate in a liquid chemical solution, or etchant, that reacts with and dissolves the exposed areas of the substrate. These etchants can be acidic, basic, or oxidizing solutions, depending on the material being etched. The regions not meant to be etched are protected by a photoresist layer, which resists the chemical reaction. Dry etching uses gases or plasmas instead of liquid chemicals to remove material from the substrate. In a vacuum chamber, reactive gases are introduced and turned into plasma by applying an electric field. The ions and reactive radicals generated in the plasma interact with the substrate surface, selectively removing material from the exposed areas. Wet etching is simpler and easier to implement, but it has higher costs associated with it and requires large quantities of etching chemicals. Dry etching is more expensive and requires complex equipment, but it is more efficient and precise^{21–24}.

1.3.3 Laser ablation

Laser ablation is a process in which a laser beam is used as the main tool for the ablation of the target material. A laser, as a highly concentrated energy source, is centered at a specific place of the target material for the evaporation of light-absorbing materials (Fig. 2). The term “ablation” means the removal of surface atoms by a multiphoton excitation process, i.e., thermal evaporation. When the laser beam is centered on a target material in an ambient (liquid or gas) medium, it increases the temperature of the irradiated spot and evaporates it. The evaporated species and the surrounding molecules thus collide with each other, which results in the excitation of the electron state coupled with light emission and generation of ions and electrons, thus forming a laser-induced plasma plume. The structure of the plasma depends upon the target material, ambient medium (liquid or gas), ambient pressure, and laser used for ablation.

The advantages of laser ablation include its broad compatibility with various workpiece materials. It can effectively process metals, polymers, ceramics, composites, semiconductors, diamond, graphite, and glass. Additionally, the force exerted during laser ablation is significantly lower than that involved in mechanical machining. This allows for the effective treatment of thin and elastic workpieces without the need for mechanical strength. Moreover, laser ablation is a non-contact machining process, resulting in no mechanical wear, and it performs well in atmospheric environments^{25–28}.

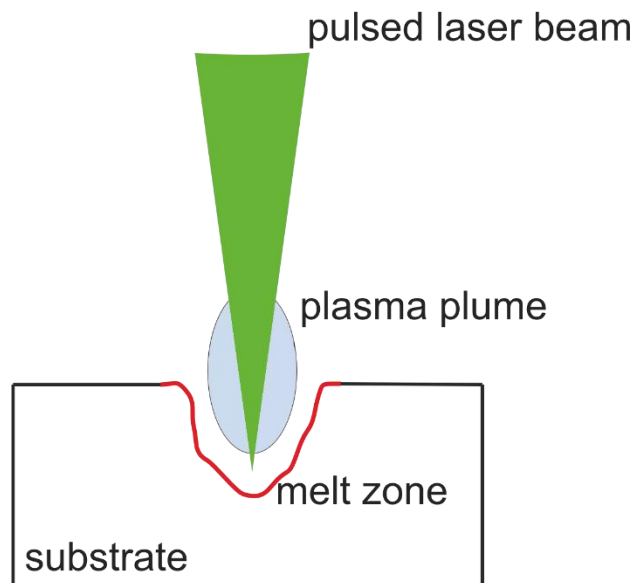


Fig. 2: Laser ablation

1.3.4 3D printing

3D printing is a form of additive manufacturing technology where a three-dimensional object is created by successive layers of material from Computer-aided design (CAD). 3D printers are generally faster, more affordable, and easier to use than other additive manufacturing technologies. They offer product developers the ability to print parts and assemblies made of several materials with different mechanical and physical properties in a single build process. Advanced 3D printing technologies can allow for the development of product prototypes from the conceptual stages of engineering design through to early-stage functional testing and small-volume production²⁹ quickly and easily.

The most common printing technique is Fused Deposition Modeling (FDM), which is an extrusion-based method where a thermoplastic filament is melted and extruded through a nozzle and then solidified by cooling³⁰ (Fig. 3). This technique works by sequentially depositing the melted material layer by layer to create three-dimensional objects. FDM utilizes thermoplastic filaments available in various materials, including ABS (Acrylonitrile Butadiene Styrene), PLA (Polylactic Acid), PETG (Polyethylene Terephthalate Glycol), and others. Although this technique is straightforward, effective, and compatible with a range of materials, the fabricated structures can be more susceptible to compressive stress fractures due to insufficient fusion between adjacent layers³¹. Additionally, achieving microchannels with appropriate transparency and dimensions poses significant challenges³².

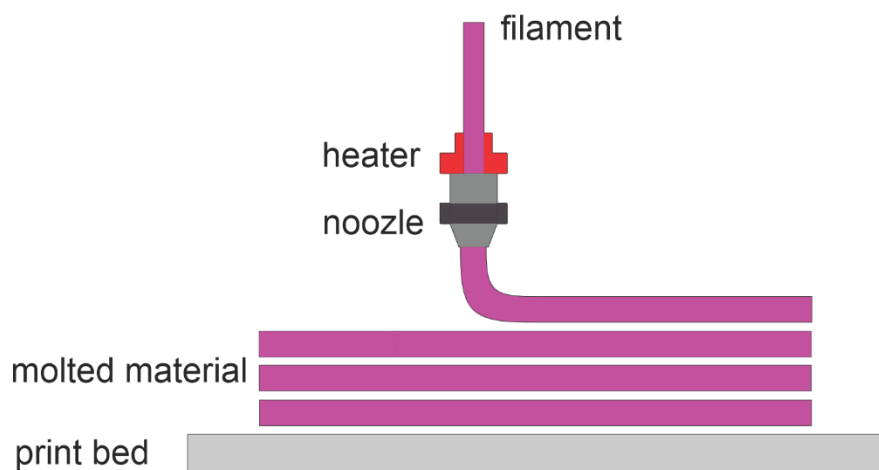


Fig. 3: FDM printing

Vat polymerization, which includes stereolithography (SLA) and digital light processing (DLP), is one of the most widely used 3D printing approaches for manufacturing fine features (Fig. 4). This technique utilizes UV light to cure resin and create three-dimensional printed microfluidics. The method consists of three main components: a light source that serves as the energy input to activate reactive species and facilitate reactions; light-sensitive precursor materials with reactants known as photoinitiators,

which respond to light exposure; and a printing platform that acts as a reaction container to hold the mixture and maintain the necessary environment for the reaction. In SLA, a focused light-emitting diode (LED) laser is directed toward a set of coordinates, hardening the photopolymer layer by layer by tracing the geometry of the design. The printing process breaks the 3D model into multiple coordinate sets for each layer. In contrast, the DLP method employs a stationary UV light source from a DLP projector to cure the resin. A build plate moves in small increments to expose the liquid polymer to the light, solidifying it layer by layer until the model is complete. After polymerization, the liquid polymer is drained from the vat, leaving the solid object behind. Then object is post-processed, which mostly includes IPA washing and UV curing. Both approaches have advantages and disadvantages but mostly differences are set by manufacturers, not technologies itself³³.

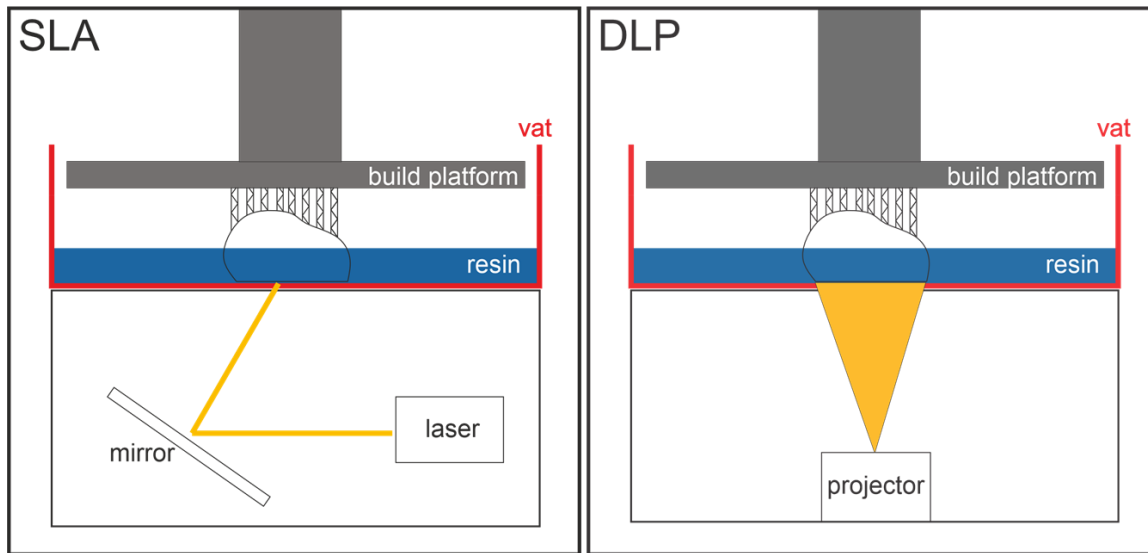


Fig. 4: SLA and DLP printing

Two-photon polymerization (2PP) is an advanced 3D printing technique that allows for the fabrication of microstructures with high spatial resolution at the nanoscale. This process utilizes the nonlinear two-photon absorption phenomenon, which enables the precise curing of photosensitive materials through the simultaneous absorption of two photons by a photoinitiator in the resin (Fig. 5). 2PP relies on the principle of two-photon absorption, where two photons of lower energy are absorbed simultaneously by a photoinitiator molecule, resulting in a higher-energy state that initiates the polymerization process. This nonlinearity allows for better spatial confinement, which is crucial for high-resolution applications. A laser beam, typically a femto-second pulsed laser, is focused into a small volume within the photopolymer resin. The high intensity of the laser at the focal point allows for the initiation of polymerization only at that specific location, enabling the creation of complex three-dimensional structures. The fabrication process occurs layer by layer. This high-resolution three-

dimensional printing approach was utilized for prototyping fabrication and increases the resolution of 3D printing into nano dimensions^{34–36}.

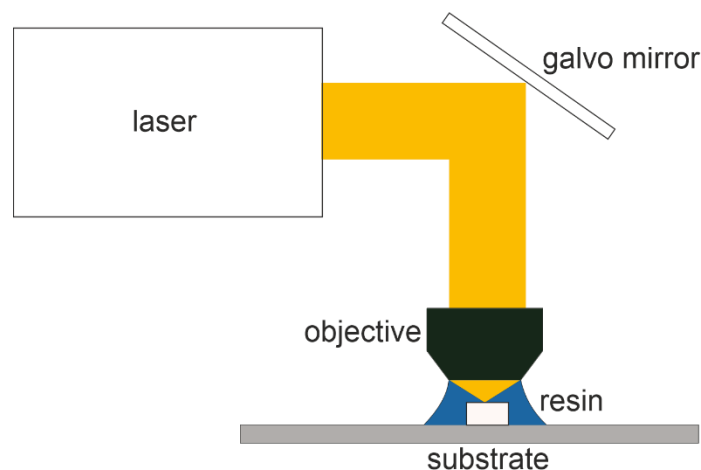


Fig. 5: Two-photon polymerization

1.3.5 Soft lithography

By all previously described methods and others (injection molding, EBL, NIL, hot embossing)^{37–39} is possible to fabricate a functional prototype of the microfluidic chip. For replication of the same design, there is a patterning method which is called soft lithography. It is a versatile and widely used technique for fabricating microstructures and microfluidic devices. This method employs elastomeric materials, typically polydimethylsiloxane (PDMS), to create flexible molds that can replicate intricate designs with high precision. Soft lithography is particularly advantageous due to its low cost, ease of use, and ability to produce complex features without expensive equipment. The process generally involves several key steps (Fig. 6). The first step is to create a master mold. The second step is mold creation. A liquid PDMS prepolymer is mixed with a curing agent (typically in a 10:1 ratio) and poured over the master mold. The mixture is then cured at increased temperatures, allowing it to solidify and form a flexible mold that captures the fine details of the master pattern. After curing, the PDMS mold is peeled away from the master, resulting in a negative replica of the original features. The third step involves device fabrication. The PDMS mold can then be used to create microfluidic devices by filling it with a liquid polymer or other materials. For example, PDMS can be bonded to a flat substrate to form microchannels. The channels can be functionalized with various surface coatings to control fluid behavior or facilitate specific biochemical interactions. The final step is assembly. The molded PDMS is often bonded to glass or other substrates to create a sealed microfluidic device. This is typically done by oxygen plasma treatment, which activates the surfaces and promotes bonding upon contact. Soft lithography enables the rapid prototyping of microfluidic

devices, allowing researchers to design and test new configurations quickly. Additionally, PDMS offers several advantages in microfluidics, including optical transparency, gas-permeable, biocompatibility, low-cost, flexibility for creating complex geometries and ease of surface modification to tailor interactions with fluids and biomolecules^{40,41}. The flexibility and simplicity of soft lithography make it an essential tool in the field of microfabrication, facilitating the development of innovative microfluidic systems⁴².

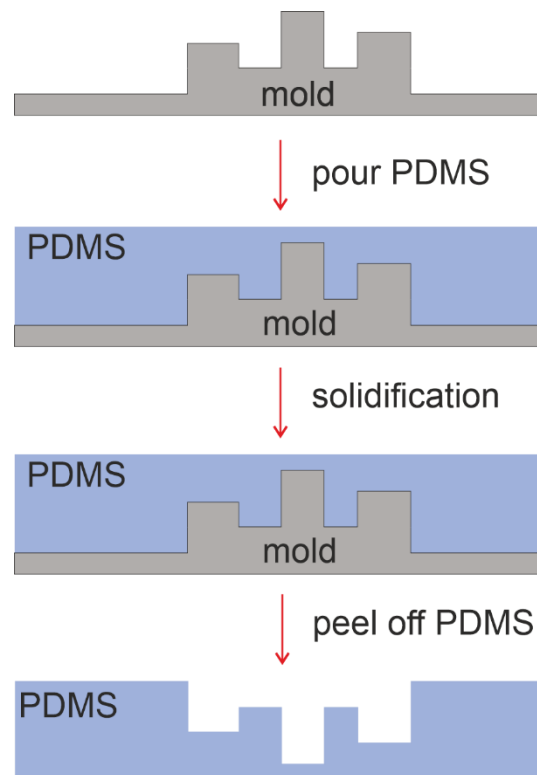


Fig. 6: Soft lithography process

1.4 Organoids

1.4.1 Stem cells

Stem cells are a unique cell type characterized by their remarkable ability to sustain self-renewal through replication and differentiate into specific tissue types⁴³. Unlike most cells in the body that serve specific functions, stem cells remain undifferentiated, meaning they have not yet developed into a specialized cell type. Stem cells were initially identified by McCulloch and colleagues in 1963⁴⁴. First human pluripotent stem cell line, a line of human embryonic stem cells (hESCs), was established in 1998⁴⁵ and, derived from the inner cell mass of the blastocyst. These cells are pluripotent, which enables them to differentiate into cells from all three germ layers⁴⁶. Human induced pluripotent stem cells (iPSCs) technology was first established in 2007⁴⁷. These cells show qualities very similar to hESCs, with the ability to differentiate into any cell type. Additionally, for therapeutic purposes, iPSCs can be derived from a patient's own cells and thus reduce the problem of immune rejection. Induced pluripotent stem cells represent a significant advancement in stem cell research, offering a powerful tool for understanding human biology and developing innovative therapies. Overall, stem cells represent a promising field of modern medicine and it is becoming more influential due to its crucial roles in areas such as tissue engineering⁴⁸, organ regeneration⁴⁹, disease modelling⁵⁰ and drug development⁵¹.

1.4.2 3D culture - Organoids

Today, the extensive use of model systems proves their value in biomedical research today. However, differences between animal species and humans have become major obstacles to the effective use of animal models in drug discovery. On the other hand, a major disadvantage of human-derived 2D cell lines is their lack of hierarchical structure, dimensionality, cellular diversity, and cell–cell or cell–matrix interactions, which limits their ability to accurately mimic the cellular functions found in tissues⁵². 3D cell culture platforms can address the limitations of both 2D cultures and animal models, creating conditions that mimic the *in vivo* environment⁵³. This 3D culture helps replicate the complexity of healthy or diseased tissues, providing a better understanding of the underlying cellular and molecular mechanisms. The 3D system improves the phenotypic stability of primary cells, enables long-term expansion, and supports differentiation into multiple lineages of iPSCs, offering significant advantages over current methods^{54,55}. The ability to achieve high levels of cell organization, cell–cell interactions, and ECM components across various cell types is closely tied to methods and technologies designed to create 3D cell models that replicate the complexity of tissues and organs.

The 3D culture system is formed by suspending cells in a liquid medium while avoiding direct contact with the plastic surface. This can be achieved using scaffold or scaffold-free techniques. Scaffolds are biological or synthetic hydrogels that resemble the natural ECM and provide structural support and ECM signals to the cells⁵⁶. Scaffold-free techniques involve growing spheroids-like cell structures in a 3D environment, where no scaffold or surface is needed for cell attachment. Spheroid culture methods, mainly the low adhesion surface modification and hanging drop techniques, use different approaches to gather cells for aggregation^{57,58}. Scaffold-free 3D culture utilizes the natural ability of cells to self-organize, forming 3D structures without the need for a scaffold, allowing cells to remain closely bound or aggregate naturally. The most widely used scaffold-free methods are the hanging drop and low-adhesion surface techniques. The hanging drop method involves placing a drop of cell suspension upside down, typically on the lid of a culture dish. The culture medium within the drop supports cell growth, causing the cells to aggregate at the top. The low-adhesion surface technique, on the other hand, modifies the culture surface to prevent cells from attaching, encouraging them to remain suspended and form aggregates. There are several modifications to this method, but the most common is an approach that uses a low adherent 96-well plate. Putting cells into U or V-shaped wells results in the formation of 3D spheres.

These spheroids are dense cellular structures composed from one or more cell types and can be highly effective platform for reproducing characteristics of tumor tissue and therapeutic screening. Also, they mimic chaotic proliferation of cell clusters which can be unsuitable for modelling of highly ordered 3D structures. Additionally, spheroids can be formed from pluripotent stem cells which is called embryoid body (EB). EBs are 3D aggregates of PSCs that undergo initial developmental specification, similar to the process in the pre-gastrulating embryo⁵⁹. EBs can further differentiate into organized tissues, similar to a teratoma. However, their growth *in vitro* allows patterning factors to guide specific identities. While not all organoid methods use an initial EB stage, they all involve tissue patterning and reaggregation to form a 3D self-organized tissue or organoid⁶⁰.

These 3D constructs harbor diverse cell types of original organs and mimic the derived organs in both architecture and function to a great degree, but they are also highly heterogeneous. Therefore, each organoid is unique, with tissue regions arranged in a relatively random manner, likely due to the absence of embryonic axis formation. For example, brain organoids display various brain regions that develop similarly to those *in vivo*⁶¹ but are not consistently organized relative to one another because of the lack of anterior-posterior and dorsal-ventral axes. Organoids derived from human PSCs have been established for the brain, liver, and retina, among others. In this work, we focus on cerebral organoids and their cultivation methodology.

1.4.3 Cerebral organoids

Currently, the study of human brain development and the pathogenesis of neurological diseases primarily relies on animal models. However, the human brain differs from those of other species, featuring more complex structures and advanced cognitive and mood functions. As a result, the development of 3D brain organoid cultivation technology offers a new approach to investigating the formation and functional networks of the human brain.

The generation of cerebral organoids (COs) starts with hESCs or iPSCs (Fig. 7). The stem cells aggregate into EBs, which are cultured in a medium that directs differentiation toward the neural lineage. These are then embedded in an extracellular matrix or transferred to a proliferative medium that supports progenitor expansion⁶². An organoid is established upon forming radially organized neural progenitors in ventricular zone-like rosettes. During the first few weeks of culture, human COs mainly consist of symmetrically expanding progenitor regions. The first neurons begin to appear around the ventricular zone after approximately 20 days. Organoids can be cultivated for long periods; postnatal characteristics develop after >1 year⁶³.

The cellular composition of COs is influenced by the diversity of the initial progenitor pool. Unguided protocols, which lack extracellular signaling molecules, lead to a mixture of regional identities⁶¹. Guided protocols involve the use of morphogens to promote or limit specific cell fates, leading to regulated CO with more uniform cell populations⁶⁴. These approaches can be utilized to generate various COs based on the specific requirements. Duration of organoid culture and protocols for an experiment depend on whether the focus is on early progenitor biology⁶⁵ and disease susceptibility⁶⁶, neuronal maturation, migration and activity, or late non-neuronal populations⁶⁷.

However, there are multiple challenges with these models that should be considered. Organoids grow rapidly over extended periods of culture, and their size can exceed the limit for passive oxygen diffusion, which leads to the formation of necrotic tissue in the core. To improve nutrient supply, vascularized organoids have been developed^{68–70}, nevertheless, these techniques currently lack the scalability to replace regular organoid cultures and require improvements.

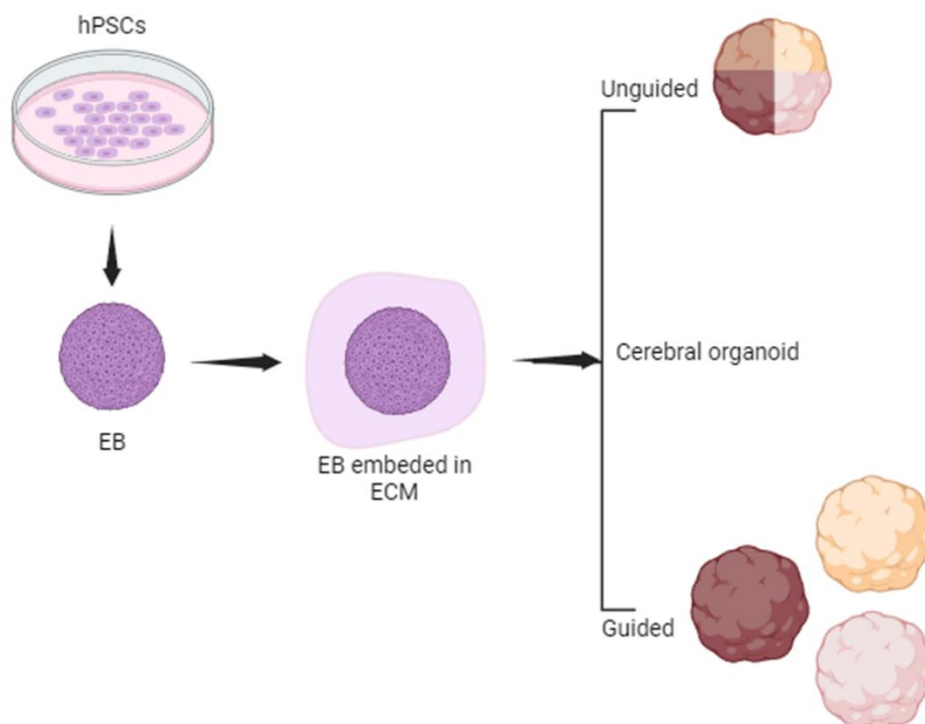


Fig. 7: Schematic illustration of Cerebral organoid generation

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1.4.4 Cerebral organoids cultivation methods

Current techniques for aggregating cells and creating organoids primarily involve non-adherent wells, with the 96-well plate (96WP) being the most commonly used approach⁶². In this method, hESCs or iPSCs are dissociated and seeded into low-adhesion 96WP with standard culture media. Aggregation occurs, followed by the addition of a neural induction medium to promote neural differentiation. The organoids are then embedded in hydrogel (e.g., Matrigel, Geltrex) and allowed to continue differentiating. This robust approach often employs U- or V-shaped wells⁷¹ and can be applied in both 96WP and 384WP⁷². Some protocols further enhance neural induction by transferring organoids into larger wells, such as 24WP."

Initial aggregation can also be performed using commercially available Aggrewell 800 plates⁷³. In this study, embryoid bodies (EBs) were transferred on day 5 to a 24-well ultra-low attachment plate. After 48 hours, the EBs were embedded in Matrigel and continued in a 4WP. Additionally, microwell arrays can be 3D printed, allowing customization of parameters such as well size and shape for optimized cell cultivation⁷⁴.

The next technique uses hanging drop as a readily feasible and cost-effective method to generate hPSCs aggregates⁷⁵. In the conventional hanging drop method, the cell aggregation process takes up to 2 or 3 days. After that aggregates were cultivated

in standard WP or transferred to polystyrene non-treated culture dishes and cultures were continued under dynamic conditions on an orbital shaker. Another alternative approach using a chemically defined hydrogel material and chemically defined culture medium⁷⁶ or AggreWell EB formation medium⁷⁷.

With all these methods in place, it is important to note that neurogenesis is significantly influenced by the size of the embryoid body (EB). The EB size is largely determined by the dimensions and shape of the microwells, as well as the number of cells initially seeded⁷⁸. Consequently, varying microwell shapes have proven effective in enhancing the initial formation of single-cell aggregates⁷⁹. Nonetheless, several challenges remain, such as frequent liquid handling, sudden shifts in the microenvironment from daily media changes⁸⁰, and potential mechanical stress or damage to the organoids⁸¹. Additionally, larger aggregates may suffer from nutrient deprivation, leading to necrosis in their core, even when cultured with shaking techniques^{61,82}. Organoid variability can be counteracted by creating more precisely defined culture conditions. This includes, for example, defined media and matrices, initial cell population, cell location and cell aggregation. Most, if not all, of these conditions can be consistently defined through engineering strategies and increased levels of automation⁸¹.

1.5 Microfluidics for cell cultivation

Microfluidics is a field focused on the manipulation of fluids at the microscale, providing a versatile platform for cell cultivation and tissue engineering. The development of microfluidic systems has revolutionized these fields by enabling precise control over cellular microenvironments. Microchannels within these systems can be designed to continuously deliver nutrients and oxygen, similar to capillary networks in real tissues, while efficiently removing metabolic waste conditions essential for the growth and function of complex tissues⁸³. These environments are crucial for developing constructs that support not only cell survival but also the differentiation and organization of cells into 3D tissue structures. Through microfluidics, it is possible to simulate biomechanical forces such as electrical stimuli⁸⁴, shear stress⁸⁵, and stretch⁸⁶, which are useful for promoting cell maturation and development. Additionally, microfluidic systems can generate solute gradients in the flow⁸⁷, which are valuable for studying and inducing diverse cell behaviours.

1.5.1 Microfluidics and Cerebral organoids

Microfluidics has recently emerged as a promising tool for COs formation, offering advancements in culture conditions, physiological relevance, reproducibility, and industrial scalability. Despite its potential, research in this area remains limited, with existing studies primarily categorized based on the cultivation method: passive or active, determined by the nature of the pressure source (Fig. 8).

Passive cultivation methods do not require additional pressure sources to generate fluid flow within the system. Instead, the flow is gravity-driven, influenced by external forces. For instance, one study employed a rocker system⁸⁸, where the entire setup was tilted periodically, causing the medium to flow back and forth. Initial aggregation and differentiation of the COs followed a standard protocol before they were transferred to the microfluidic system. Within the microfluidic environment, the COs were embedded in a brain-specific extracellular matrix, which, along with the flow dynamics, significantly enhanced their viability and maturation. Another approach involved the manual lifting of the entire device for medium exchange. This device consisted of individual chambers designed for CO formation, each incorporating an air-liquid interface. This configuration enabled the entire cultivation process to occur within a single culture vessel, promoting better oxygenation and reducing the formation of hypoxic cores⁸⁹. Overall, passive cultivation methods are simpler to prepare and operate. Gravity-driven flow effectively supports nutrient exchange; however, medium containers require manual handling, which limits scalability. Consequently, passive systems are less suited for the automated cultivation of larger quantities of organoids.



Fig. 8: Microfluidic cultivation method: Passive and Active

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Unlike passive systems, active microfluidic systems utilize pressure-driven flow, typically provided by pumps, to sustain and manage fluid movement. These systems interconnect various components via tubing, directing the flow of medium from a reservoir through the cultivation area and into a waste container. This approach has been employed in studies such as investigating the effects of prenatal nicotine exposure on neurodevelopment⁹⁰. One such microfluidic device featured two cultivation channels filled with extracellular matrix, in which embryoid bodies (EBs) were embedded. A perfusion channel situated between the cultivation channels improved oxygen and nutrient delivery to the COs. This study demonstrated that the microfluidic system enhanced COs quality compared to conventional methods and revealed that nicotine exposure impairs early neurogenesis. Another group developed a device using micropillar arrays to facilitate COs formation and conduct neurodevelopmental toxicity tests. Their optimized micropillar-based system streamlined the process of COs generation,

encompassing initial aggregation, differentiation, and final maturation while eliminating the need for labor-intensive manual transfers and reducing procedural complexity^{91–93}. In a separate study, microfluidics were applied to co-culture COs with vascular cells. Initially cultivated via conventional methods, the COs were later embedded in a microfluidic chip, where vascular cells were introduced. This setup enabled the creation of 3D perfused vasculature within the organoids, significantly enhancing their maturation compared to non-vascularized controls⁹⁴. Together with other studies^{95,96}, all these examples confirm the versatility of microfluidic systems in COs cultivation, where a dynamic environment enhances the viability and differentiation of COs. Importantly, active microfluidic systems offer precise control over fluid flow and, consequently, the microenvironment within the system. The ability to program flow rates enables automation, reducing or even eliminating the need for manual handling during cultivation. However, these systems are more challenging to set up and technologically complex, requiring advanced equipment and expertise for successful implementation.

In summary, microfluidics has proven to be a valuable tool for the formation and cultivation of COs. Most research emphasizes the differentiation stage, utilizing continuous flow to enhance organoid maturation, while the initial formation of EBs remains less explored. Although some studies have focused on entirely in situ organoid culture within microfluidic systems, they often lack direct comparisons with standard cultivation protocols. Crucially, there remains a significant gap in evidence demonstrating that microfluidic systems can comprehensively improve or replace standard protocols, particularly for high-throughput applications.

1.6 Computational Fluid Dynamic simulations

Computational Fluid Dynamics (CFD) is a powerful tool that predicts and analyses fluid flow behaviour by solving governing equations using numerical methods and computational power. CFD is the analysis of systems involving fluid flow, heat transfer and associated phenomena such as chemical reactions by means of computer-based simulation. The technique is very powerful and spans a wide range of industrial and non-industrial application areas (Aerodynamics, hydrodynamics, turbomachinery, electronic engineering, biomedical engineering). The main reason why CFD has lagged behind is the tremendous complexity of the underlying behaviour, which precludes a description of fluid flows that is at the same time economical and sufficiently complete⁹⁷.

There are several unique advantages of CFD over experiment-based approaches to fluid systems design: substantial reduction of lead times and costs of new designs, ability to study systems where controlled experiments are difficult or impossible to perform, ability to study systems under hazardous conditions at and beyond their normal performance limits, practically unlimited level of detail of results. CFD codes are structured around the numerical algorithms that can tackle fluid flow problems⁹⁸.

1.6.1 Mathematical model

The starting point of any numerical method is the mathematical model, i.e., the set of partial differential or integro-differential equations and boundary conditions. One chooses an appropriate model for the target application (incompressible, inviscid, turbulent, two- or three-dimensional, etc.). This model may include simplifications of the exact conservation laws⁹⁹.

The main structure of thermo-fluids examination is directed by governing equations that are based on the conservation law of fluid's physical properties. The basic equations are the three laws of conservation: Conservation of Mass (continuity equation), Conservation of momentum (Newton's second law), and Conservation of Energy (energy equation). These principles state that mass, momentum, and energy are stable constants within a closed system. In short, everything must be conserved¹⁰⁰.

1.6.2 Continuity Equation

The equation for the Conservation of Mass is specified as:

$$\frac{\partial \rho}{\partial t} + \nabla \cdot \rho \vec{V} = 0 \quad (7)$$

Where ρ is the density, $\vec{v}$ the velocity and ∇ the gradient operator.

$$\vec{\nabla} = \vec{i} \frac{\partial}{\partial x} + \vec{j} \frac{\partial}{\partial y} + \vec{k} \frac{\partial}{\partial z} \quad (8)$$

If the density is constant, the flow is assumed to be incompressible and the continuity equation reduced to:

$$\frac{\partial \rho}{\partial t} = 0 \rightarrow \nabla \cdot \vec{V} = \frac{\partial u}{\partial x} + \frac{\partial v}{\partial y} + \frac{\partial w}{\partial z} = 0 \quad (9)$$

1.6.3 Navier-Stokes Equation

Conservation of Momentum which can be referred to as Navier-Stokes Equation is given by:

$$\overbrace{\frac{\partial}{\partial t}(\rho v)}^I + \overbrace{\nabla \cdot (\rho v \vec{v})}^{II} = \overbrace{\nabla p}^{III} + \overbrace{\nabla \cdot \vec{\tau}}^{IV} + \overbrace{\rho \vec{g}}^V \quad (10)$$

Where p is static pressure, $\bar{\tau}$ is viscous stress tensor and $\rho\vec{g}$ is the gravitational force per unit volume. The Roman numerals denote: *I*-Local change with time, *II*-Momentum convection, *III*-Surface force, *IV*-Diffusion term, *V*-Mass force. If fluid is assumed to be incompressible with constant viscosity coefficient μ the Navier-Stokes equation simplifies to:

$$\rho \frac{dv}{dt} = -\nabla p + \mu \nabla^2 v + \rho g \quad (11)$$

1.6.4 Newton's First Law of Thermodynamics

Conservation of Energy is the first law of thermodynamics which states that the sum of the works and heat added to the system will result in the increase of the energy in the system:

$$dE_t = dQ + dW \quad (12)$$

Where dQ is the heat added to the system, dW is the work done on the system and dE_t is the increment in total energy of the system.

1.6.5 Discretization

To solve the governing equations, it is important to choose a suitable discretization method, i.e., a method of approximating the differential equations by a system of algebraic equations for the variables at some set of discrete locations in space and time. It can be imaged as a network (mesh) of discrete points that divides the computational domain into smaller sub-regions or control volumes. The main discretization techniques are finite difference, finite volume and finite element (Fig. 9). Other methods, like spectral schemes, boundary element methods, and lattice-Boltzmann methods are used in CFD, but their use is limited to special classes of problems⁹⁷.

Finite difference method (FDM) approximates the derivatives in the governing equations using finite differences. The continuous variables (such as velocity, pressure, and temperature) are evaluated at discrete grid points, and the derivatives are replaced by differences between these points.

Finite elements method (FEM) is a more flexible and used primarily in structural mechanics. The domain is discretized into small elements (such as triangles, tetrahedra, etc.), and the governing equations are solved by approximating the solution over each element using shape functions. FEM is well-suited for handling complex geometries and boundary conditions but can be computationally expensive for large-scale fluid dynamics problems.

Finite volume method (FVM) divide domain into small control volumes or cells. The governing equations are integrated over each control volume, and fluxes (like

mass, momentum, and energy) are computed across the surfaces of these volumes. The method conserves physical quantities (such as mass, momentum, and energy) exactly within each control volume, making it robust and widely used in fluid flow simulations, especially for complex 3D geometries⁹⁸.

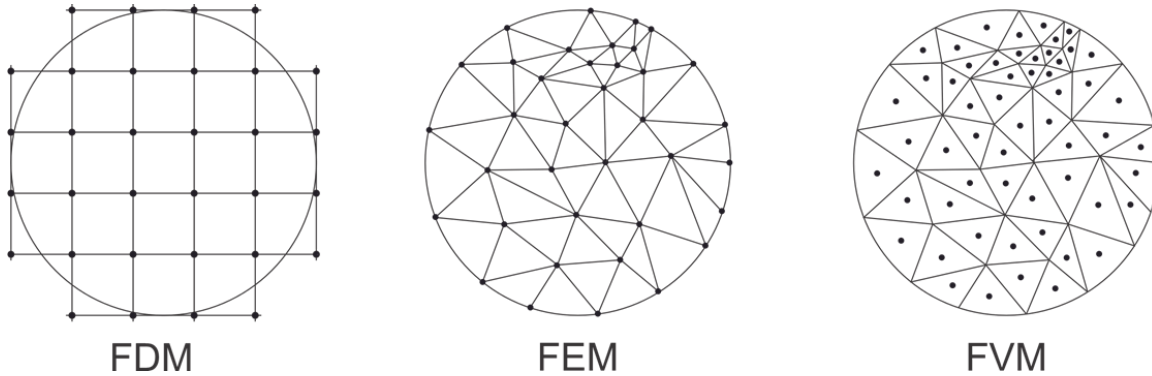


Fig. 9: Concept of discretization methods

1.6.6 Calculation strategy

A specific calculation strategy is used to solve the governing integral equations for the conservation of mass and momentum energy (when appropriate) and other scalars, such as turbulence and chemical species. Firstly, it is important to choose an adequate solver. Historically, the pressure-based solver approach was developed for low-speed incompressible flows (Mach number below 0.3), while the density-based solver approach was mainly used for high-speed compressible flows. Both solvers can be used for a broad range of flows, but in some cases one formulation may perform better (i.e., yield a solution more quickly or resolve certain flow features better) than the other. The pressure-based and density-based approaches differ in the way that the continuity, momentum, and (where appropriate) energy and species equations are solved. Both solvers use solution methods for equations¹⁰⁰.

Many solution methods developed for steady incompressible flows are implicit and use pressure equation to enforce mass conservation for either time step solving or steady solving. The most used methods are Segregated and Coupled algorithm.

The Segregated algorithm solves governing equations one after the other, which reduces the memory used due to the serial nature of the equation solution, but it also reduces the speed of convergence of the problem.

The coupled solution method solves a set of momentum coupling equations and the pressure-based continuity equation, so there is no need to solve the pressure correction equation. Since the momentum and continuity equations are solved in a closely coupled manner, the rate of solution convergence significantly improves when compared to the segregated algorithm. However, the memory requirement increases by 1.5 - 2 times that of the segregated algorithm since the discrete system of all

momentum and pressure-based continuity equations needs to be stored in the memory when solving for the velocity and pressure fields⁹⁹.

In general, segregated methods are faster per iteration, while the coupled algorithm usually requires fewer iterations to converge. For this reason, the coupled solver is usually recommended for steady-state simulations. For transient simulations, the coupled solver has the best robustness properties, especially for large time step sizes.

As mentioned above, simulation can be simulated in two variants: steady-state and transient flow. In order to account for time-dependent effects, a transient simulation should be considered. A steady-state simulation should be considered if one is only interested in the converged steady state solutions where the flow condition does not change over time⁹⁷.

1.6.7 The sequence of Computational Fluid Dynamics simulations

The sequence of steps in a Computational Fluid Dynamics (CFD) simulation follows a systematic approach, typically divided into three main stages: pre-processing, solving, and post-processing (Fig. 10).

Pre-processing in a Computational Fluid Dynamics (CFD) simulation begins with geometry design, which can either be created directly within the CFD interface or imported from external modeling platforms such as AutoCAD, SolidWorks, or Rhino. The geometry must be both simple and accurate, tailored to the simulation's purpose and expected outcomes. Simplifying the geometry to emphasize key features is often an effective strategy for expediting the pre-processing phase. Once the geometry is imported into the CFD interface, material properties are assigned. For solid materials, essential properties include thermal conductivity and wall roughness. For fluid materials, the required properties include density, viscosity, thermal conductivity, specific heat capacity, and compressibility. These attributes are critical for accurate simulation results. Boundary conditions are also defined during pre-processing. These conditions serve as initial inputs that establish the limits of the computational domain and its interaction with the surrounding environment. It connects the computational domain and its surroundings showing the velocity, pressure, or volumetric flow rate of the incoming or outgoing fluid. For steady-state simulations, these inputs remain constant over time, while in transient simulations, they vary with time. At the outlet, the boundary condition is typically set using the operating pressure to ensure accurate flow behavior throughout the computational domain.

The solving phase of a CFD simulation involves three key steps: grid generation, solver selection, and simulation calculation. Grid generation, also known as meshing or discretization, is critical to the accuracy and reliability of the simulation. In this step, the computational domain is divided into smaller segments, or cells, to enable numerical calculations. Proper grid generation requires selecting an appropriate discretization scheme and optimizing the size of the cells to balance computational cost and

result accuracy. The quality and configuration of the mesh significantly influence the accuracy, stability, and convergence of the simulation. A fine mesh may capture detailed features more accurately, but it increases computational demands. Poor-quality meshes can lead to numerical errors, slow convergence, or even divergence of the solution. High-quality meshes have well-shaped cells that are as uniform as possible. Subsequently, the choice of solver (calculation strategy) and method plays a crucial role in determining the uncertainties and accuracy of simulation results.

Once the simulation configuration is finalized, the iterative calculation process begins. In this process, the governing equations are repeatedly solved to compute flow variables such as velocity, pressure, and temperature. The goal is to achieve solution convergence, a state where the changes in the computed variables from one iteration to the next become negligible. Convergence indicates that the solution is stable and sufficiently accurate for analysis. Once the solution has converged, the gathered data should be presented using diverse visualization techniques to facilitate analysis. While commercial CFD software offers a range of visualization options, it is crucial for users to choose the method that best aligns with the objectives of their analysis¹⁰¹.

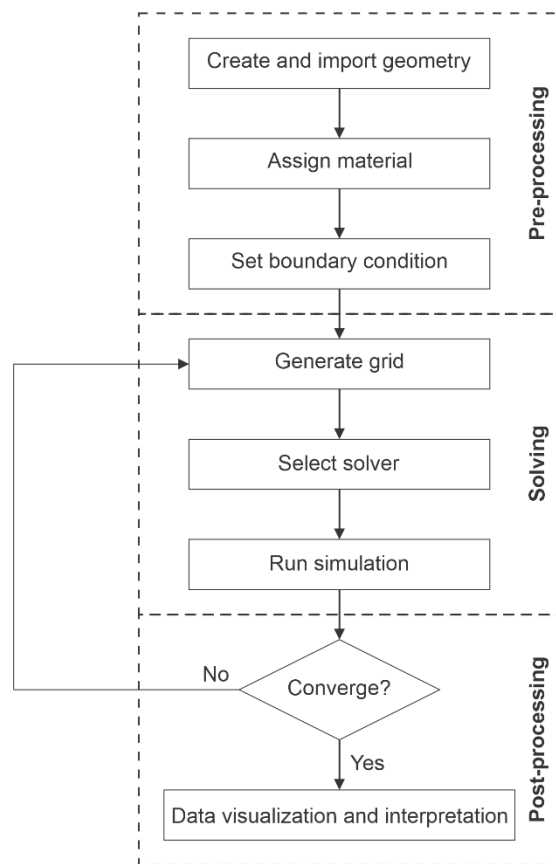


Fig. 10: The sequence of CFD simulations

1.7 Microfluidic system for cell isolation and separation

Single-cell multi-omics technologies characterize cell states and activities by integrating various omics methods, such as transcriptome, genome, epigenome, epitranscriptome, proteome, or metabolome, all from the same single cell. These technologies allow for the simultaneous identification of multiple molecule classes within a single cell, providing deeper biological insights than analyzing each molecular level from separate cells. Two key aspects are crucial for the routine application of single-cell multi-omics analysis. First, cells must be detectable and isolable, even if they are very rare. Second, there must be automated and parallelized preparation of multi-omics data from individual cells. However, recent years have seen challenges in detecting and isolating specific cells, such as tumor cells from patients, using current detection markers and techniques. The process also relies on the quantitative and physical separation of cells while ensuring high-quality recovery of cellular information. Therefore, combining reliable separation and recovery methods with innovative microfluidic circuits is essential for effectively implementing single-cell multi-omics analyses. Those microfluidic systems can be used to process a sample collected from liquid biopsies (Fig. 11). The system can detect, measure and manipulate cells, which will be physically separated and transferred for a single multi-omics analysis.

As mentioned above (chapter 1.3), most microfluidic chips are fabricated from conventional silicone materials. On the other hand, for repetitive use of the microfluidic chip, researchers developed an idea to use silicon semiconductor substrates. This material has greater compatibility with molecular biological analysis than conventional silicone materials and has an excellent ability to create nano- and microstructures. Semiconductor substrates also offer several benefits over silicone-based methods: (1) they do not absorb biomolecules¹⁰², (2) they can be sterilized and made DNA/RNA-free, making them reusable¹⁰³, (3) they have excellent wettability with aqueous solutions¹⁰⁴ and (4) they exhibit negligible channel expansion effects due to their mechanical properties¹⁰⁵. These qualities make silicon ideal for microscale fluidic circuits and for cost-effective implementation of molecular biological analysis methods. Furthermore, silicon-based microchannels can be produced in large quantities at a low cost¹⁰⁶.

At the end of the thesis, we present results from collaboration with OTH University in Regensburg, where we developed a novel manufacturing method based on silicon technology, which enables the production of microfluidic chips with high reproducibility in under 20 minutes. This process can create microchannel dimensions of around 50 μm quickly, resulting in microchannel geometry well-suited for processing individual cells. Results from this research stay are summarized in the final section of the chapter (4.6) results.

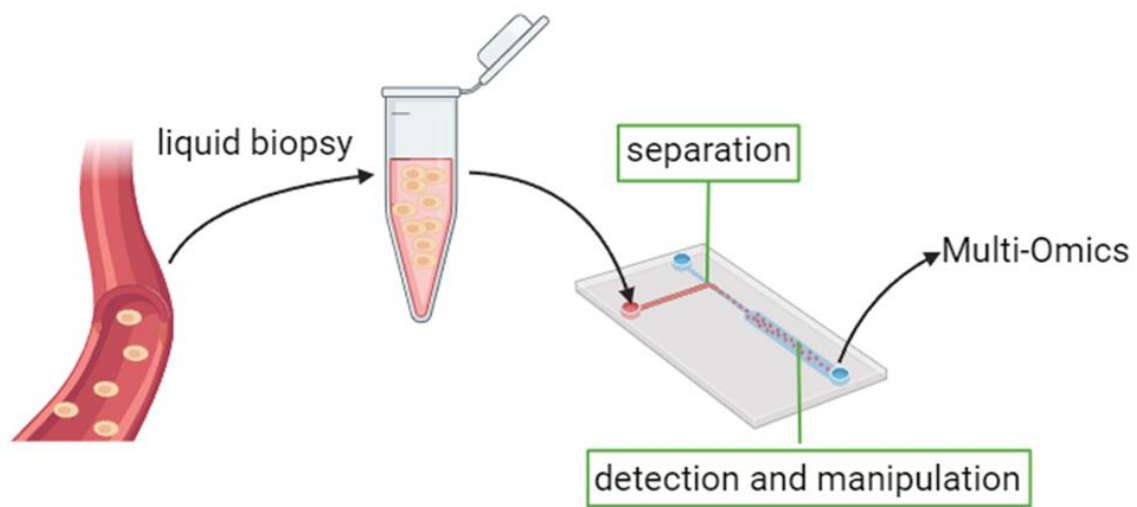


Fig. 11: Microfluidics incorporated for Multi-Omics analysis

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2 Goal of thesis

Microfluidic systems are becoming essential in cell biology and stem cell engineering, with increasing research highlighting their use for cultivating cells and mimicking tissue functionality. By precisely controlling the microenvironment, these systems replicate physiological and pathological conditions. The expanding microfluidics market offers tailored solutions for scientific research, yet the technology remains largely in the prototyping stage, limited by expensive, single-use fabrication processes and a lack of standardized methods. To tackle these challenges and drive progress in the field, this thesis is guided by the following four objectives and their respective specific aims:

1) **Streamline Microfluidic System Fabrication and Design**

Develop an efficient and flexible fabrication protocol for microfluidic systems that allows to modify designs easily and complete the process from design to ready-to-use chip within a few days. Utilize computational simulations to predict system performance, reducing the time and cost associated with experimental optimization.

2) **Optimize Microfluidic Systems for Cell Cultivation and Organoid Differentiation**

Design and refine microfluidic systems for uniform cell aggregation and cultivation. Adapt these systems to support cerebral organoid differentiation by leveraging microfluidic advantages and optimizing protocols for enhanced maturation and uniformity.

3) **Demonstrate Versatility for Other Organoid Types**

Validate the adaptability of the designed microfluidic system for cultivating different types of organoids without altering the chip design.

4) **Create a Reusable Microfluidic System**

Establish a fabrication process for reusable microfluidic systems, improving current protocols for simple and rapid prototyping with customizable properties.

3 Material and Methods

3.1 Fabrication of chip

The microfluidic chip was fabricated using a stereolithography (SLA) 3D printer (Form 2, Formlabs). The 3D models of the casting molds were designed using Fusion 360 (Autodesk), and the molds were printed from UV-cured resin (FLGPCL03, Clear). The chip consists of two parts – a top and a bottom. The bottom part contains a flow channel (height: 800 μm , width: 2600 μm) with 48 U- and V-shaped microwells (diameter: 1000 μm , depth: 1000 μm) and a vacuum channel (height: 800 μm width: 500 μm) (Fig. 12). The top and bottom parts of the chip were cast from PDMS prepolymer (1673921, Sylgard 184, Dow) mixed at a ratio of 10:1 (w/w) with PDMS precursor and curing agent. The mixture was degassed for 10 min, then poured into a printed 3D mold and baked at 80 °C for 30 min in an oven. The PDMS was then peeled off from the mold. The top layer was cast with a thickness of 5 mm and punched for tubing (800/100/120, Smith Medicals). The layers were assembled and bonded with a PDMS mixture. The whole chip was baked at 80 °C for 30 min, followed by ethanol cleaning and UV light sterilization for 30 min. Before use, the chip was washed with 0.1 M phosphate-buffered saline (PBS) and coated with 2% BSA in PBS overnight.

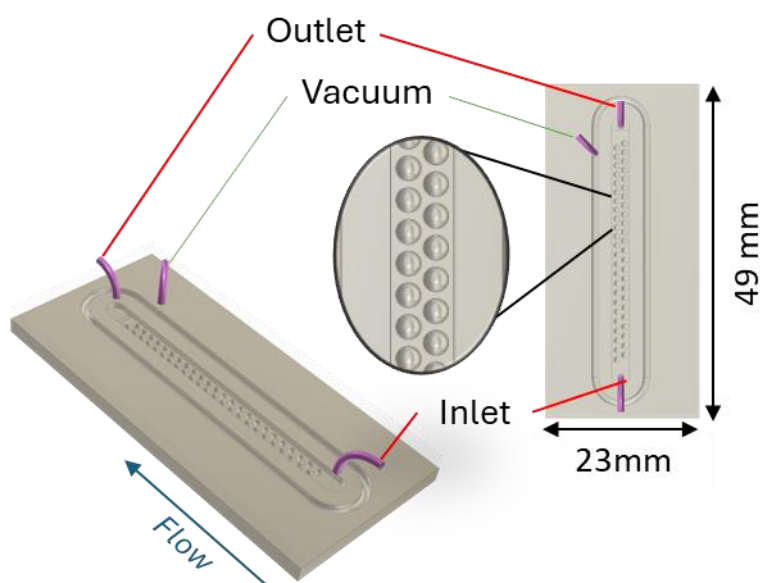


Fig. 12: Desing of microfluidic chip for COs formation

3.2 Numerical simulation for dextran diffusion

Numerical simulations of the dextran diffusion were performed using Ansys Fluent software (Ansys, Inc.). The equations were solved for the transient state flow by Navier-Stokes equations and the multi-component equations using the finite volume solver of ANSYS Fluent 2023 R1. The geometry of the agarose block was designed in Ansys SpaceClaim 2023 R1 and split into tetrahedral control volumes (elements). For simulation, a pressure-based solver was used with a coupled method and second-order spatial discretization. For boundary conditions, walls were set as stationary, with no slip conditions. Diffusion through agarose was modeled as free diffusion. The specific parameters set for the simulation are listed in Tab. 1.

Parameter	Value	Ref.
Density of culture media [kg/m ³]	998	107
Dynamic viscosity of culture media [Pa.s]	0.001003	107
Dextran diffusion coefficient in agarose [m ² /s]	1×10^{-10}	108
Density of agarose [kg/m ³]	1640	109
Viscosity of agarose [kg/m.s]	0.25	110
Dextran molecular weight [kDa]	10	N/A
Agarose molecular weight [g/mol]	306	N/A
Pressure in outlet channel [Pa]	101325	N/A
Number of mesh elements [-]	3×10^5	N/A

Tab. 1: Parameters for simulation of dextran dynamic diffusion in agarose block

3.3 Numerical simulation for lactate diffusion

Numerical simulations of the medium flow and lactate diffusion were performed using Ansys Fluent software (Ansys, Inc.). The equations were solved for the transient state flow by Navier-Stokes equations and the multi-component equations using the finite volume solver of ANSYS Fluent 2023 R1. The geometry of the chip and WP was designed in Ansys SpaceClaim 2023 R1 and split into tetrahedral control volumes (elements). For simulation, a pressure-based solver was used with a coupled method and second-order spatial discretization. For boundary conditions, walls were set as stationary, with no slip conditions. Due to the low Reynolds number ($Re \ll 1$), the flow was considered as laminar. The outlet was set to pressure outlet at a standard pressure. The simulations focused on lactate production and diffusion in wells together with interaction between wells and flow channel. The compositions and dimensions of the microfluidic devices analyzed in the numerical simulation were identical to those fabricated and used in the cell experiments. Spheroid lactate production was determined based on previous work^{111,112} using data on production by a single cell and the number

of cells. The number of cells per spheroid was derived from the volume of the spheroid, 450 μm in diameter, and the volume of the single cell. The specific parameters set for the simulation are listed in Tab. 2.

Parameter	Value	Ref.
Density of culture media [kg/m^3]	998	107
Dynamic viscosity of culture media [$\text{Pa}\cdot\text{s}$]	0.001003	107
Lactate diffusion coefficient in culture medium [m^2/s]	5.9×10^{-11}	113
Density of organoids [kg/m^3]	1020	114
Medium flow rate [$\mu\text{l}/\text{h}$]	100/50	N/A
Volume of a single cell, 10 μm in diameter [μm^3]	513	N/A
Lactate molecular weight [g/mol]	90	N/A
Lactate production per cell [$\text{pmol}/24\text{ h}$]	20	111,115
Pressure in outlet channel [Pa]	101325	N/A
Number of mesh elements [-]	Chip_U: 5.08×10^5 Chip_V: 4.81×10^5 WP: 4.09×10^5	N/A

Tab. 2: Parameters for simulation of dynamic diffusion in a microfluidic chip

3.4 Propagation of human embryonic stem cells for microfluidic system optimization

Human embryonic stem cells of the CCTL14 line (Center of Cell Therapy Line 14, <https://hpscereg.eu/cell-line/MUNie007-A>) were used for the optimization of the microfluidic system. The line was cultured at 37 °C with 5% CO₂ and propagated on cell culture dishes coated with Matrigel (356277, hESC-Qualified, Corning) in a mouse embryonic fibroblast (MEF)-conditioned medium (enriched with factors produced by MEF), supplemented by 10ng/ml of FGF2. Cells were passaged using TrypLE™ (Life Technologies, 12605010).

3.5 Cerebral organoids

3.5.1 Propagation of human pluripotent stem cells for Cerebral organoids

As lines of pluripotent stem cells, we used human embryonic stem cells (hESCs; line H1 and H9; characterized in ⁴⁵) and induced pluripotent stem cells (hiPSCs) line BWT3; (MUNli017-A), line M8(UQi00A-A), line WTF80(MUNli010-A), line M67(derivated from skin fibroblasts)¹¹⁶, line Neo1(derived from neonatal fibroblasts)¹¹⁷, line AM13(derivated from skin fibroblasts)¹¹⁸. All lines were cultured at 37 °C with 5% CO₂

and propagated on cell culture dishes coated with Matrigel (356277, hESC-Qualified, Corning). H1, H9, BWT3, WTF80, M8, Neo1 in mTeSR™1 medium (85857, StemCell technologies) supplemented with 0.5% ZellShield (13-0050, Minerva Biolabs). Cells were passaged using 0.5 mM EDTA in sterile D-PBS. AM13 was maintained in an MEF-conditioned medium.

3.5.2 Media for differentiation of cerebral organoids

Following cell culture media were used for the differentiation of hPSCs to cerebral organoids:

Aggregation medium: mTeSR™1(85857, StemCell Technologies) was supplemented with 20 μ M Rho-kinase inhibitor Y-27632 (ROCKi; S1049, Selleckchem).

Neural induction medium (NIM): DMEM-F12 (21331046, Gibco) was supplemented with 1% N2 (17502001, Gibco) (v/v), 1% Glutamax (35050061, Gibco) (v/v), 1% MEM-NEAA (11140035, Gibco) (v/v), 1% ZellShield, heparin (H3149, Merck; final concentration 1 μ g·ml⁻¹).

Cerebral organoid differentiation medium without vitamin A (COI): DMEM-F12 and Neurobasal (21103049, Gibco) were mixed in a 1:1 ratio and complemented with 0.5% N2 supplement, 0.025% Insulin (I9278, Merck), 1% Glutamax, 0.5% MEM-NEAA and 1% ZellShield, 100 μ M 2-mercaptoethanol (M3148, Merck), and 1% B27 supplement without vitamin A (12587010, Gibco).

Cerebral organoid differentiation medium with vitamin A (COII): DMEM-F12 and Neurobasal were mixed in a 1:1 ratio and complemented with 0.5% N2 supplement, 0.025% insulin, 1% Glutamax, 0.5% MEM-NEAA and 1% ZellShield, 100 μ M 2-mercaptoethanol, and 1% B27 supplement with vitamin A (17504044, Gibco).

3.5.3 Generation of cerebral organoids in a 96-well plate

Cerebral organoid formation in 96WP followed the Lancaster protocol⁶² with minor modifications^{119,120}. Briefly, cells were harvested using Accutase (A1110501, Gibco). They were then resuspended in aggregation medium and seeded into a low adherent poly-HEMA (P3932, Merck) coated 96WP (M9686-100EA, Greiner CELLSTAR) at a concentration of 2000-9000 cells per well in 150 μ l of the medium, centrifuged at 200 g for 2 min and incubated. The EBs were formed, and the medium was exchanged after 48 h by gentle aspiration and the addition of fresh 100 μ l mTeSR™1 medium without ROCKi. The differentiation media were changed depending on the timing and size/morphology of cell aggregates. After EBs were larger than 350–400 μ m, a NIM medium was added. Approximately over the next seven days, the organoids grew to a size of 900-1000 μ m and were then embedded into 10 μ l droplets of Geltrex™

(A1413302, Gibco). The droplets were maintained in a 6 cm cell culture dish (12 droplets per dish) with COI medium exchanged every other day until rosettes were clearly observable in the peripheries of organoids around day 7. Then COII medium was applied, and the dishes were placed on an orbital shaker (Rotamax 120, Heidolph) at 50 rpm. Organoids were maintained until day 55.

3.5.4 Generation of cerebral organoids in a microfluidic device

The formation of cerebral organoids in the chip corresponded to the protocol for the 96WP. The microfluidic chip was loaded with cell suspension to achieve a concentration of 2000-9000 cells per well in the Aggregation medium. The cells loaded into the microfluidic chip were centrifuged at 200 g for 2 min, then allowed to aggregate initially at 37 °C for 10 min, and finally, the chip was connected to a syringe pump (Fusion 4000, Chemyx). Two experimental conditions were set up with flow rates of 100 µl/h and 50 µl/h. Similarly to the 96WP protocol, the Aggregation medium was replaced with mTeSR™1 after 48 h, and then the NIM medium was used when EBs were 350–400 µm in size. Cell culture continued until the aggregates reached a size of 900-1000 µm, approximately at day 10 (±1 day). Then, organoids were removed from the microfluidic chips, embedded in Geltrex droplets, and cultured until day 55 with COI and COII medium.

3.5.5 Histochemistry

For hematoxylin and eosin staining, sections were incubated for 10 min in hematoxylin, washed two times in warm water, and incubated in eosin for 5 min. Subsequently, sections were washed with cold tap water, dehydrated in alcohol for 10 min, and clarified in xylene for 10 min.

3.5.6 Immunofluorescence

Organoids were fixed in 4% (v/v) formaldehyde solution for 1 h, washed 3 times with 0.1 M PBS, embedded into liquid 3% low-temperature gelling agarose (A9414-25G, Merck), embedded in a paraffin block and sectioned. Sections 7 µm thick were deparaffinized by xylene and a descending series of alcohols and washed with dH₂O. Then, sections were incubated with Target retrieval solution pH 6 for 30 min in 96 °C (S2031, Dako) to unmask the antigenic epitope, permeabilized with 0.2% (v/v) Triton X-100 in PBS and blocked with 1% (w/v) bovine serum albumin in 0.2% Triton X-100 for 1 hr. The samples were then incubated with primary antibodies (listed in Appendix A) overnight, then washed with PBS and incubated with secondary antibodies AF488 (A11070, ThermoFisher) or AF568 (A21069, ThermoFisher) at a 1:250 dilution for 1 hr. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, 62248, ThermoFisher) for 5 min and washed with PBS. The final mounting of samples was

performed in the Mowiol mounting medium (0713.1, Roth). Six organoids from a 96WP, U-shaped, as well as V-shaped microwells of the microfluidic chip were analyzed per each time point from each condition.

3.5.7 Microscopy, image analysis, and calculating the wrinkling index

Brightfield images of the organoid size and morphology were captured by a Cell[^]R microscope system (Olympus) and DMIL microscope (DMIL LED, Leica). Immunofluorescence images were acquired with LSM880 laser scanning confocal microscope (Carl Zeiss AG). Automated image acquisition of whole sections was performed with TissueFAXS (TissueGnostics). Image analysis of organoid diameter, area, and wrinkling index was performed using Fiji (ImageJ) and Imaris software (RRID: SCR_007370). On day 14, the wrinkling index was calculated as follows:

$$W_i = L_G / L_F \quad (13)$$

Where L_G is the contour perimeter of the organoid and L_F is the length of the outer convex contour of organoids⁷⁴.

3.5.8 Gene expression by qPCR analysis

On days 9 and 55, individual organoids were lysed in RNA Blue (R011, Top-Bio), and RNA was isolated by chloroform extraction and isopropanol precipitation according to the standard protocol¹²¹. RNA concentration was measured by a spectrophotometer (mySpec, VWR). Reverse transcription was performed using Transcriptor First Strand cDNA Synthesis Kit (04896866001, Roche) according to the manufacturer's instructions. qPCR with specific primers and SYBR Green I master (cat. 04707516001, Roche) was performed on a LightCycler 480 Instrument II system (Roche). Results were normalized to the expression of endogenous control RPL13A. Primers used for qPCR are listed in Appendix B. Six organoids from 96WP, U-shaped, as well as V-shaped microwells of the microfluidic chip were pooled and analyzed per condition for each time point.

3.6 Retinal organoids

3.6.1 Propagation of human pluripotent stem cells for Retinal organoids

As lines of pluripotent stem cells, we used human induced pluripotent stem cells line M8 (UQi00A-A). The line was cultured at 37 °C with 5% CO₂ and propagated on cell culture dishes coated with Vitronectine in E8 medium (Gibco, Cat. A1517001). Regular enzymatic passage was performed using TrypLE™ (Life Technologies, 12605010).

3.6.2 Media for differentiation of retinal organoids

Following cell culture media were used for the differentiation of iPSCs to retinal organoids:

Aggregation medium: Essential 8™ Medium (A1517001, Gibco) supplemented with 20uM ROCK inhibitor.

The differentiation medium: Growth factor-free CDM (gfCDM) supplemented with 15% KOSR, 41% Iscove's modified Dulbecco's medium (IMDM), 41% HAM's F12, 1% GlutaMAX, 1% Chchemically defined lipid concentrate, 1% Pen/Strep and 225 2-mercaptoethanol. On day 6, 2,25nM of recombinant human BMP4 was added.

Maintenance medium: DMEM/F12 supplemented with 10% FBS, 1% GlutaMAX, 1% N2 supplement 0,5 uM Retinoic Acid, 0,1 mM Taurine and 1% Pen/Strep.

3.6.3 Generation of retinal organoids in a 96-well plate

Cells were harvested using 0.5 mM EDTA in sterile D-PBS. Then cells were resuspended in aggregation medium and seeded into a low adherent poly-HEMA (P3932, Merck) coated 96WP (M9686-100EA, Greiner CELLSTAR) at a concentration of 2000 cells per well in 150 µl of the medium. After two days(D0), the medium was changed for the differentiation medium, and the medium was exchanged after 48 h by gentle aspiration and the addition of fresh medium. At D6, 2,25 nM BMP4 was added into the differentiation medium for 3 days. Then, the medium was switched back to GfCDM medium. On day 19, organoids were transferred from WP to PM6, where maintenance medium was exchanged every 3 days.

3.6.4 Generation of retinal organoids in a microfluidic device

The formation of cerebral organoids in the chip corresponded to the protocol for the 96WP. The microfluidic chip was loaded with cell suspension to achieve a concentration of 2000 cells per well in the Aggregation medium. The cells loaded into the microfluidic chip were centrifuged at 200 g for 2 min, then allowed to aggregate initially at 37 °C for 10 min, and finally, the chip was connected to a syringe pump (Fusion 4000, Chemyx). Two experimental conditions were set up with flow rates of 100 µl/h and 50 µl/h. Similarly to the 96WP protocol, the Aggregation medium was replaced with differentiation medium after 48 h, and then at D6 in the differentiation medium was added 2,25 nM BMP4 for 3 days. Then the medium was switched back to GfCDM medium. On day 19, organoids were transferred from WP to PM6, where maintenance medium was exchanged every 3 days.

3.6.5 Immunofluorescence

On day 21, the organoids were fixed in 4% (v/v) formaldehyde solution for 1 h, washed 3 times with 0.1 M PBS. After washing, 30% sucrose was added overnight. Then, sucrose with organoids was transferred into the plastic mold for OCT molding. Half of the sucrose was aspirated and OCT was added. Next, the mold was put on a shaker for 15 minutes. The second half of sucrose was aspirated and the mold was filled with OCT. By needle, organoids were positioned in the centre of the mold. After that, the mold was put on dry ice for fast freezing. Molds were stored at -80°. The block of OCT was cut into 5µm thick slices and placed on glass slides. Slices were dried for 20 minutes at room temperature. Then, slides were gently washed three times with PBS to remove OCT and a blocking buffer (0.3% Triton-x with 1% BSA) was added for 1 hour. Next, the primary antibody was diluted by antibody diluent (0.3% Triton-x with 1% BSA) and added for overnight incubation at 4°. After that, the slides were washed three times with an antibody dilutant. Then, a diluted secondary antibody was added for 1 hour. Finally, the slides were washed three times with PBS and mounted with Vectashield.

3.6.6 Gene expression by qPCR analysis

Analysis of retinal organoids by qPCR was performed as described for cerebral organoids.

3.7 Microfluidic chip for water/oil droplet generation

3.7.1 Chip fabrication

The microfluidic chip was fabricated using a laser ablation (Quintel, Evergreen EVG00200). The 2D design was designed using Fusion 360 (Autodesk). Chip was ablated from a silicon wafer (Thickness: 525 µm, Orientation: 100, Type: P, Dopant: B, Resistance: 10-200Ω, Polish: DSP). The flow channel had a height of 60 µm and the T-junctions area (dispersed and continuous phase) had a width of 60 µm. Channels were smoothed by Wet etching. Firstly, the chip was first etched by HF 10% for 2 minutes and washed by IPA in an ultrasonic bath for 5 minutes. Final etching was done by TMOH 20% for 3 minutes.

Then, the chip was cleaned by SC1 cleaning protocol¹¹⁴ and closed with borosilicate glass (Thickness: 250µm) by anodic bonding at 400°C and voltage 1000 V. For coating, the chip was fixed into the holder by screws (35N/cm²), and the tubes were connected to the holder by fitting. Syringes were filled with coating solutions and connected to tubes. By pumps (NE-1002X) chip channels were filled with coating solutions. Then, the chip was disconnected from the holder, and the coating solution was blown away by air. This process was repeated three times.

3.7.2 Droplet generation

The chip was fixed into the holder. Two Hamilton syringes (HAM80075, Hamilton) were filled with water for the dispersed phase, and one Hamilton syringe (HAM80075, Hamilton) was filled with a mixture of mineral oil (Sigma M8410) with 0.5% Span80. Then, syringes were connected to pumps (NE-1002X). Firstly, oil was introduced into channels and after that, water was pumped into chip.

4 Results

As depicted in the chapter “Goals of the thesis”, the primary objective of this work was to design, fabricate and test the microfluidic system for the generation and growth of stem cells-derived cerebral organoids. Importantly, having this technology in hands, we have also performed a proof-of-concept experiment where we aimed to differentiate retinal organoids from iPSCs via the same microfluidic chip setup. Finally, in collaboration with OTH University in Regensburg, we aimed to develop a microfluidic system designed for the separation of single cells. These efforts, described in the following sections, collectively highlight the versatility and potential of microfluidic systems, not only in advancing organoid research but also in expanding the toolkit for precision single-cell analysis and differentiation studies.

4.1 Chip fabrication

4.1.1 Chip design software

We aimed to develop a simple cultivation tool that facilitates initial aggregation, followed by the cultivation and differentiation of 3D cell structures. The microfluidic chip consists of two primary components: channels and wells. The channels enable nutrient exchange, while the wells serve as chambers where the 3D cell structures are formed (Fig. 13). This closed system is connected via tubing to a syringe on one end and a waste reservoir on the other. The syringe is mounted on a pump, allowing precise control of fluid flow, which can be adjusted to meet specific cultivation requirements.

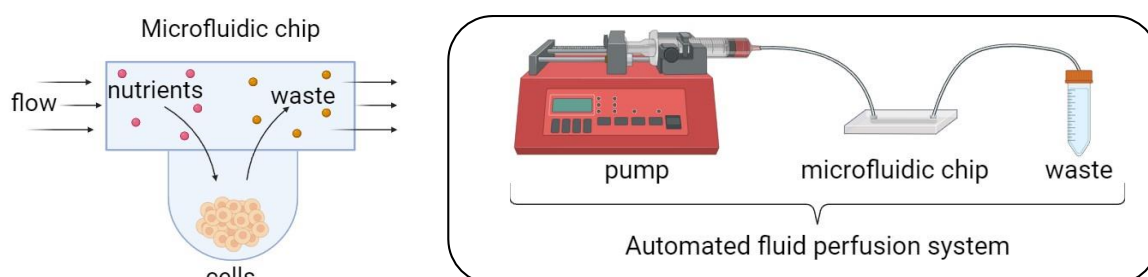


Fig. 13: Idea of automated microfluidic system for cell aggregation

Created by author with Biorender

The design concept for the microfluidic chip (Fig. 13) was developed using the student version of Fusion360 software. Each version of the microfluidic system was iteratively refined in this software. Initially, a model of the chip itself was created. From this, a negative mold was generated, around which the PDMS casting mold was designed (Fig. 14). This 3D design was exported in multiple formats to facilitate easy modification and preparation for 3D printing.

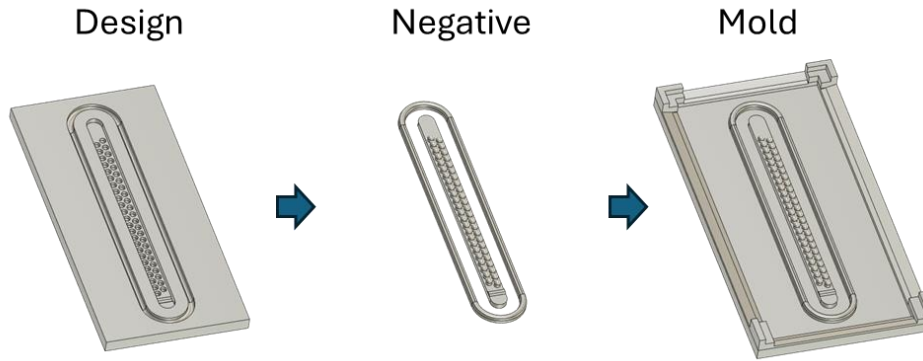


Fig. 14: Creating of the master mold from the design of the chip

4.1.2 SLA printing and optimization

To achieve optimal fabrication results in surface smoothness and well quality, the printing settings were carefully optimized. The initial parameter adjusted was the printing tilt of the mold, a critical factor for overall surface smoothness. To test this, prisms with various tilt angles (90° , 65° , 45° , 35° , 25° , and 15°) were fabricated. This stage also included testing two different printing materials: clear resin (FLGPCL03) and white resin (FLGPWH02) (Fig. 15). Clear resin was chosen for its superior post-processing capabilities, while white resin was selected for its favourable mechanical properties.

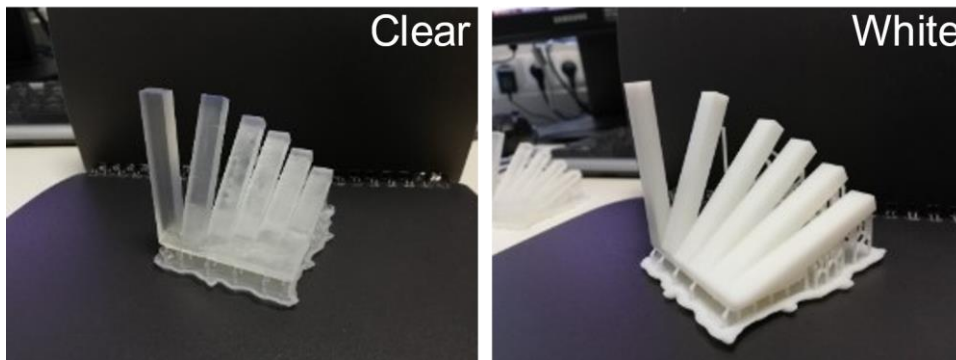


Fig. 15: Angle of tilt for two printing materials. On the left is FLGPCL03 (Clear) and on the right is FLGPWH02 (White).

Based on both prints, a tilt angle of 45° produced the smoothest surface. Regarding material choice, clear resin yielded a smoother surface compared to white resin. In the next step, pillars (negative wells) were added to the surface to observe the effects tilt angle on pillar geometry. Pillars were fabricated at angles of 40° , 43° , 46° , 49° , 52° , and 55° to include a range of possible configurations (Fig. 16A).

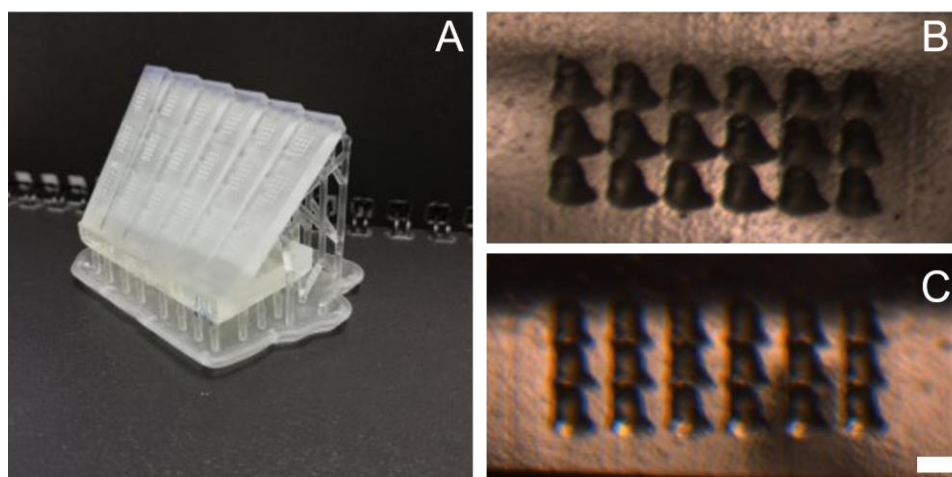


Fig. 16: Fabricated pillar in different tilt angles (scale bar: 400 μ m)

Microscopic images revealed that a tilt angle of 55° caused teardrop-shaped formations around the pillars (Fig. 16B). In contrast, a tilt angle of 40° yielded the best results, with no interconnections between pillars (Fig. 16C). To ensure surface smoothness and minimize the teardrop effect, a 40° tilt angle was chosen.

To further analyze the impact of pillar size (diameter, height) and distribution on fabrication quality and detail, a large plate containing pillars was designed and printed with a 40° tilt angle (Fig. 17A). The specific dimensions of the printed pillars are provided in Tab. 3.

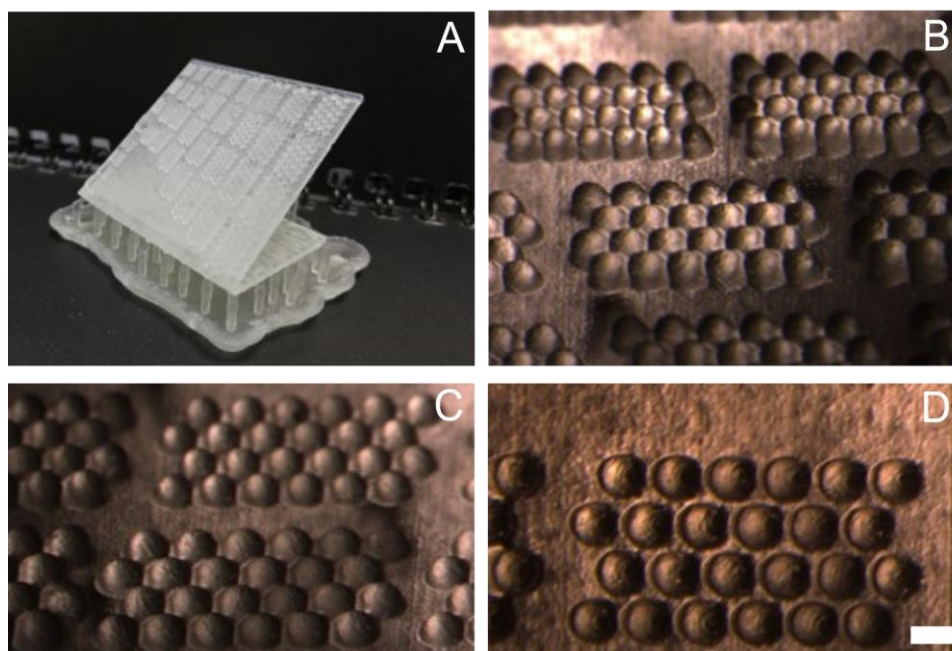


Fig. 17: Printed pillars on large plate, macro and microscopic observation (scale bar: 600 μ m)

Distance [μm]	100	200	100	200	100	200	100	200	100	200
	d=200	d=200	d=400	d=400	d=600	d=600	d=800	d=800	d=1000	d=1000
	h=200	h=200	h=400	h=400	h=400	h=400	h=400	h=400	h=500	h=500
	d=200	d=200	d=400	d=400	d=600	d=600	d=800	d=800	d=1000	d=1000
	h=400	h=400	h=600	h=600	h=600	h=600	h=600	h=600	h=600	h=600
	d=200	d=200	d=400	d=400	d=600	d=600	d=800	d=800	d=1000	d=1000
	h=600	h=600	h=800	h=800	h=800	h=800	h=800	h=800	h=800	h=800
	d=200	d=200	d=400	d=400	d=600	d=600	d=800	d=800	d=1000	d=1000
	h=800	h=800	h=1000	h=1000	h=1000	h=1000	h=1000	h=1000	h=1000	h=1000

Tab. 3: List of printed pillars

Pillars were printed with spacings of 100 μm and 200 μm. A 100 μm spacing yielded unsatisfactory results, as all pillars became interconnected. Images in Fig. 17B and Fig. 17C show pillars with spacings of 600 μm and 800 μm and a height of 600 μm. Due to the small gap and high energy in this region, the resin polymerized, filling the spaces between pillars. A 200 μm spacing between pillars produced promising results in four cases, with a maximum pillar height of 500 μm and a diameter-to-height ratio not exceeding 2:1. Pillars with a diameter and height of 400 μm successfully maintained a gap between them (Fig. 17D). Based on these results, we concluded that a minimum spacing of 200 μm is required for smaller pillars, with larger pillars greater distance is required.

4.1.3 PDMS molding

According to the standard molding protocol, PDMS is poured onto the master mold, with solidification time dependent on temperature. Higher temperatures reduce the PDMS curing time. This same protocol was applied for molding using the 3D-printed mold. Based on this approach, a specific design was created to form a closed master mold in combination with a glass slide (Fig. 18). PDMS was poured into the SLA mold, and a glass slide was positioned to create a smooth, flat surface. The master mold with PDMS was then placed in an oven to solidify.

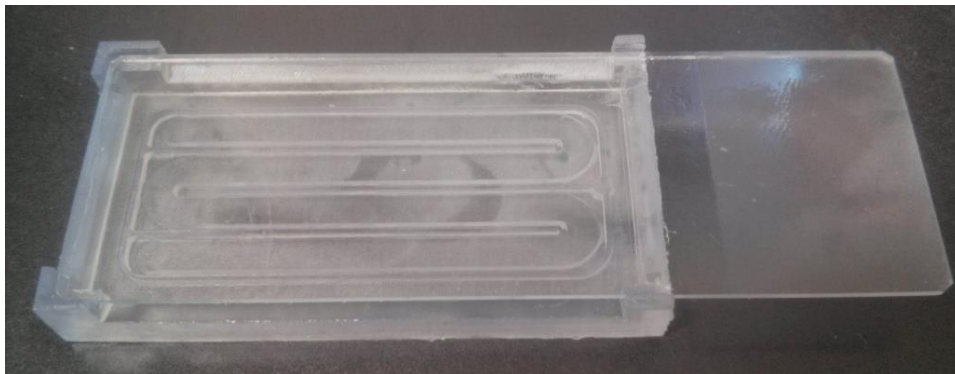


Fig. 18: SLA mold with a glass slide

A problem occurred due to leftover uncured resin on the surface or inside the printed mold, which prevented PDMS from solidifying. This made pre-treatment of SLA molds essential prior to PDMS molding. Additionally, the wide variety of resins available meant that no single protocol could be applied universally. To address this, a protocol was developed to optimize the treatment of SLA-printed molds. UV post-curing, temperature, and solvent treatments were tested. Results indicated that resin adsorption is a gradual process; while acceleration is possible, the best outcomes were observed after an extended period (over one day). Based on these findings, a post-treatment protocol was established. It includes thorough rinsing with IPA, UV curing for 15 minutes, and baking at 60°C for 24 hours.

In some cases, the initially molded PDMS remained sticky and uncured. To address this, the curing time and temperature were optimized (Tab. 4). It was found that curing at 60°C or lower required 24 hours for complete solidification, while curing at 80°C or higher achieved full curing in just one hour.

Time/ Temp.	1h	2h	3h	4h
60°C	No	No	No	OK/Sticky
70°C	OK/Sticky	OK/Sticky	OK	OK
80°C	OK	OK	OK	OK
90°C	OK	OK	OK	OK
100°C	OK	OK	OK	OK
110°C	OK	OK	OK	OK

Tab. 4: Optimization of PDMS curing

4.1.4 Connection of two PDMS layers

Typically, connecting PDMS to PDMS is achieved through plasma treatment, which creates a watertight covalent bond. For successful bonding, both surfaces must be nearly perfectly smooth and free of defects, similar to a silicon wafer or glass slide. However, SLA printing did not yield PDMS molds with sufficiently smooth surfaces for effective connection. To address this issue, uncured PDMS was used as an adhesive by applying a thin layer to both PDMS surfaces. The layers were then connected and placed in an oven for permanent bonding.

4.1.5 Thickness of PDMS for microscopy

During the cultivation process, it is essential to validate the conditions within the PDMS chip and monitor the cell structures. To assess the environment inside the chip, microscopy methods with appropriate magnifications were employed. Literature¹²² has indicated that the thickness of the PDMS layer affects light transmission and high-

magnification observations. Based on that, a PDMS chip with a thickness of 200 μm was designed and fabricated. This thickness ensures that the PDMS layer is located beneath the wells, which serve as the primary focus for observation (Fig. 19).

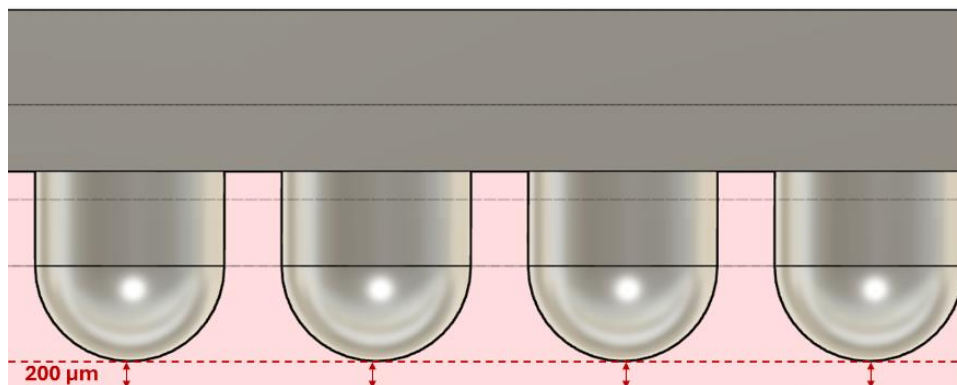


Fig. 19: Thickness of PDMS under wells

After fabrication, the observation capabilities were evaluated (Fig. 20). Cell aggregates were placed in the wells for testing. Four different magnifications were used for microscopy. Ultimately, focusing on the cell aggregates during cultivation was not an issue.

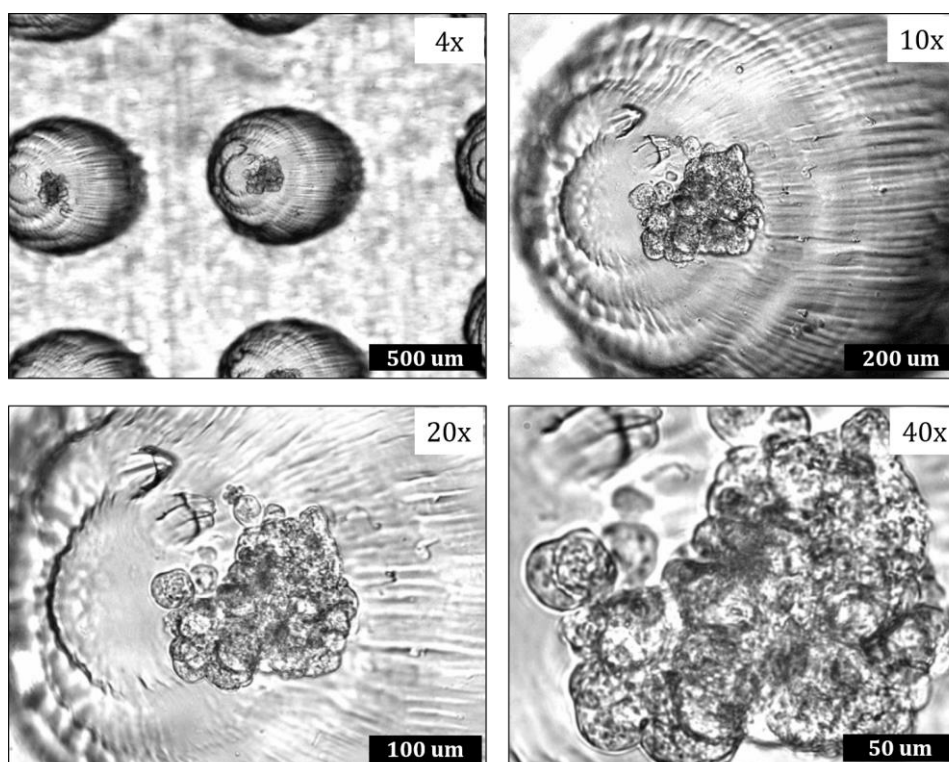


Fig. 20: Observation and focus capabilities for different magnifications

4.1.6 Degassing of microfluidic system

Connection and test running of the complete automatic system (Fig. 13) showed generation of bubbles inside the flow channel. It was not possible to determine the primary source of these bubbles. There were two possibilities: first, bubbles could have been generated in the tube or syringe and then introduced into the chip through the tubing; Second, gas might have permeated the chip through the semi-permeable PDMS. To identify the source of the bubbles and resolve the issue, a following experiment was designed (Fig. 21A). A pump was connected to two chambers to capture the bubbles, and a control experiment without exhaust was also conducted.

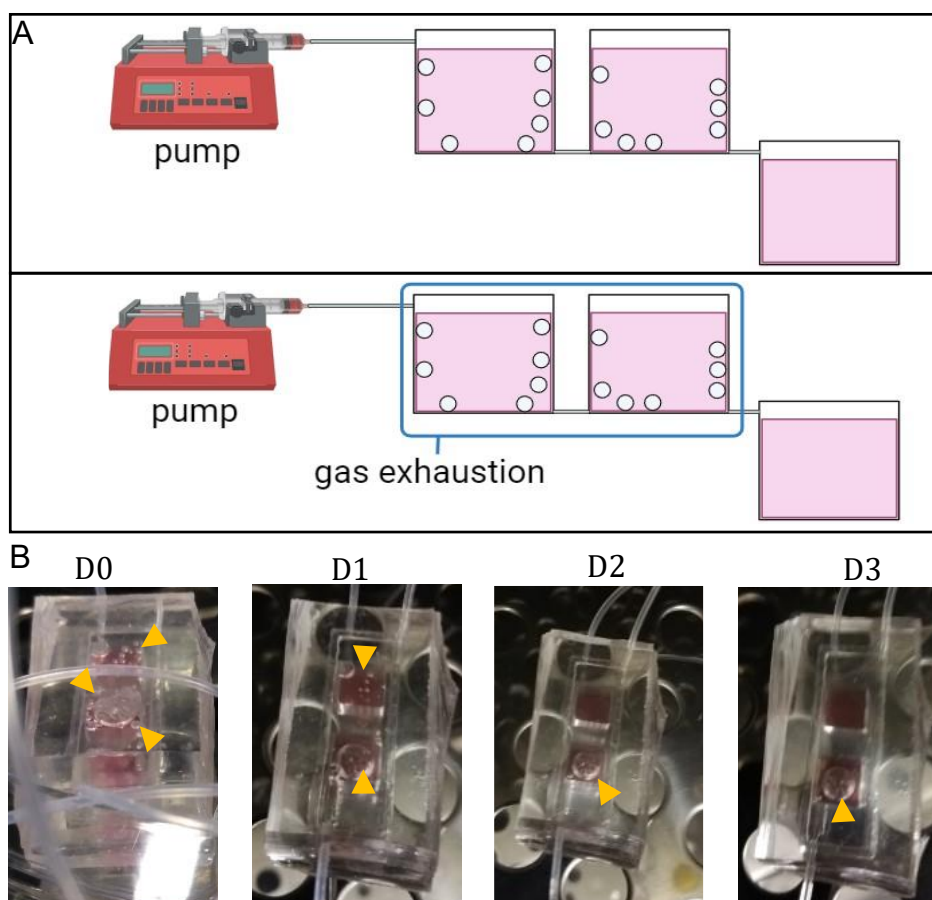


Fig. 21: Scheme of gas exhaustion experiment and results during days

During the experiment, it was discovered that bubbles formed on the walls of the chambers, indicating that gas was penetrating through the PDMS. Additionally, a large bubble was transferred into the first chamber. The medium flowed for one day without gas exhaustion (D0) (Fig. 21B). On day D1, gas exhaustion was activated. Observations from the images showed that bubble formation on the walls of the chambers

ceased. However, the largest bubble remained in the first chamber. Due to its size, the exhaustion system was unable to remove this bubble over two days.

To simplify the microfluidic system, a new microfluidic chip was fabricated with an exhaustion channel surrounding the main cultivation channel (Fig. 22). The differences between the chip with the exhaustion channel and the one without are also illustrated. In subsequent experiments, it was found that the exhaustion channel continued to function even without active gas exhaustion. The exhaustion channel, equipped with an opening for gas, was sufficient to prevent bubble formation in the main channel.



Fig. 22: Chip with exhaustion system

4.1.7 Depth and diameter of wells

The diameter and depth of wells affect SLA printing, but more importantly, it is essential to understand how these parameters influence cell cultivation. Based on the data in (Tab. 3), suitable pillar/well configurations for printing were selected. For the cultivation test, wells with a diameter of 400 μm and depths of 200, 400, 600, and 800 μm were prepared. After seeding, the cells were cultivated for three days, after which the cell aggregates were measured. The graph (Fig. 23) did not show a significant difference in aggregate size among the 200, 400, and 600 μm wells. However, the 800 μm well exhibited smaller aggregates compared to the others. Since the depth was twice the diameter, it may have impacted the nutrient supply to the cells.

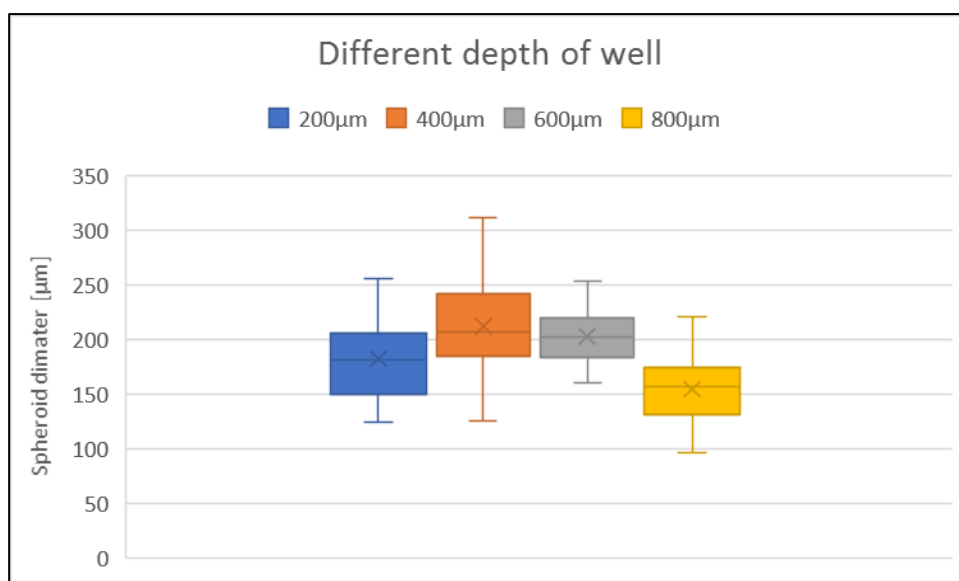


Fig. 23: Impact of well depth on cell aggregate size

To analyze the effect of diameter, wells with diameters of 400, 600, and 800 μm were prepared, each with a depth of 400 μm. The cells were cultivated for three days. The assumption was that wells with larger diameters would initially accommodate more cells. The graph (Fig. 24) supported this assumption; however, there was not a significant difference in cell numbers between the 400 μm and 600 μm wells.

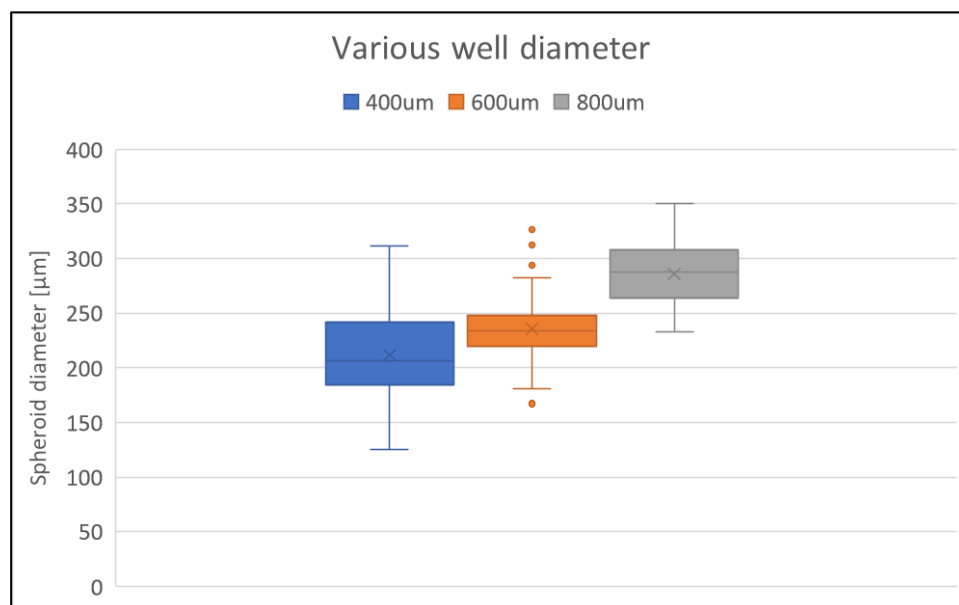


Fig. 24 Impact of well diameter on cell aggregate size

4.1.8 Surface modification to increase cells clustering

The quality of SLA printing depends on various factors, one of which is the printing resolution. For our printing process, a Form 2 printer with a laser spot size of 140 microns was used. In macro-observations, this resolution provides good surface smoothness with optimal settings. However, the curvature or inclination of specific design features in areas smaller than 1 mm can impact the printing results. For our fabrication process, the shape of the pillars and wells is critical, as it creates microtopography on the surface (Fig. 25A). This microtopography negatively affects PDMS molding, as PDMS can become embedded in the surface irregularities and tear away during demolding. Additionally, it impacts cell cultivation and adhesion, causing cells to cluster on the walls of the well rather than at the bottom, which can compromise the homogeneity of the cell culture. To mitigate these negative effects, the 3D-printed master mold was coated with varnish. A thin layer of varnish was applied, resulting in a smoother surface (Fig. 25B). Although more layers of varnish were applied, it was found that after the second layer (Fig. 25C), additional applications did not significantly improve smoothness.

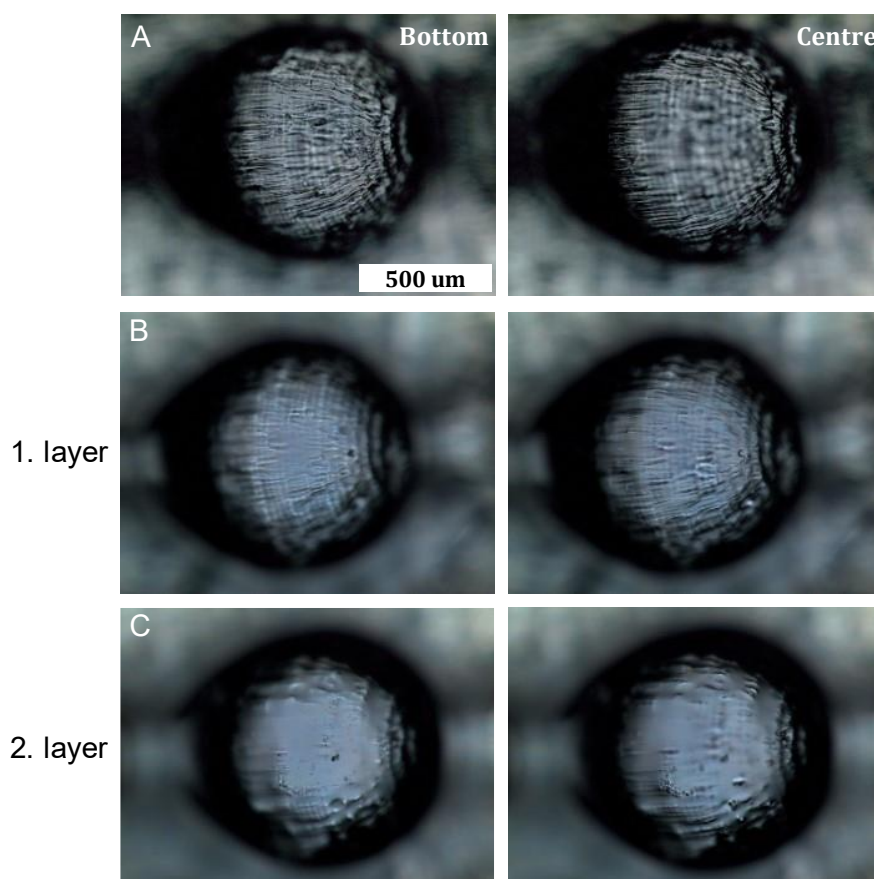


Fig. 25: Microtopography on wells surface

The smoothing step during cultivation reduced the total number of aggregates to 12 ± 2 per U-shaped well; however, clustering remained inadequate, likely due to the adhesive properties of PDMS. To address this issue, as described by Azizipour et al.¹²³, the PDMS surfaces of the channels and microwells were coated with bovine serum albumin (BSA) to form an anti-fouling layer. This modification inhibited cell adhesion to the walls during culture and significantly improved cell clustering into a single aggregate (Fig. 26). Similar results were also observed with V-shaped wells.

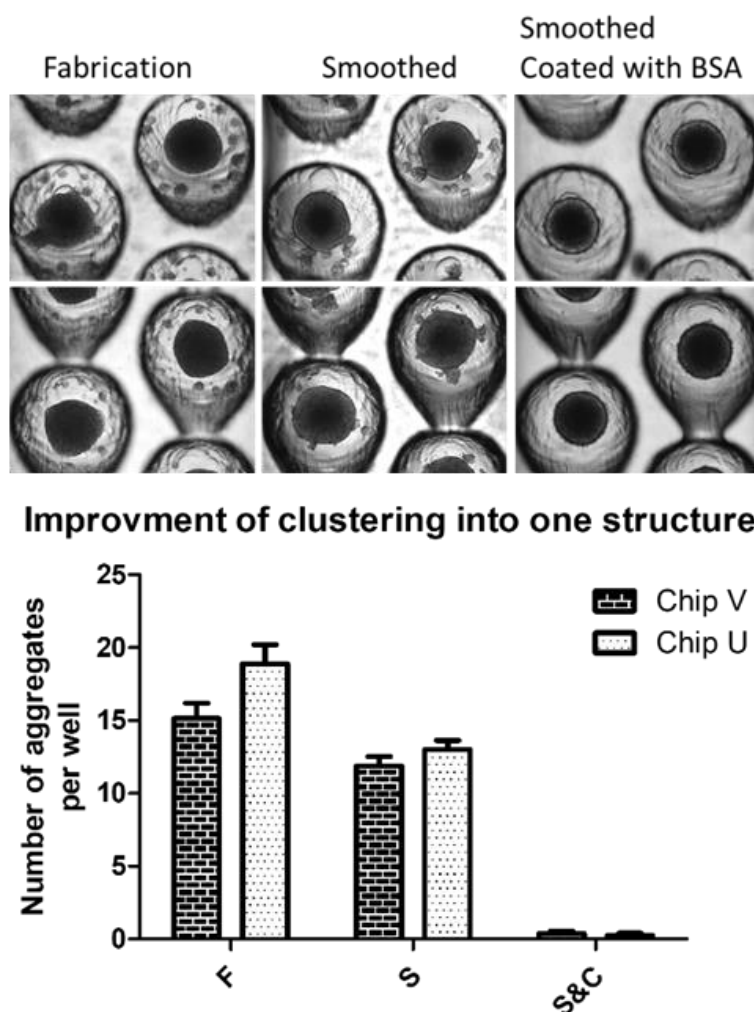


Fig. 26: Surface modification for cell clustering

4.1.9 V-shaped well optimization

During the optimization process and the seeding of cells into the wells, the formation of multiple cell aggregates was observed. The geometric shape of the well is another important aspect that affects this phenomenon. Initially, a standard U-shaped well was used; however, it does not allow for adjustments to the steepness of the walls. To address the issue of multiple aggregates while providing a means to modify the geometry, V-shaped wells were designed. Four types of wells with different tilt angles were created for optimization (Fig. 27). The well with a 60-degree angle ensured that cells settled in the middle, but the shape of the well influenced the morphology of the organoids. The well with a 30-degree angle also produced positive results, although small satellite aggregates appeared around the organoids. The best angle-to-formation ratio was observed with wells at 40 and 50 degrees. Based on these findings, a well with a 45-degree angle was ultimately designed.

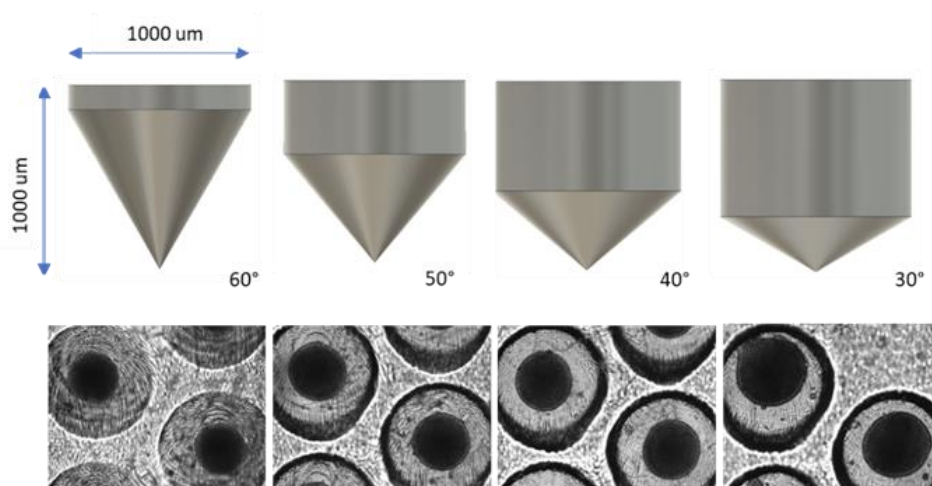


Fig. 27 Cultivation in V-shaped wells

4.1.10 Chip centrifugation

In the standard protocol for seeding cells in a 96WP (V-bottom), the plate is centrifuged to facilitate the accumulation of cells at the bottom and promote faster spheroid formation. To concentrate as many cells as possible at the bottom of the well and eliminate the formation of satellite aggregates, centrifugation was also applied to the chip. In both cases, spheroids were observed without any satellite aggregates (Fig. 28).

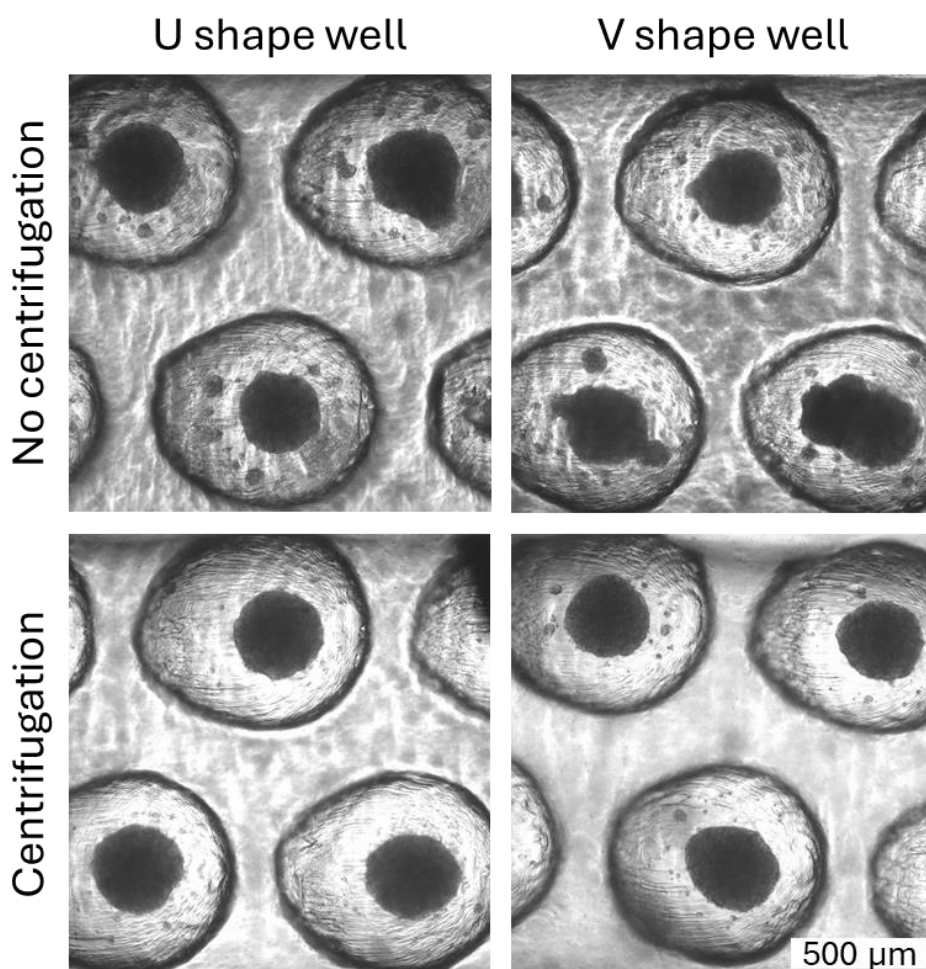


Fig. 28: Chip centrifugation after seeding

4.1.11 Different shapes of wells

The geometry of well shape is an important aspect of cell cultivation, as it influences the formation of organoids both initially and during the cultivation process. During the optimization steps, both U-shaped and V-shaped wells were utilized. This experience indicated that there are additional opportunities to modify the original designs or create new ones. Consequently, we decided to develop a library of designs from which users could select specific options for cultivation (Fig. 29) In addition to the U

and V shapes, a hybrid design known as MVU was created, aimed at achieving perfectly round aggregates from the outset. Functional elements were incorporated at the bottom of the well, where the tilt angle helps position the cells in the middle of the curved bottom. The VU design was intended for larger organoids, featuring steep walls for better seeding and a U-shaped bottom. Additionally, pyramidal and hexagonal shapes were designed to enhance space utilization while minimizing dead space around the wells, both featuring a V-shaped bottom.

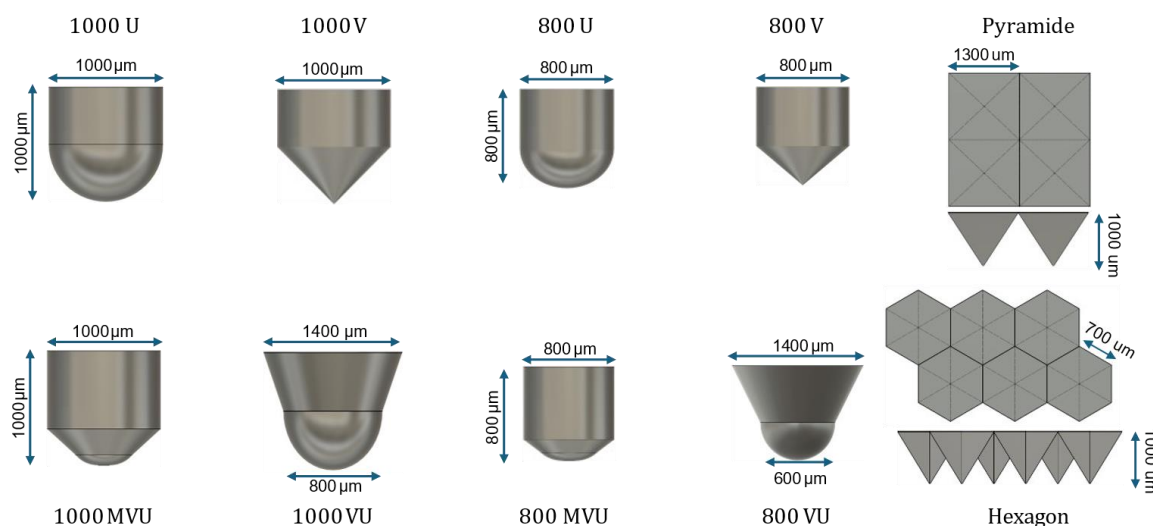


Fig. 29: Various shapes for cultivation well

During cultivation with cells U- and V-shaped wells produced results similar to those obtained previously (Fig. 30). Although smaller diameters resulted in fewer cells entering the wells, there was no visible difference in outcomes. Pyramidal wells addressed the issue of dead space around the wells; however, the corners became ideal spots for cell adhesion and aggregate formation. Additionally, it was observed that aggregates adapted well to the geometry of the wells. The MVU design performed comparably to the U and V shapes, making it difficult to determine whether the geometry had a better impact on aggregation. Aggregates in the VU shape were larger than expected and functional. In the hexagonal wells, the aggregates were the smallest, yet they maintained a round shape with no additional aggregates forming around them. Based on these findings, U and V shapes were selected for future experiments, as they demonstrated comparable or better performance than other shapes. Moreover, these shapes are commonly used in standard cultivation, facilitating direct comparison with the microfluidic system.

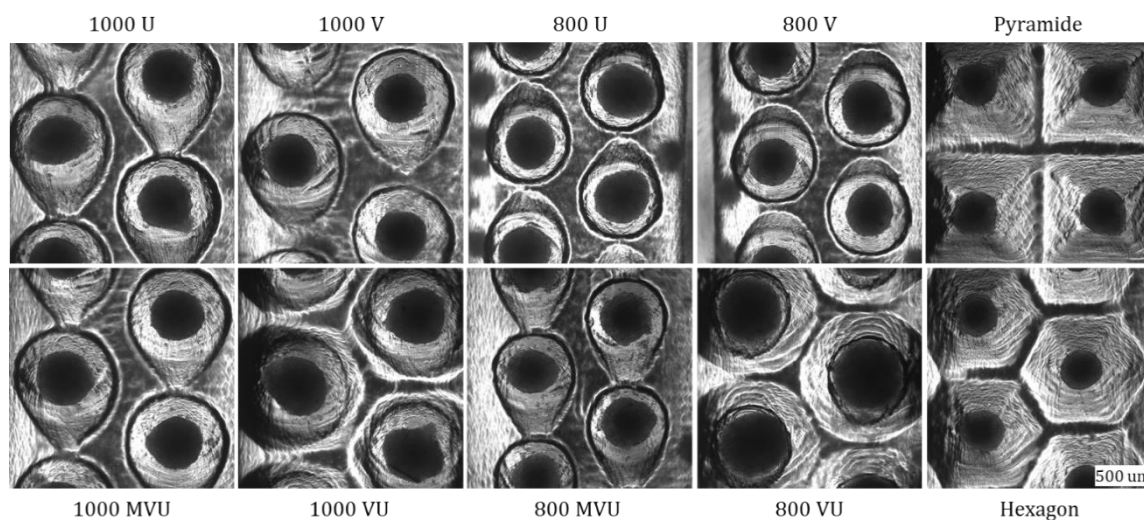


Fig. 30: Cultivation in various wells geometry

4.2 Optimization of function

4.2.1 Cells seeding to provide uniform deployment of cells

The geometry of the wells and surface modifications facilitate the successful aggregation of cells into a single structure. Additionally, the spacing between the microwells was designed in a staggered zig-zag arrangement to minimize dead space and maximize the number of cells that settle into the wells after seeding. To form homogeneous cell clusters, an even distribution of cells is essential. This was achieved through the gentle injection of cells into the channel and subsequently into the wells during the seeding process (Fig. 31).

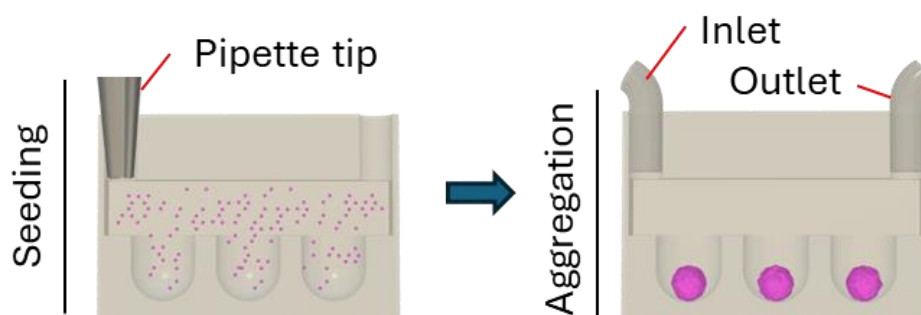


Fig. 31: Cell seeding into chip

The original number of cells was calculated based on the number of cells per well. However, during the optimization process, it was observed that not 100% of the cells settle into the wells due to the presence of dead spaces at the inlet, outlet, and around the

wells. As a result, it was necessary to increase the original cell count. To determine the correct number of cells, different seeding densities were compared (110%, 120%, 150%, and 200%). The cells were cultivated for 24 hours, after which the sizes of the aggregates were measured and compared (Fig. 32). From the graphs, it appears that a seeding density of 150% is the minimum required to ensure a homogeneous distribution of cells in the channel. Conversely, a twofold increase in cell number seems excessive, as the size of the resulting aggregates in the chip increases significantly. Therefore, for this channel size with a specific number of wells and appropriate seeding techniques, the ideal cell count should be 1.5 to 2 times larger than the original number.

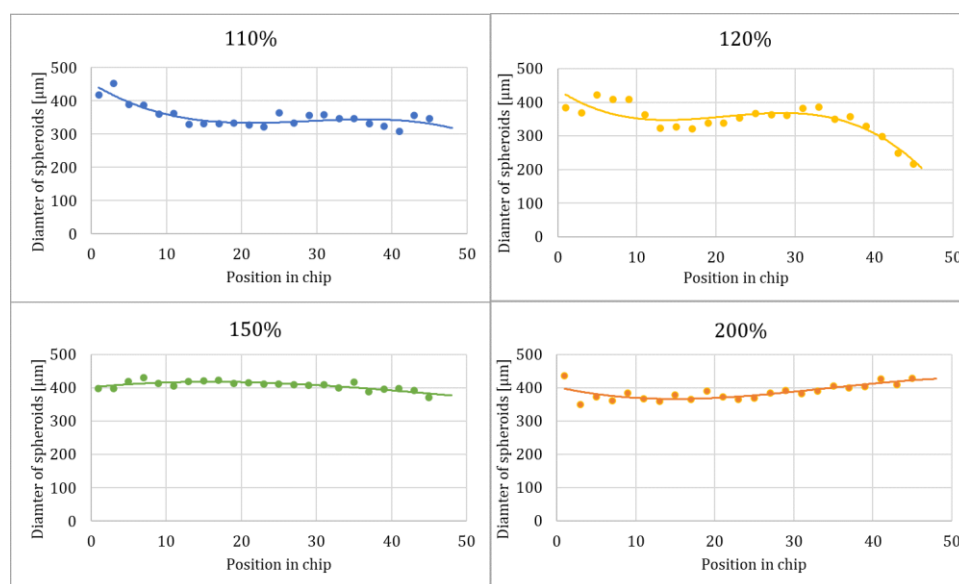


Fig. 32: Number of cells seeded to form uniform spheroids

4.2.2 Impact of ROCK inhibitor and cell number on the aggregate shape

Rho kinase inhibitors are a series of compounds that specifically target Rho-associated protein kinase. These inhibitors enhance the survival of human embryonic stem cells (hESCs) when they are dissociated into single cells by preventing dissociation-induced apoptosis (anoikis)¹²⁴, thereby increasing their cloning efficiency. Additionally, they improve embryoid body formation using forced-aggregation protocols. In our protocol for the generation of cerebral organoids, published by Lancaster et al, a Rho kinase inhibitor was used during the first six days of cultivation. The Lancaster protocol⁶² typically employs a concentration of 50 µM, while other protocols use a concentration of 20 µM¹²⁵. We compared these concentrations alongside different cell numbers per well. The first experiment was conducted in a 96WP (V-bottom). On day 5, spheroids were transferred to a flat dish for shape analysis (Fig. 33). Results from day 5 of cultivation showed that, from a top view, the spheroids treated with 20 µM exhibited a

wrinkled shape. Side view analysis indicated that the 20 μM spheroids formed a flatter structure. Notably, a higher number of cells also tended to form flatter structures.

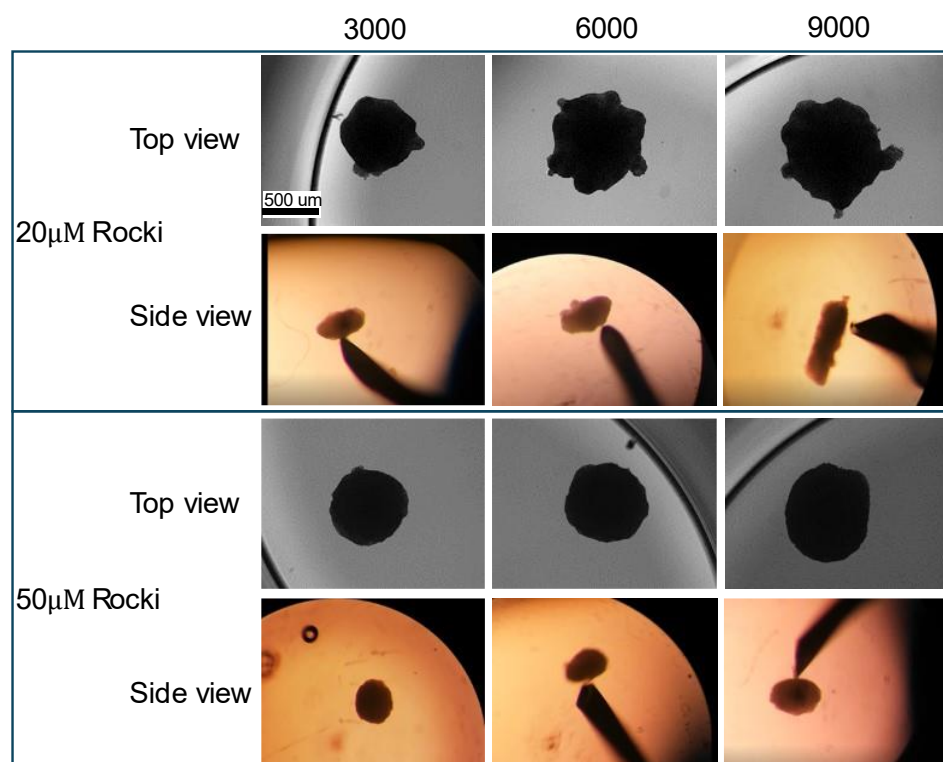


Fig. 33: Shape of spheroids after 5 days of cultivation in WP

The second experiment was conducted in a chip, and based on the results from the 96WP experiment, only 50 μM of Rho kinase inhibitor (ROCKi) was used. In the chip, the shapes of spheroids were tested in U and V wells (Fig. 34). From a top view, there was no noticeable difference in the morphology of the spheroids. Side view observations showed similar results for the number of cells seeded per well, with spheroids in the V wells exhibiting shapes that conformed to the geometry of the wells. Overall, the number of cells seeded per well plays a crucial role in maintaining a round spheroid shape, and a higher concentration of ROCKi contributes to achieving this result.

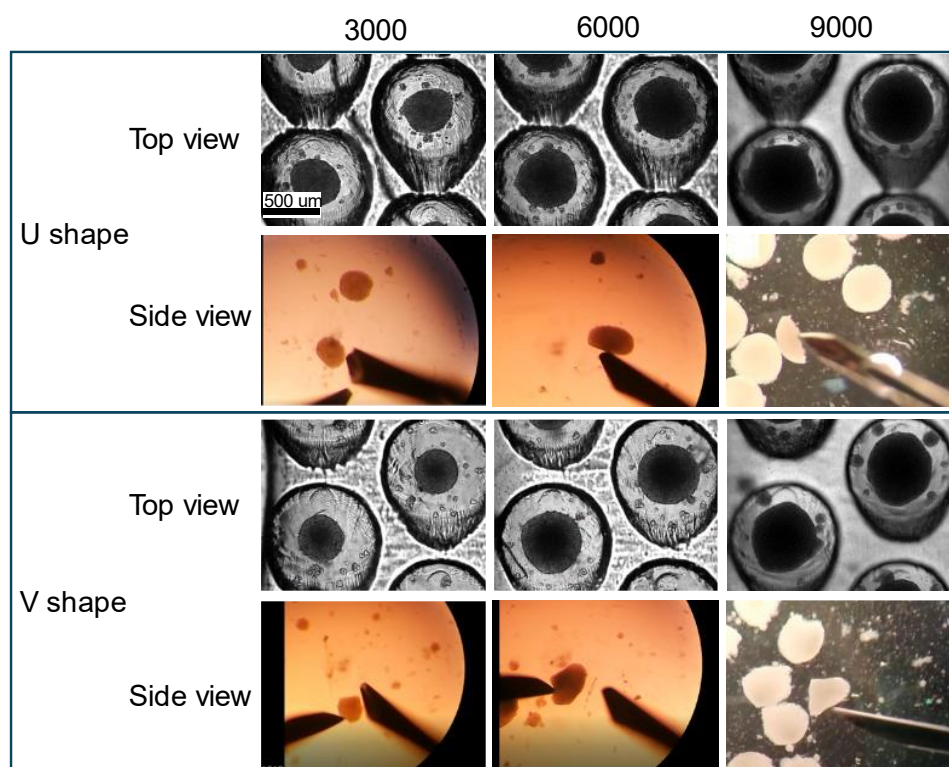


Fig. 34: Shape of spheroids after 5 days of cultivation in chip

Another experiment was added to the chip study to investigate how the number of cells affects the size of aggregates after three days of cultivation in U and V-shaped wells. Four cell concentrations were compared: 9000, 6000, 3000, and 2000 cells per well (Fig. 35). For both U and V-shaped wells, there was no significant difference in aggregate size. The smallest difference in diameter was observed between the 3,000 and 2,000 cell conditions, indicating that a seeding density of 2,000 cells may lead to a more uniform round shape of aggregates compared to 3,000 cells.

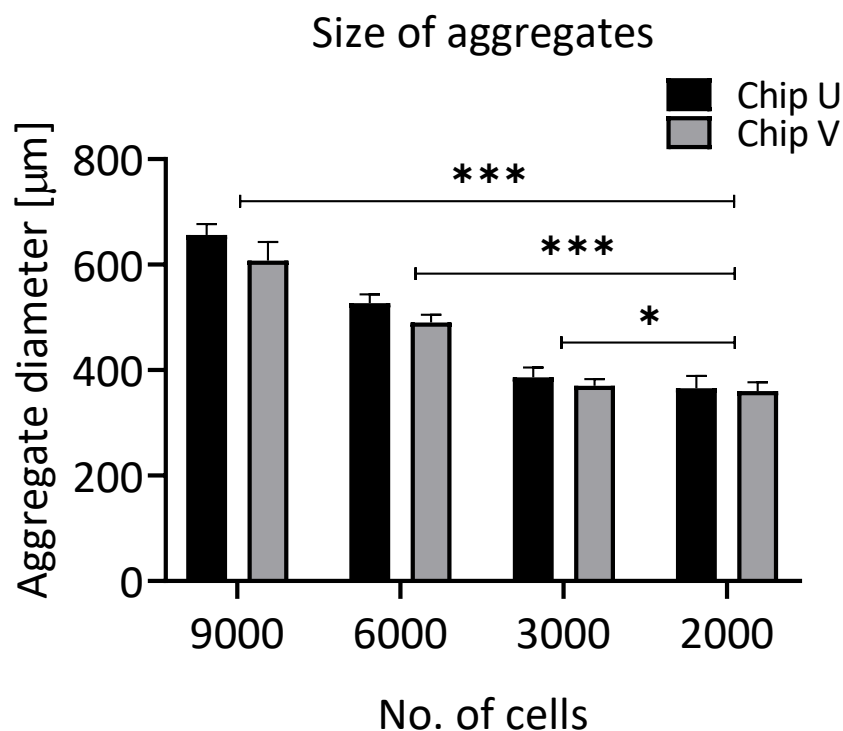


Fig. 35: Aggregate diameter for different number of cells in chip

4.2.3 Optical aberration in V shaped 96-well plate

To assess the functionality of the microfluidic chip, the system was compared to the commonly used cultivation protocol in a 96WP. During the experiments, the diameter of aggregates from the WP was larger than those from the chip, despite the cell seeding densities per well being the same. To further investigate this, the sizes of aggregates in the 96WP (V-bottom) were compared to those in a cell culture dish with a flat bottom. A total of 70 aggregates were analyzed, revealing differences in diameter (Fig. 36A). From the measured values, a power trendline was established (Fig. 36B) along with the equation for converting to the real size of the organoid.

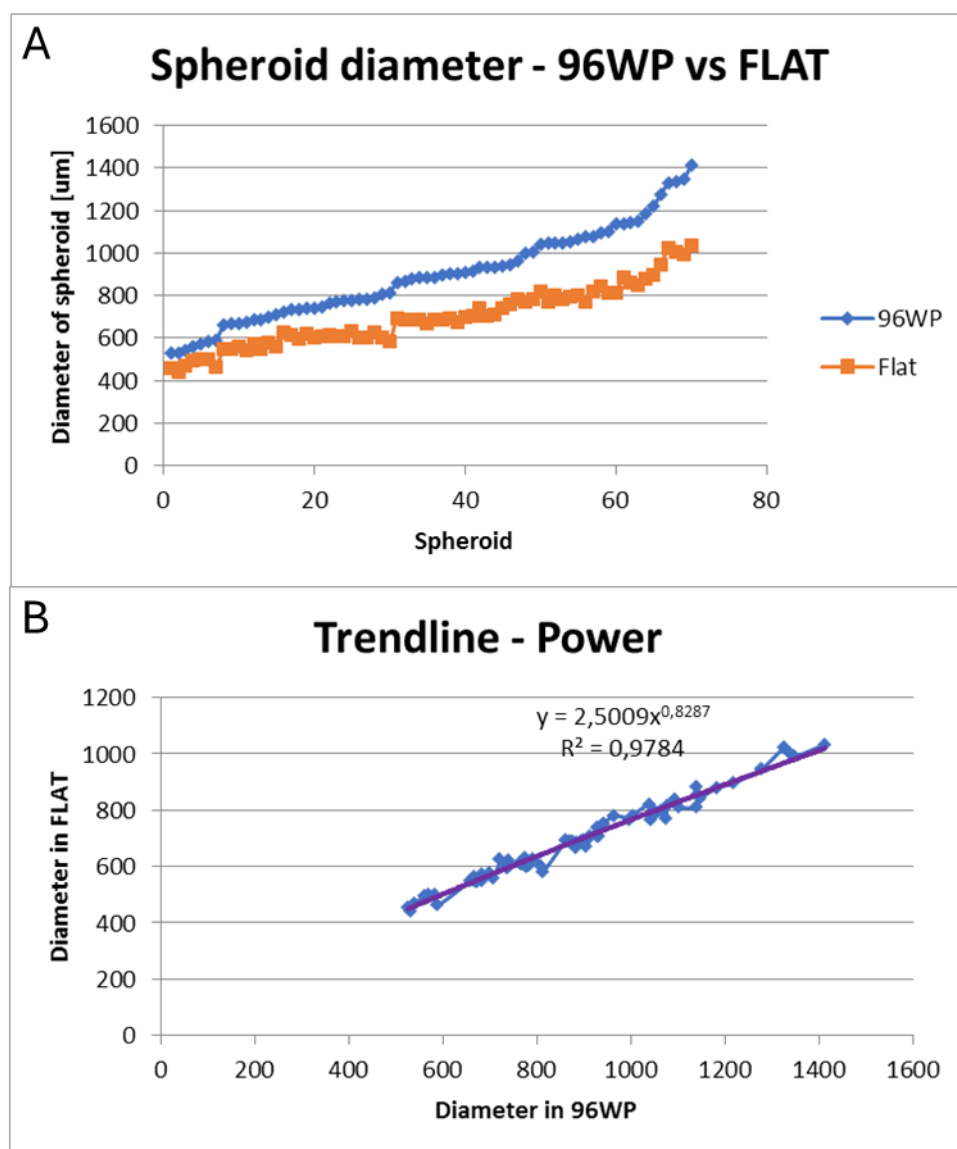


Fig. 36: Comparison of aggregates and power trendline

Conversion equation

$$y = 2,5x^{0.8287} \quad (14)$$

Where the x is measured diameter of aggregate in WP.

4.3 Simulations

Optimizing various parameters for cultivation was essential to understand the key factors before conducting experiments. However, this optimization process can be time-consuming. Therefore, simulations are more suitable for gaining insights into various conditions and investigating dynamic environments. This approach is more cost-effective and efficient, allowing for the simultaneous adjustment of multiple parameters.

4.3.1 Simulations validations

Before using simulations for cultivation in microfluidic system, it was necessary to validate simulation protocol and methods. For this purpose, a simple model was created to compare simulation results with laboratory experiments. As the model, we designed an experiment where dextran diffusion was observed.

Based on literature¹⁰⁸, the properties of the materials and diffusion parameters were established and simulations were conducted in an agarose block (Fig. 37). Three different concentrations of dextran were compared, revealing that the mass fraction in the agarose gel decreases with lower concentrations of dextran. Notably, reducing the concentration to 0.1 mg/ml significantly affected the mass fraction of dextran in the agarose block. Fluorescence measurements of the gradient demonstrated results consistent with the predicted gradients from the simulations. The intensity of fluorescence from dextran decreased with lower concentrations. Additionally, fluorescein was introduced into the simulations to compare the mass fraction of molecules at specific time points. The simulations confirmed that molecules with lower atomic mass exhibit higher diffusivity; at every time point, fluorescein penetrated further into the agarose block than dextran. After 24 hours, the mass fraction of fluorescein decreased linearly, while simulations for dextran indicated differences in mass fraction between the beginning and further regions of the agarose block. The graph after 24 hours showed an exponential decrease in the mass fraction of dextran.

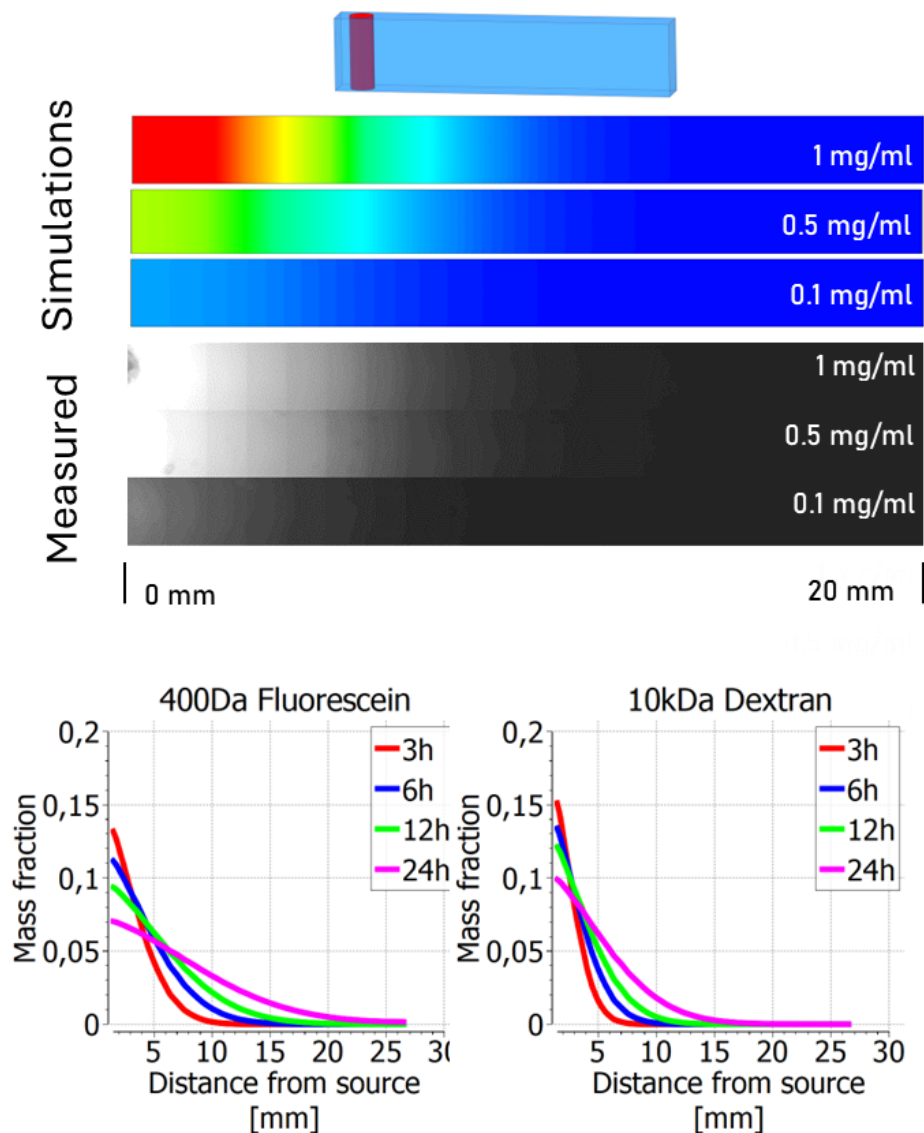


Fig. 37: Gradient and diffusivity of Dextran and Fluorescein in simple agarose block

Next, a defined amount of medium was added on top of the agarose block (Fig. 38). The introduction of the medium led to dispersed diffusion of dextran, resulting in a reduction of dextran concentration within the agarose block. This phenomenon was consistently observed across all concentrations tested. Fluorescence measurements of the gradient mirrored the predicted outcomes from the simulations. The intensity of dextran fluorescence decreased with lower concentrations, and the model incorporating the medium exhibited lower intensity compared to the model without it. Additionally, the model with medium demonstrated a reduced mass fraction of dextran in the agarose block. In this scenario, the same trend was observed for both molecules, similar to the model without medium; however, the differences in their behaviour were more pronounced. The graphs confirmed the earlier findings, indicating that at specific

distances, the mass fraction of molecules was lower in the model with medium compared to the initial model.

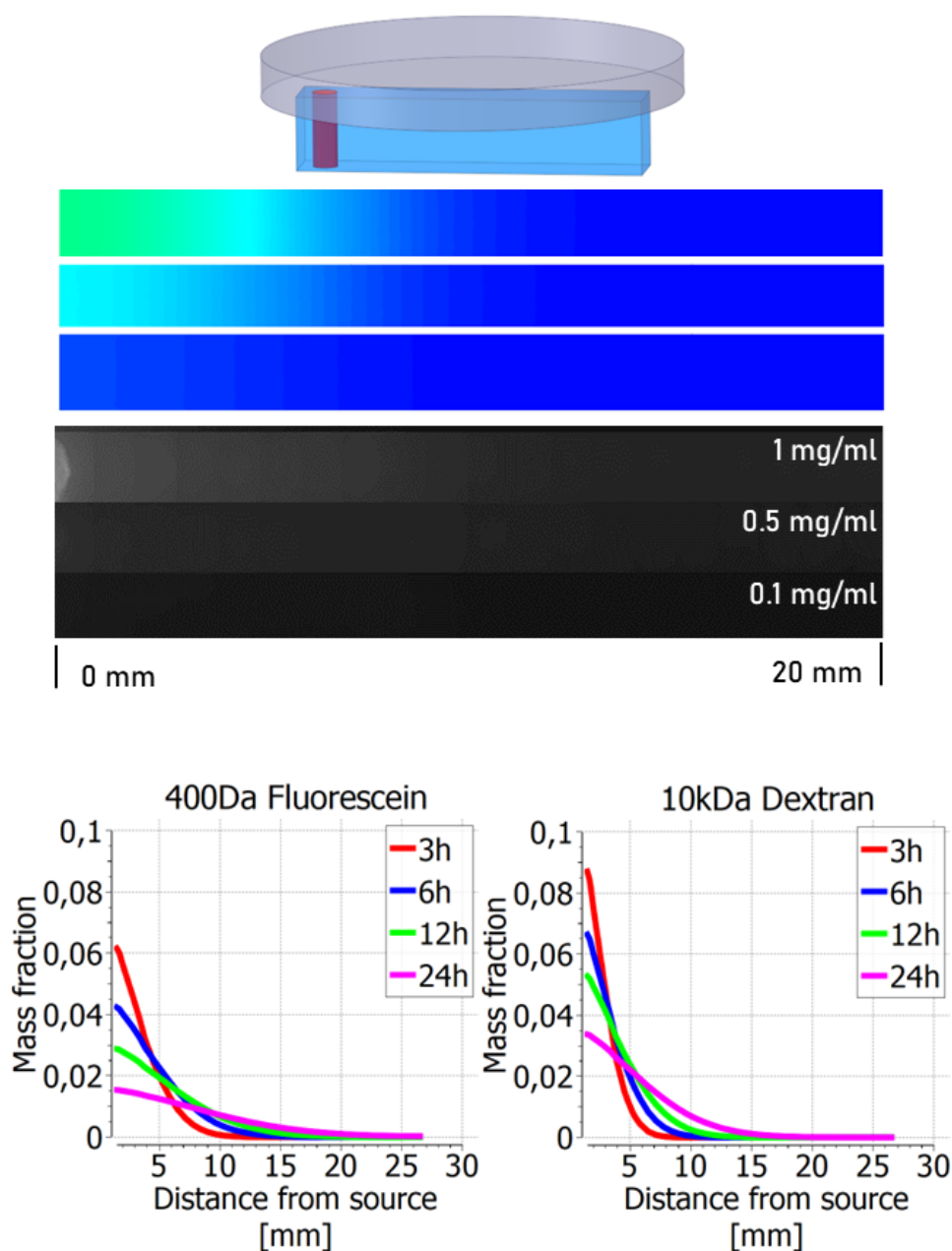


Fig. 38: Gradient and diffusivity of Dextran and Fluorescein to agarose block with medium

Additionally, after a verification of accuracy, simulations were employed to predict diffusion from a 3D printed agarose through water over a 24-hour period (Fig. 39). The images illustrate specific time points of diffusion. Initially, molecules accumulate in the source. According to the simulation, after 6 hours, the molecules are expected to diffuse into the area with a specific structure. This simulation enables the prediction of

molecular diffusion and identifies key time points for observing morphological changes. The distance between structures and the volume of specific substances can be adjusted to achieve the desired effect.

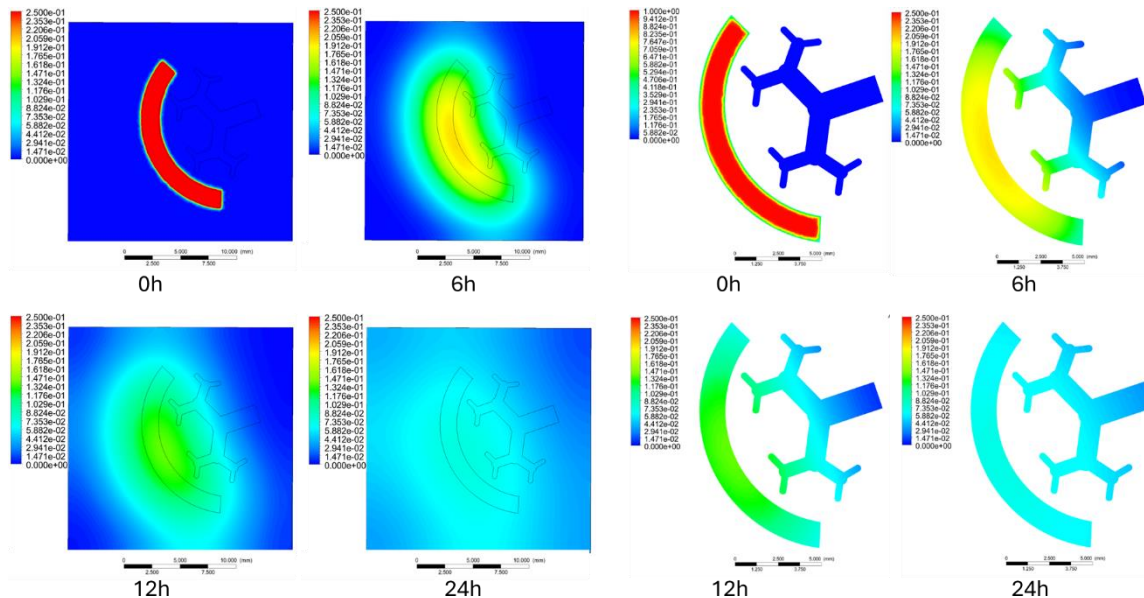


Fig. 39: Simulation of 24-hour diffusion from 3D printed source through water

4.3.2 Diffusion of lactate from cell structures in microfluidic conditions

After simulation validation, we continued with simulation in a microfluidic environment, where we investigated the role of flow rate in medium exchange and metabolite diffusion. The medium exchange simulations were inspired by findings from Berger et al.⁹⁶ and Cho et al.⁸⁸, which highlighted the impact of microfluidic flow on lactate concentration and nutrient/oxygen transfer. Our simulations aimed to analyze lactate concentration and evaluate the reduction in required culture medium volume compared to conventional 96WP. We also added a microfluidic chip with half of the flow (Chip50%) and compared to the chip with the original flow (Chip100%) and 96WP.

In the simulated model, microfluidic chips with microwells contained spherical cell aggregates releasing lactate into the surrounding area. Aggregates with a diameter of 450 μm were modelled to represent densely packed cells occupying less than half the microwell diameter, corresponding to approximately day 4 of cell culture. Lactate diffusion was visualized over time during a continuous exchange with fresh medium, allowing for the establishment of a steady-state lactate concentration around the aggregates (Fig. 40). We compared four flow rates (1000, 100, 50, and 10 $\mu\text{l/h}$) in both U- and V-shaped microwells. The results showed that after 30 minutes of simulation, lactate accumulated in all microwells. After three hours, the lactate concentration in the Chip100% and Chip50% variants, along with their diffusion and removal, reached

equilibrium due to the continuous supply of fresh medium and the removal of metabolites. The analyzed outlier variants, chip with 10 times higher flow (Chip1000%) and chip with 10 times lower flow (Chip10%), equilibrated the average lactate concentration within 0.5 and 8 hours, respectively (Fig. 40).

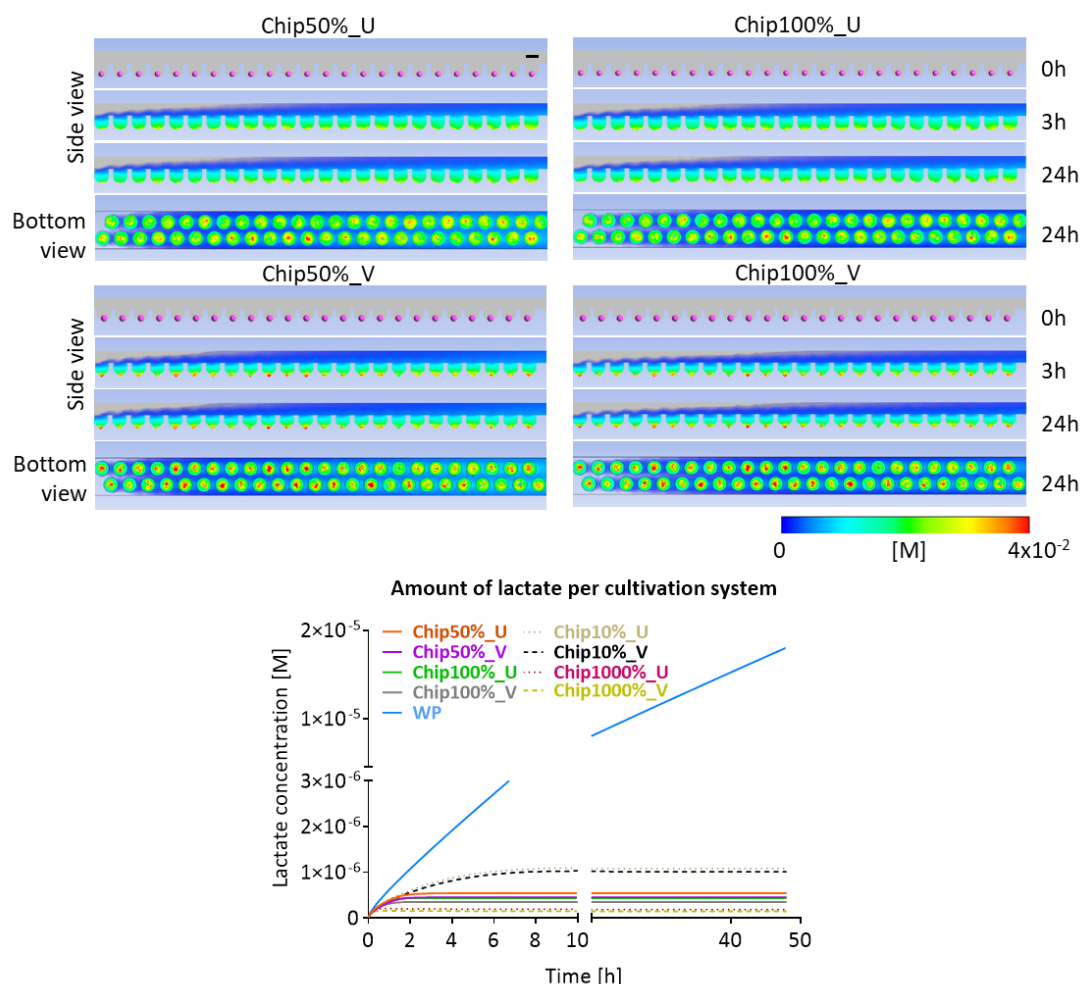


Fig. 40: Effect of microwell shapes on diffusion and accumulation of lactate in the chip in time

Although there are differences in local lactate concentrations between chips with V-shaped and U-shaped wells, as well as between the first and last microwells on the chip (Fig. 41), the average lactate levels in the chips are much lower than those in a 96WP. At a flow rate of 10 $\mu\text{l/h}$, the lactate concentration difference between the first and last wells was about 33%, and the average lactate in Chip10% was over 10 times lower than in the static conditions of the 96WP. For a flow rate of 1000 $\mu\text{l/h}$, this difference decreased to 10%, and the average lactate concentration was 100 times lower than in the 96WP. At a flow rate of 50 $\mu\text{l/h}$ (Chip50%), the steady-state lactate concentrations were slightly higher than at 100 $\mu\text{l/h}$ (Chip100%), reaching up to 0.54×10^{-6} M. This is partly due to the microfluidic chip having a smaller microwell volume of 0.75

μl compared to the 150 μl volume of the 96WP. Importantly, while lactate levels in a standard 96WP change with each media swap, the continuous flow in the chip maintains a stable lactate concentration (and likely other metabolites and media supplements) throughout the cell culture period.

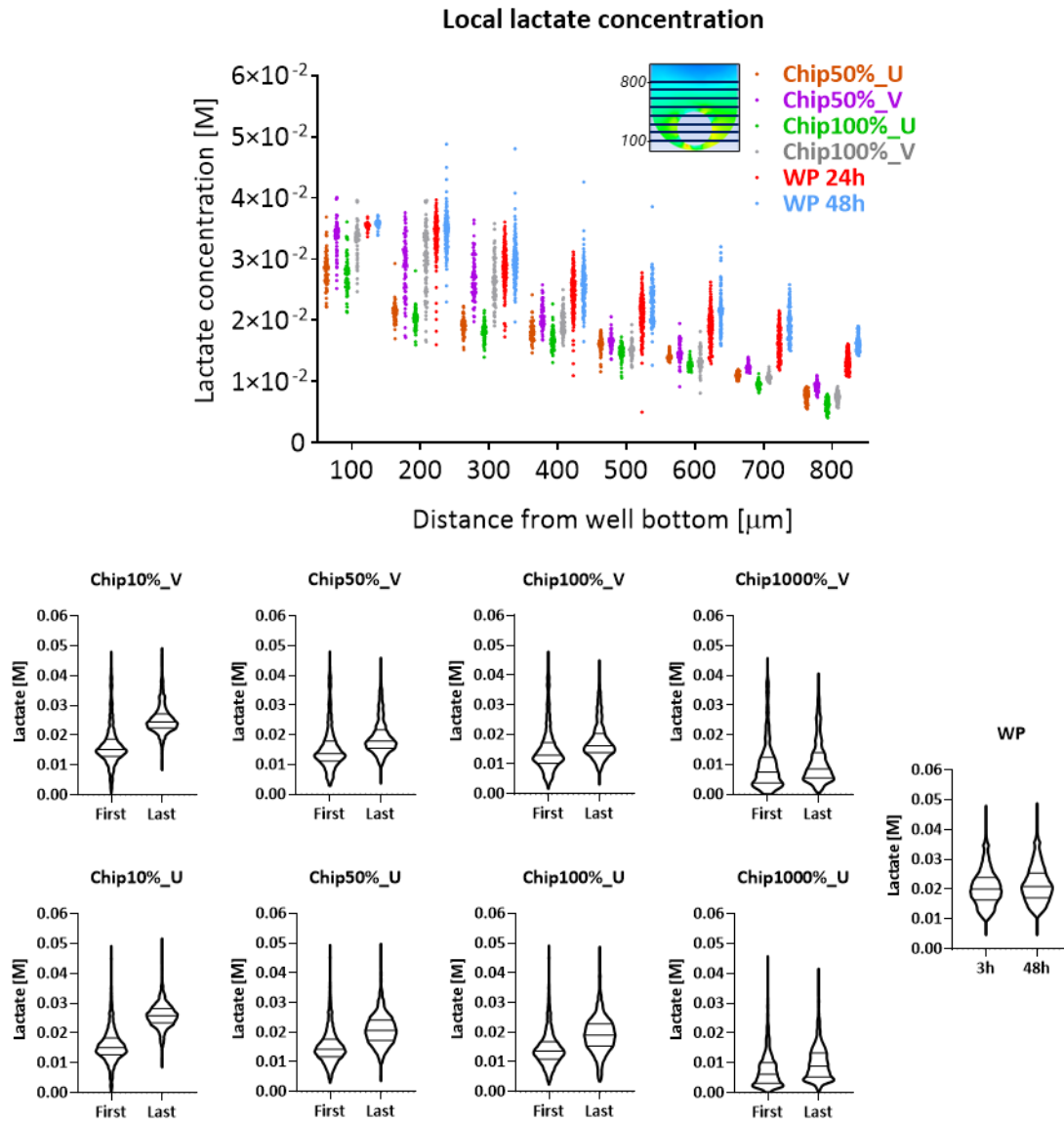


Fig. 41: Local lactate concentration in wells around the organoid

The shape of the microwells affects how lactate diffuses and its local concentration around cell aggregates. When comparing U-shaped and V-shaped microwells on the chip, or V-shaped microwells with V-shaped 96WP, the V-shaped microwells had about 50% higher lactate concentration between the bottom of the microwell and the centre of the aggregate (Fig. 41 and Fig. 42). This is because the aggregate settles

higher in the V-shaped microwells, limiting space for lactate to diffuse. Above 250 μm , which represents the upper half of the organoid, there was a noticeable increase in lactate diffusion into the flow channel in both chip conditions, but the lactate concentration remained higher in the 96WP. Despite this, the overall trend in lactate concentration changes was similar between the plate and chip. This similarity is due to the larger volume of the 96WP, which allows lactate to diffuse into it, while the smaller volume in the microwells is continuously refreshed through the flow channel. Overall, this simulation showed that our newly designed microfluidic chip can support the long-term growth of cell aggregates effectively.

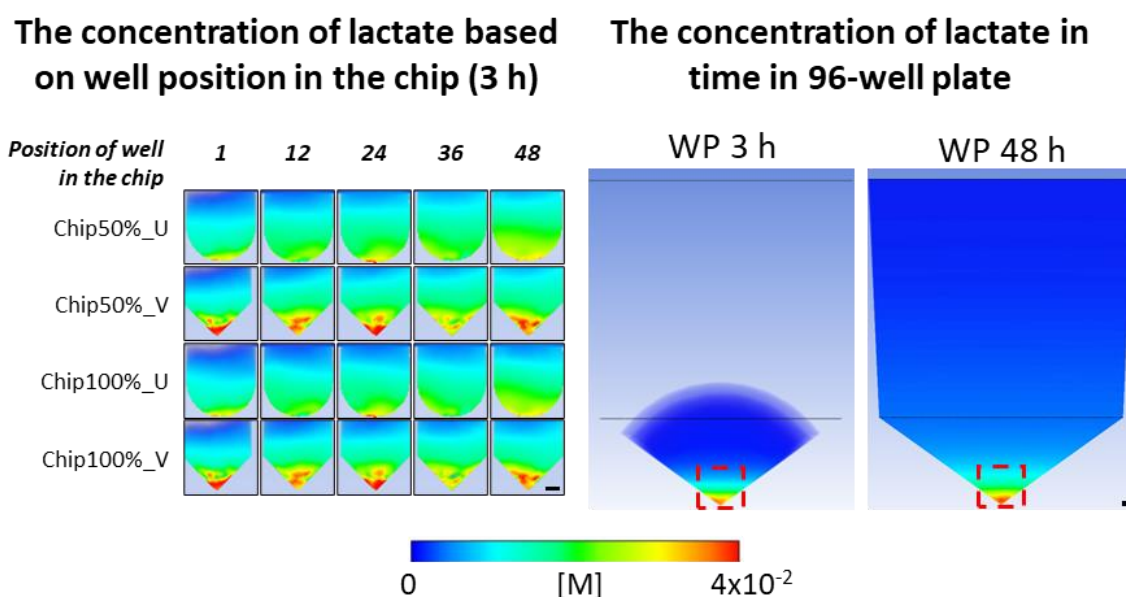


Fig. 42: Visualization of the lactate concentration in all cultivation systems

4.3.3 Shear stress simulation

Cultivating cells in a microfluidic chip with continuous fluid flow can lead to problems with shear stress. High flow rates might disturb the microwells and negatively impact the initial cell aggregation phase. For a specific maximum fluid flow rate of 100 $\mu\text{l/h}$, complex simulations were created to study how flow speed and well size affect spheroid surfaces. The simulations showed the impact of fluid flow on spheroids and the shear stress on their surfaces at 100% flow in a U-shaped well. The maximum calculated shear stress on the surface of the cell aggregate at a 100 $\mu\text{l/h}$ flow rate was $3.6 \times 10^{-6} \text{ N/m}^2$ (Fig. 43). This value is much lower than the harmful level of 1 N/m^2 , which is known to negatively affect cell survival^{126,127}.

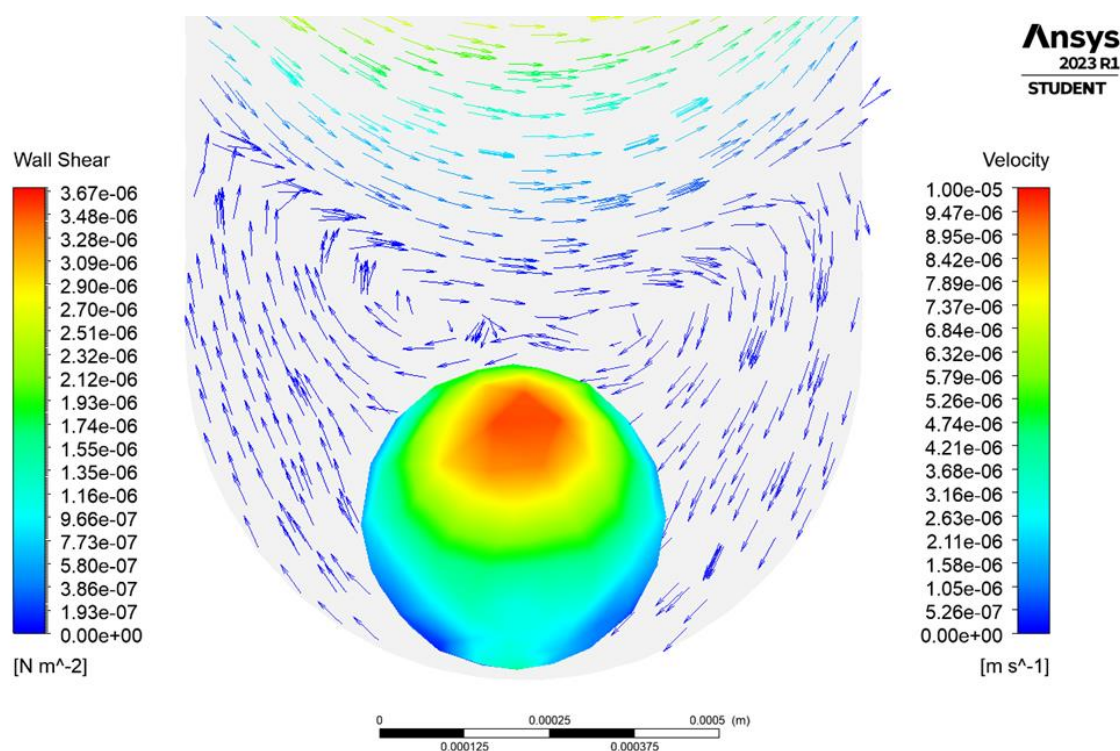


Fig. 43: Simulations of fluid flow inside wells and shear stress created on spheroid surface

4.4 Cerebral organoid cultivation

After finalizing the design of the microfluidic chip and conducting simulations to optimize its function, the device's performance was validated through extensive cell culture experiments. These experiments included initial setups to establish cultivation parameters, select appropriate cell lines, and conduct experiments with cerebral organoids. Detailed analyses were performed on organoid size, morphology, gene expression profiles, and immunohistochemistry, allowing for a reliable comparison between the chip and conventional 96WP. The optimization of cultivation parameters was carried out simultaneously with the optimization of chip fabrication and function, adjusting the parameters based on the specific cultivation needs.

4.4.1 Setting the medium flow for the cultivation of organoids

The standard cultivation protocol (Fig. 44) was based on Lancaster's original method. After cell seeding, aggregates were grown for 6 days in an expansion medium and then transferred to 48WP with a neural induction medium. On day 11, the organoids were moved to Geltrex drops and cultured in a petri dish. They were kept in a differentiation medium for cerebral organoids for 4 days, then switched to a differentiation medium with Vitamin A until day 50. Organoids were also differentiated in a microfluidic

system with U-shaped wells. The chip flow rate was calculated based on the medium usage from the standard protocol for transferring between 96WP and 48WP, as described in the methods. A flow rate of 100 $\mu\text{l/h}$ was used, and based on simulation data, a second chip was added with the flow rate decreased to half. After 11 days, the organoids were transferred to Geltrex droplets and cultivated using the same methods as before.

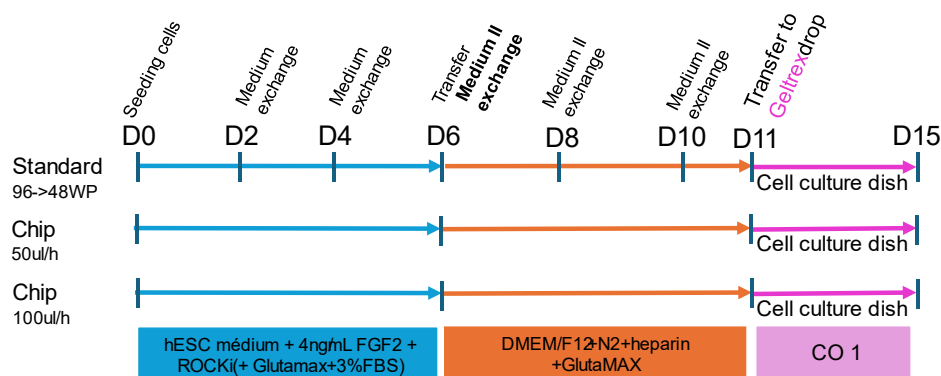


Fig. 44: Protocol for the cultivation of organoids with different flow rates

On day 2, spheroids are formed, with those in the WP being slightly larger in diameter than those in the chip, although their shapes are the same (Fig. 45). By day 6, after transferring spheroids from 96WP to 48WP, there was no difference in size or morphology between the WP and the chip, and this similarity continued until day 11. After transferring to Geltrex drops, similar morphology and shape of the organoids were observed by day 15. This confirmed that using half of the original flow rate is suitable for cultivating the organoids.

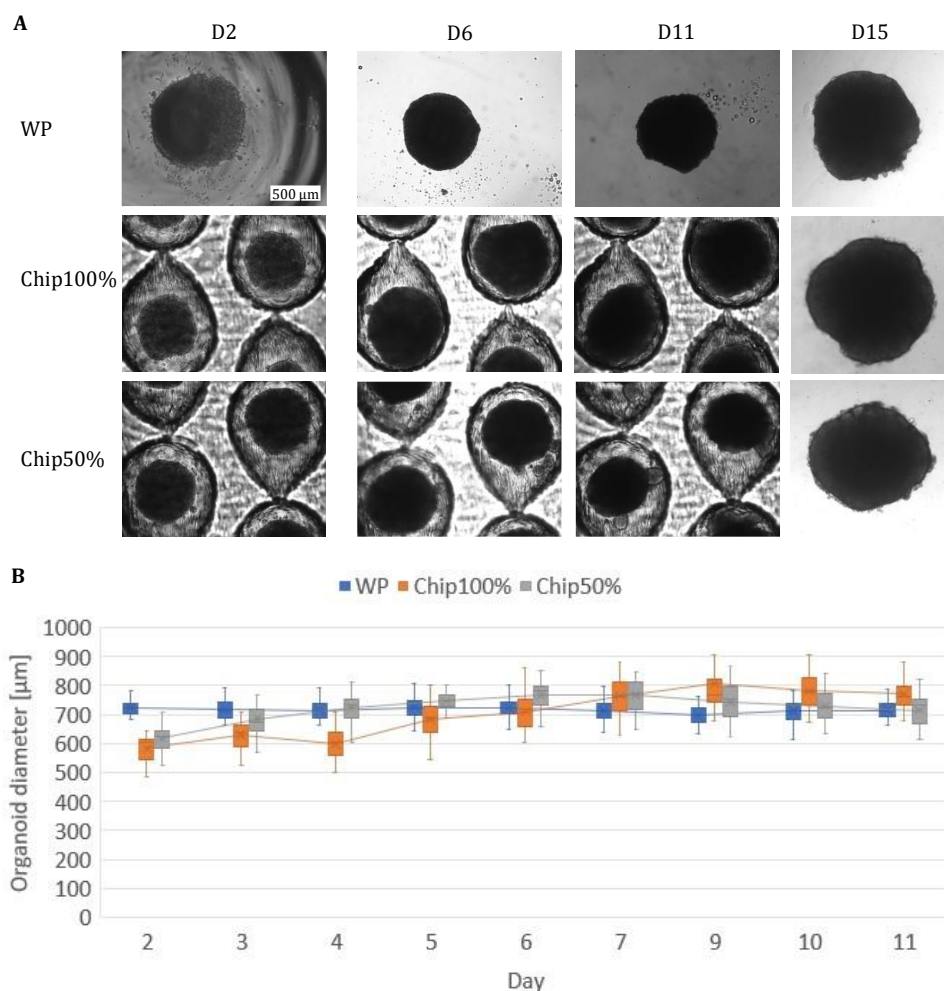


Fig. 45: Difference in size and morphology for different flow rate

4.4.2 Transformation of cultivation protocol

During the optimization process and cultivation following the standard Lancaster protocol, it was found that ROCKi is effective in the first 2 or 3 days, as it aids in cell clustering. Based on this information, an experiment was designed to compare two different protocols (Fig. 46). The first protocol involved switching to an expansion medium without ROCKi on day 2. The second protocol changed to a neural induction medium on day 2. Each protocol included three approaches: the standard protocol, the standard protocol without transferring to 48WP, and the chip method.

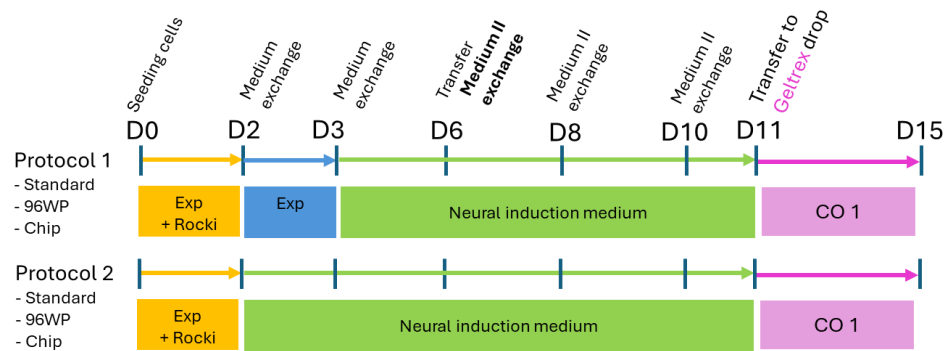


Fig. 46: Scheme of cultivation protocols

Initially, the cultivation was compared using the standard protocol with and without the 48WP. For the 48WP, a twice as large volume of media was used, but images showed no impact of the smaller volume in the 96WP on organoid diameter. However, it was noted that organoids in the WP had a larger diameter, which was later determined to be due to optical aberrance (described in chapter 4.2.3). There was no significant difference in shape or morphology (Fig. 47). After transferring the organoids into Geltrex, their sizes were comparable. When comparing the sizes between protocols, organoids cultivated using Protocol 1 were larger starting from day 10. By day 15, the organoids from Protocol 1 also exhibited a more wrinkled shape.

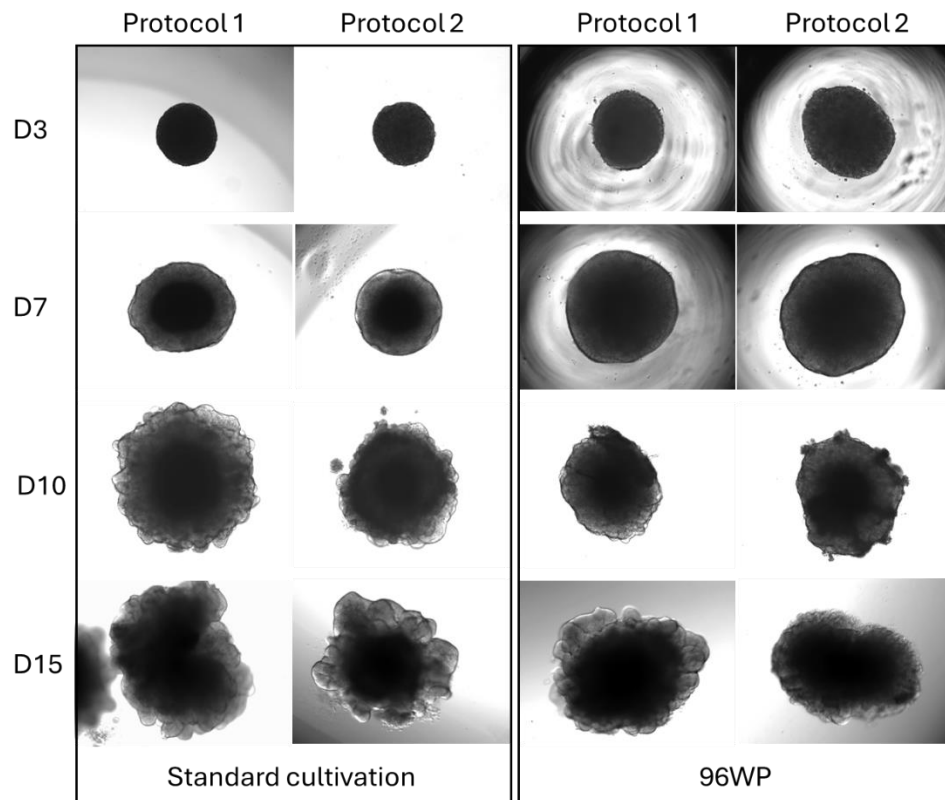


Fig. 47: Morphology of organoids by two different protocols and cultivation approaches

For the next steps, Protocol 1 was chosen to cultivate organoids in the chip, which was then compared with standard cultivation (Fig. 48). Both U-shaped and V-shaped wells were used in this experiment. Organoids in the chip were smaller from the beginning due to unoptimized seeding densities. The morphology in U-shaped wells was similar to that of standard cultivation, but in V-shaped wells, wrinkling was observed by day 7, likely due to the shape of the well. After transferring to Geltrex, the morphology was similar to that of standard cultivation. Protocol 1 and cultivation in 96WP were chosen for organoid cultivation because the results were comparable to standard methods, and handling the organoids was easier.

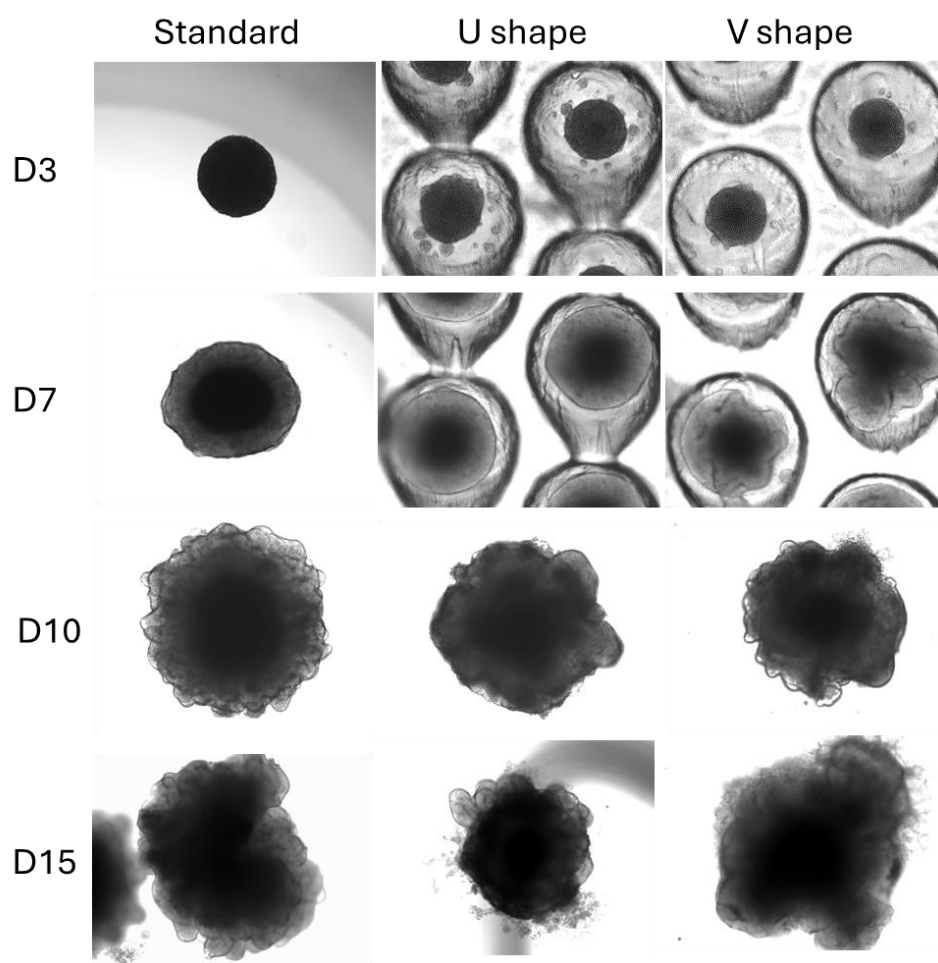


Fig. 48: Cultivation in chip according to Protocol 1

4.4.3 Selection of cell lines

For the successful cultivation and differentiation of cerebral organoids, it is essential to have not only a functional protocol and effective cultivation methodology but also a suitable cell line that can differentiate into neural tissue. For our microfluidic technology, finding a cell line that could adapt to an environment different from 96WP was crucial. Factors like material, microenvironment, and continuous flow significantly influenced the living cells. During the optimization process of cultivation protocols and microfluidic chip setup, nine pluripotent cell lines were tested (Fig. 49). All cell lines are detailed in the methods section (chapter 3.5.1). To demonstrate the functionality of the microfluidic system, we aimed to test one cell line from human embryonic stem cells (hESCs) and one from induced pluripotent stem cells (iPSCs). There were two main challenges. The first was the failure of initial clustering, as cell lines WTF80, M8, and NEO1 were unable to form aggregates upon initial cell seeding to the chip. The second challenge was the inability to differentiate; cell lines CCTL14, H9, and AM13 formed aggregates but started to disintegrate after being transferred to Geltrex around day 20. The cell line M67 did not fully differentiate into cerebral organoids. The best results were observed with the hES cell line H1 and the iPS cell line BWT3. Therefore, these two cell lines were chosen for cerebral organoid differentiation using microfluidic chips.

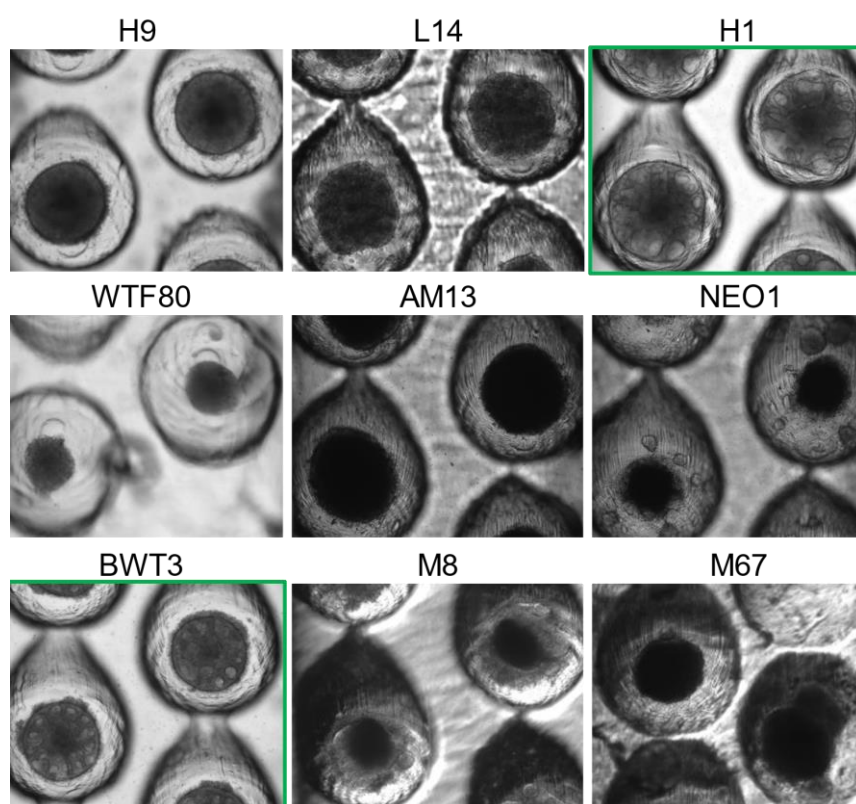


Fig. 49: Cell lines tested for cultivation and differentiation

4.4.4 Cerebral organoids cultivated by microfluidic chip

After finalizing the microfluidic chip design, conducting *in silico* simulations and cultivation optimizations, comprehensive cell culture experiments were performed to validate the device's performance. These experiments included detailed analyses of organoid size, morphology, gene expression profiles, and immunohistochemistry, which provided a reliable comparison of the chip and the conventional WP method. In all subsequent experiments, two human pluripotent stem cell lines, H1 and BWT3, were seeded, with each line having two biological replicates (marked as Experiment 1 - "E1" and Experiment 2 - "E2"). The initial seeding concentration was 2000 cells per well. Importantly, compared to the standard WP format that involves regular media changes every 24–48 hours (indicated by a "drop" sign in Fig. 50), our designed chip automates the culture procedure during the initial 9–11 days, eliminating the need for direct pipetting of fresh media onto the organoids during manual medium changes (Fig. 50). This automation is thus not only less labor-intensive but also reduces the risk of organoid damage and contamination. Additionally, to demonstrate that using a microfluidic chip can reduce the amount of media required for cultivation, an experimental condition with half the flow rate was implemented.

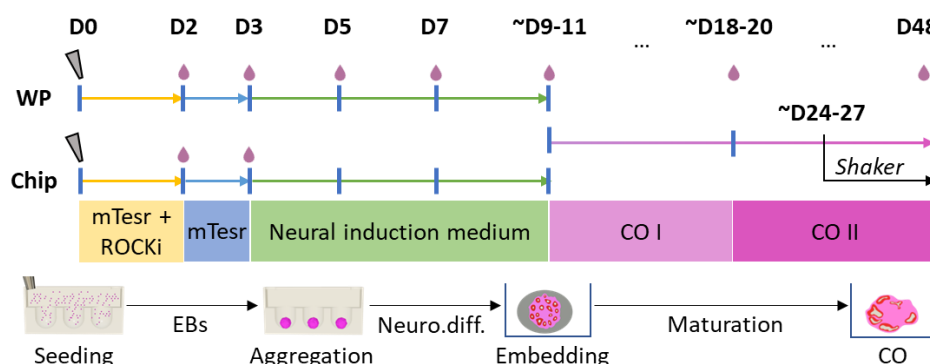


Fig. 50: Functional cultivation protocol for cerebral organoids differentiation

Upon initiation of differentiation, we first compared the size and morphology of the cell aggregates over a period of 10 days (± 1 day based on the size of the organoids). Brightfield images confirmed the proper formation and growth of the aggregates in both the microfluidic chip and WP. On day 3, we observed the formation of homogeneous aggregates with consistent sizes in both V-shaped and U-shaped microwells (Fig. 51), regardless of their relative position in the microfluidic chip or the flow rate.

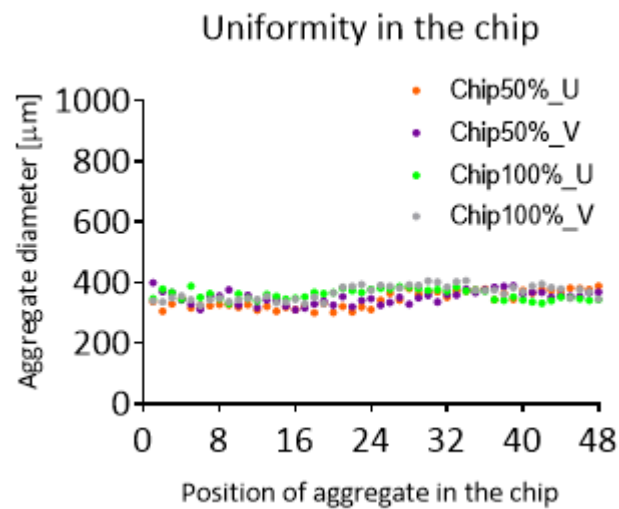


Fig. 51: Uniformity of aggregates in chip

Neural induction was observed between days 3 and 5, characterized by bright and smooth edges, followed by the formation of distinct bright layers with luminal-like structures forming within the cell aggregates between days 6 and 9 (Fig. 52).

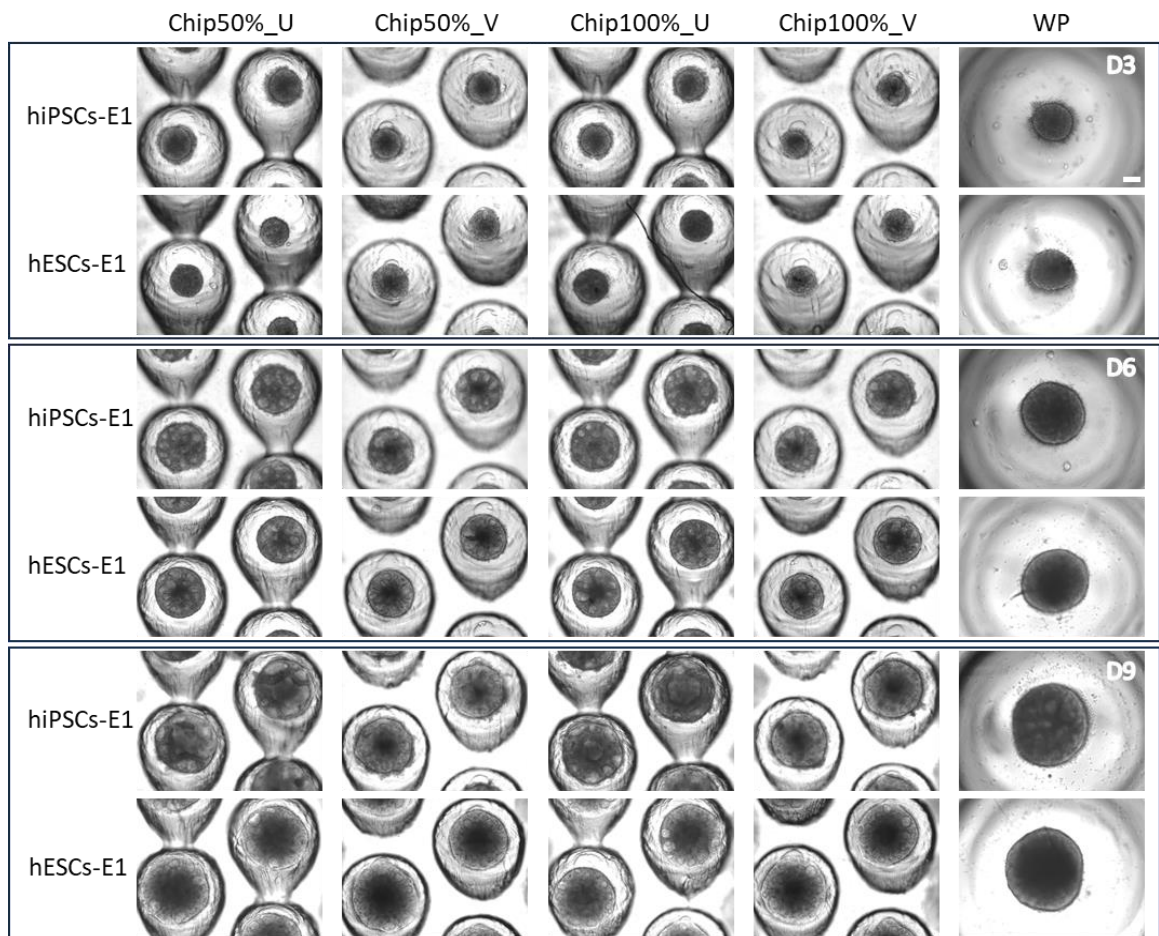


Fig. 52: Morphology of cerebral organoids in microfluidic chip

When the size of the aggregates was measured, they achieved similar dimensions during the neural induction phase in all experimental conditions, even when the flow rate in the microfluidic devices was reduced by 50% (Fig. 52). The only minor differences were observed between V-shaped and U-shaped wells, with aggregates cultured in V-shaped wells being slightly smaller in three out of four biological replicates, likely due to denser cellular packing. Subsequently, after 9–11 days of *in vitro* culture, the differentiating aggregates were removed from their respective cell culture vessels (chip or WP) and embedded into Geltrex droplets. From this point onwards, we determined increased variation in the size of individual organoids, both in the microfluidic chips and WP (Fig. 53). Importantly, we noticed no major differences between aggregates grown in the chip and those grown under the standard WP format during the 48-day period and we thus concluded that the newly designed chip supports the growth of hPSC-derived aggregates when assessed by the standard morphological criteria.

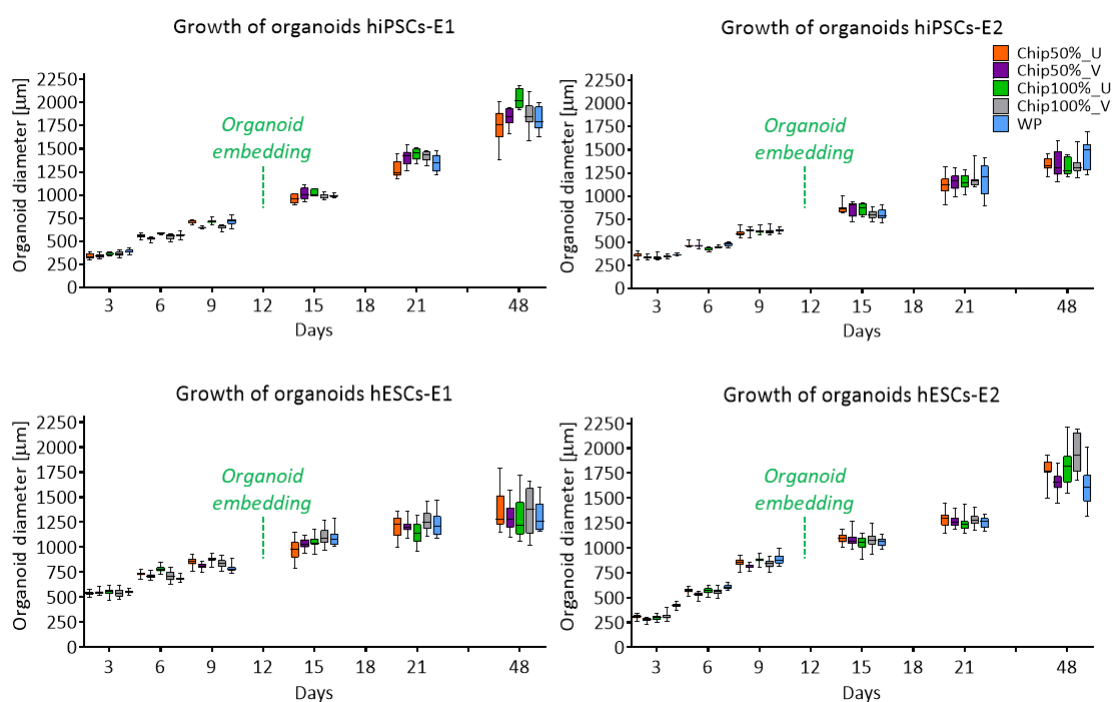


Fig. 53: Size of aggregates for each cell line experiments

To further complement the standard morphological criteria evaluated in 9-day-old cerebral organoids grown in chip and WP, we aimed to evaluate the extent of early-stage neural differentiation by the assessment of the commonly used neurodifferentiation-related markers, including PAX6 and N-cadherin/CDH2 as well as the expression of SOX1 and SOX2 genes. Experimental data from immunohistochemistry (IHC) analysis show that all tested conditions within the chip and WP adequately support the early onset of differentiation (Fig. 54), and PAX6 expression and N-cadherin expression were similar in all tested conditions.

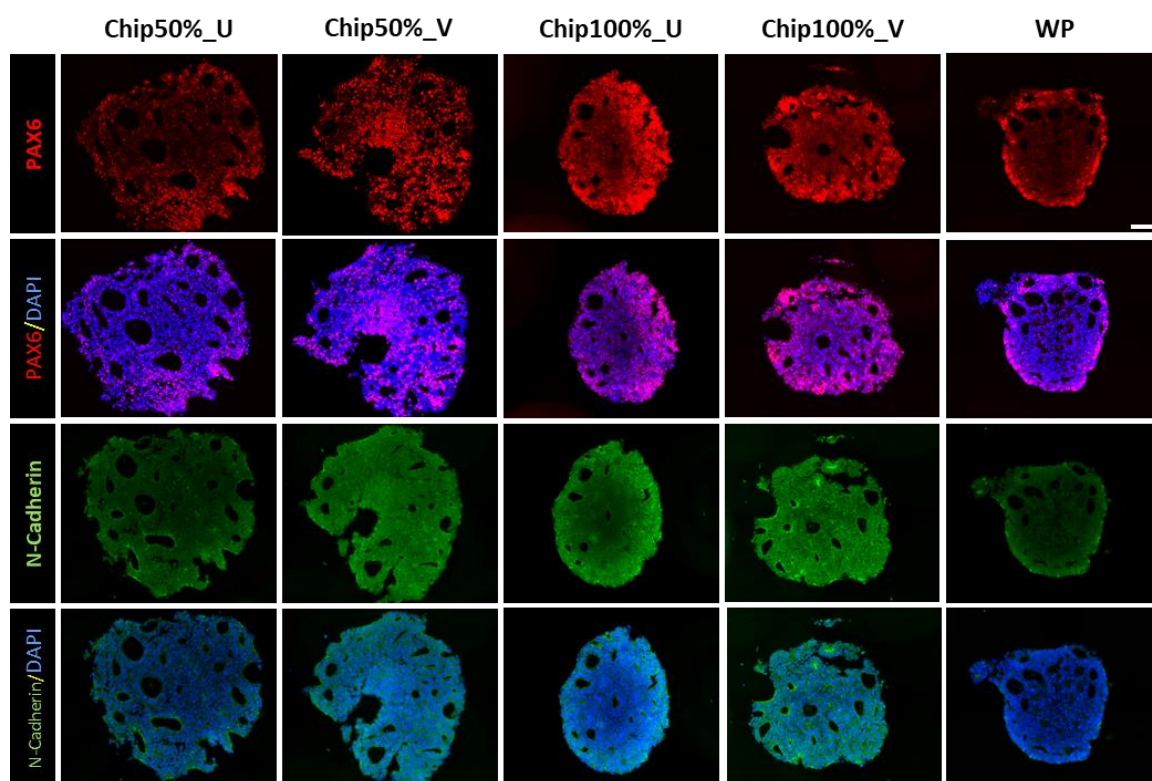


Fig. 54: Immunofluorescence analysis of early-stage neural differentiation

Gene expression analysis by qPCR revealed significant variability in the expression levels of the *PAX6*, *CDH2*, *SOX1*, and *SOX2* genes across both individual batches of cell lines and experimental conditions, including variations between the microfluidic chip and WP settings. Notably, the expression of the *SOX2* gene remained consistently stable across all experimental conditions, indicating the successful initiation of early neural differentiation in the developing cerebral organoids across all tested samples (Fig. 55).

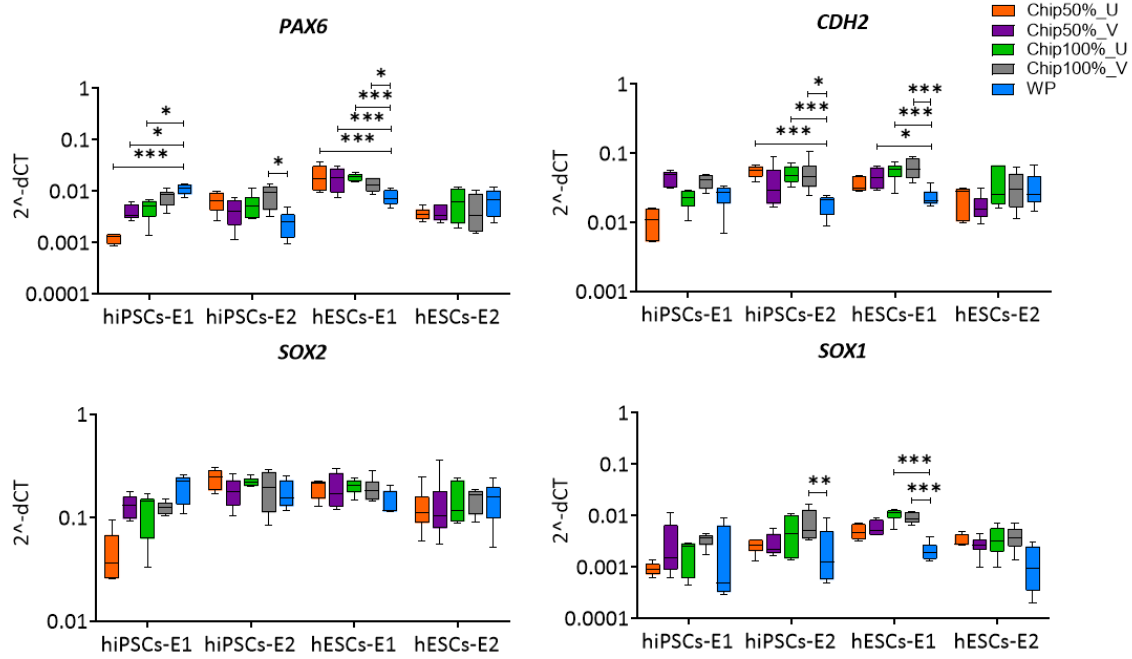


Fig. 55: qPCR of early-stage neural differentiation

To further assess other biological parameters in the developing 9-day-old cerebral organoids, we first examined the expression of the proliferation marker Ki-67 and apoptotic marker cleaved caspase 3 (cCasp3). Ki-67 was widely expressed in all organoids, with the expression intensity decreasing towards the organoid center (Fig. 56). cCasp3-positive cells showed a low expression pattern with comparable levels between all tested conditions (Fig. 56). Although no significant differences were found between culture conditions, our analysis of the percentage of cCasp3-positive cells revealed lower values in Chip100%_U and Chip100%_V with less variability than in the other cases. Overall, the percentage of apoptotic cells in Chip conditions was found to be similar or lower than the percentage observed in WP conditions, as shown in Fig. 56.

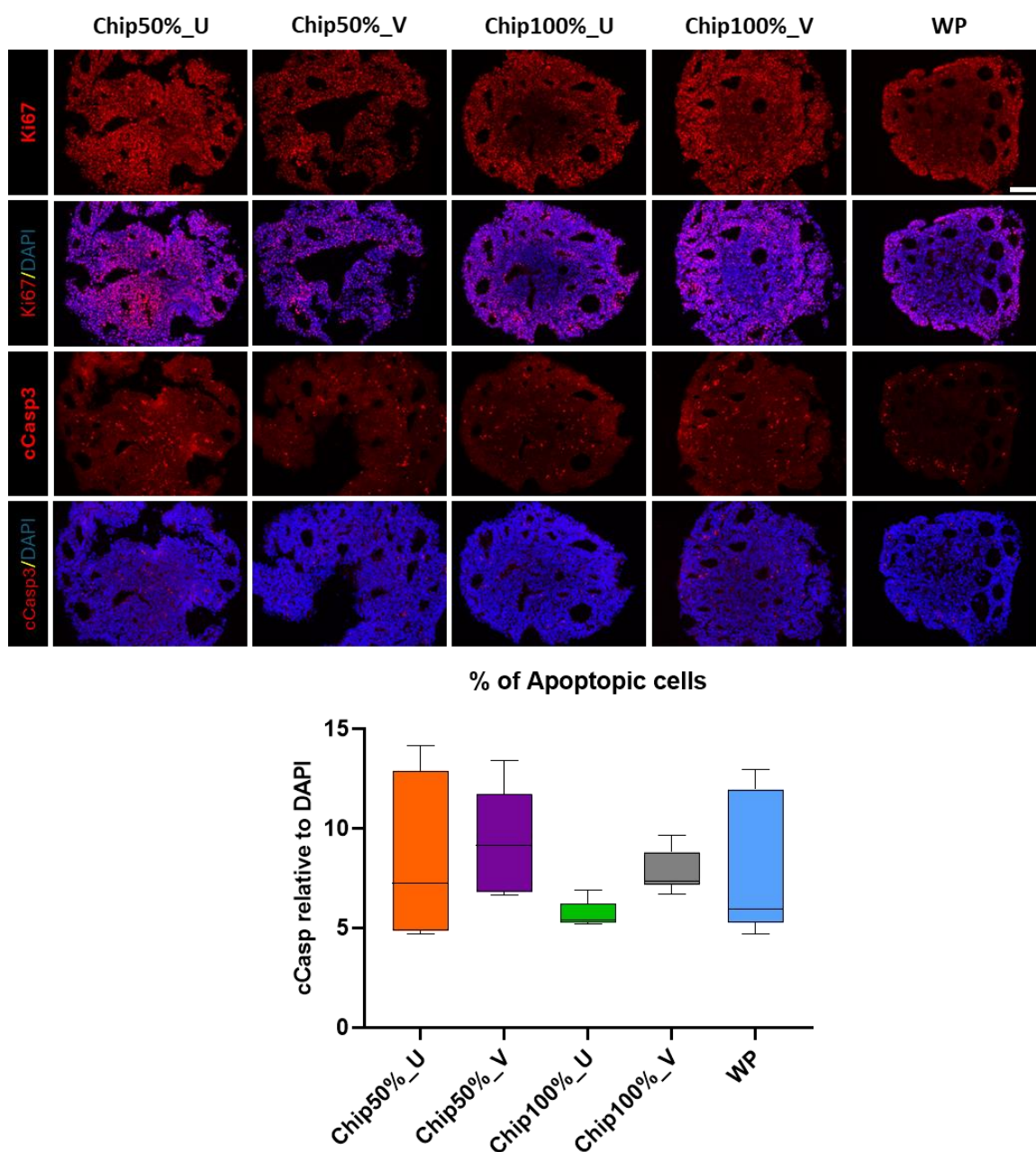


Fig. 56: Immunofluorescence analysis of proliferation and apoptosis

Additionally, considering the nutrient supply and oxidative stress in static (WP) and dynamic (Chip) organoid culture environments, we analysed the expression of two markers, glucose transporter 1 (GLUT1) and 8-hydroxyguanine (8-OHG). Glucose transport is vital for cellular energy production and various cellular processes, with impaired glucose transport resulting in decreased energy production and altered cellular behavior. Our results from IHC analysis show that GLUT1 expression was comparably low in organoids produced in chip with 100% and 50% flow irrespectively of U-

or V-shaped wells (Fig. 57), indicating favorable glucose metabolism in all tested conditions. Similarly, the expression of 8-OHG, a biomarker of oxidative DNA and RNA damage, was comparably low in chips with 100% and 50% flow rates irrespectively of U- or V-shaped microwells (Fig. 57). The examined biological parameters across various chip conditions thus consistently confirmed the successful initial development of organoids within the chip without any hindered cell growth, significant oxidative DNA damage, or impaired glucose transportation.

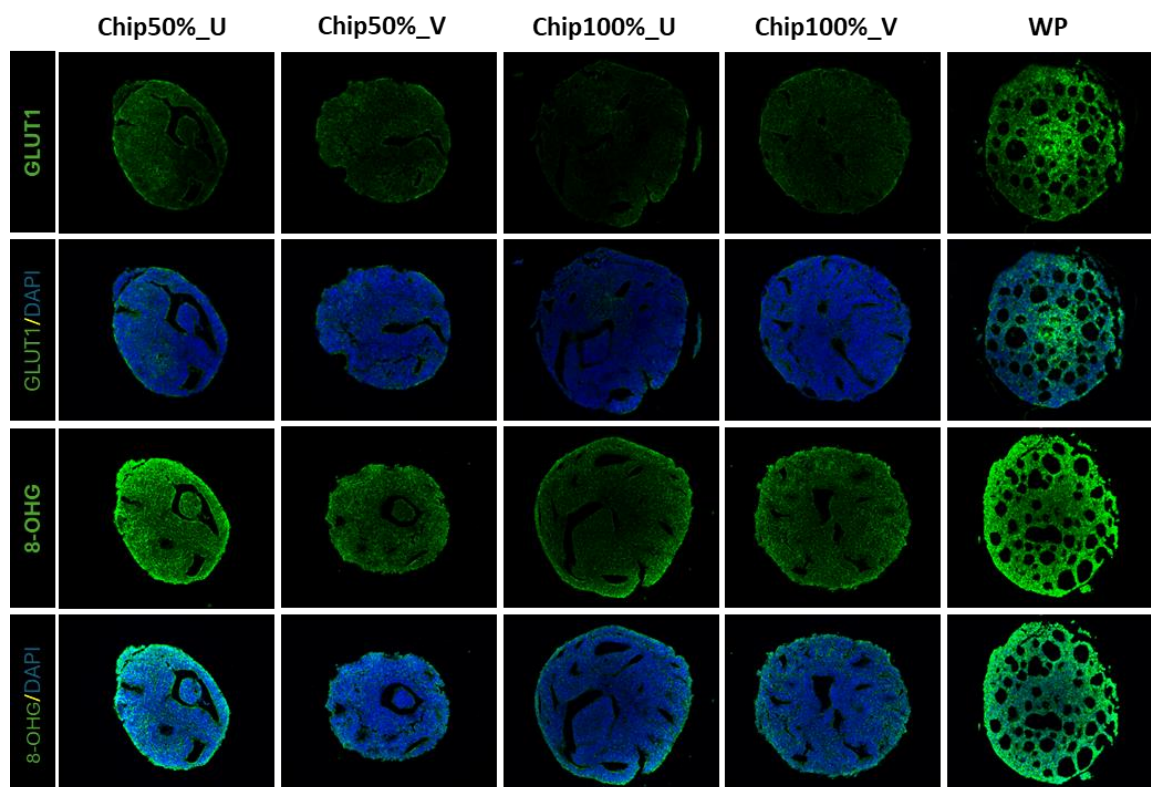


Fig. 57: Immunofluorescence analysis of oxidative stress and nutrient supply

To this point, we analyzed the organoids cultured until day 10 (± 1) inside the closed microfluidic chip. After this period, and reaching the diameter of $\sim 1000 \mu\text{m}$, organoids were, according to the original protocol, embedded in a surrogate extracellular matrix (e.g. Geltrex). This embedding promotes further neuroepithelial expansion, and if neural identity is properly accomplished, organoids generate large neural tube-like buds on the edges that also appear bright⁸¹. Experimental data (Fig. 58) shows that, our 14-day-old organoids also developed a wrinkled outer layer of neuroectoderm with an orderly arrangement, which appeared more translucent in bright-field microscopy.

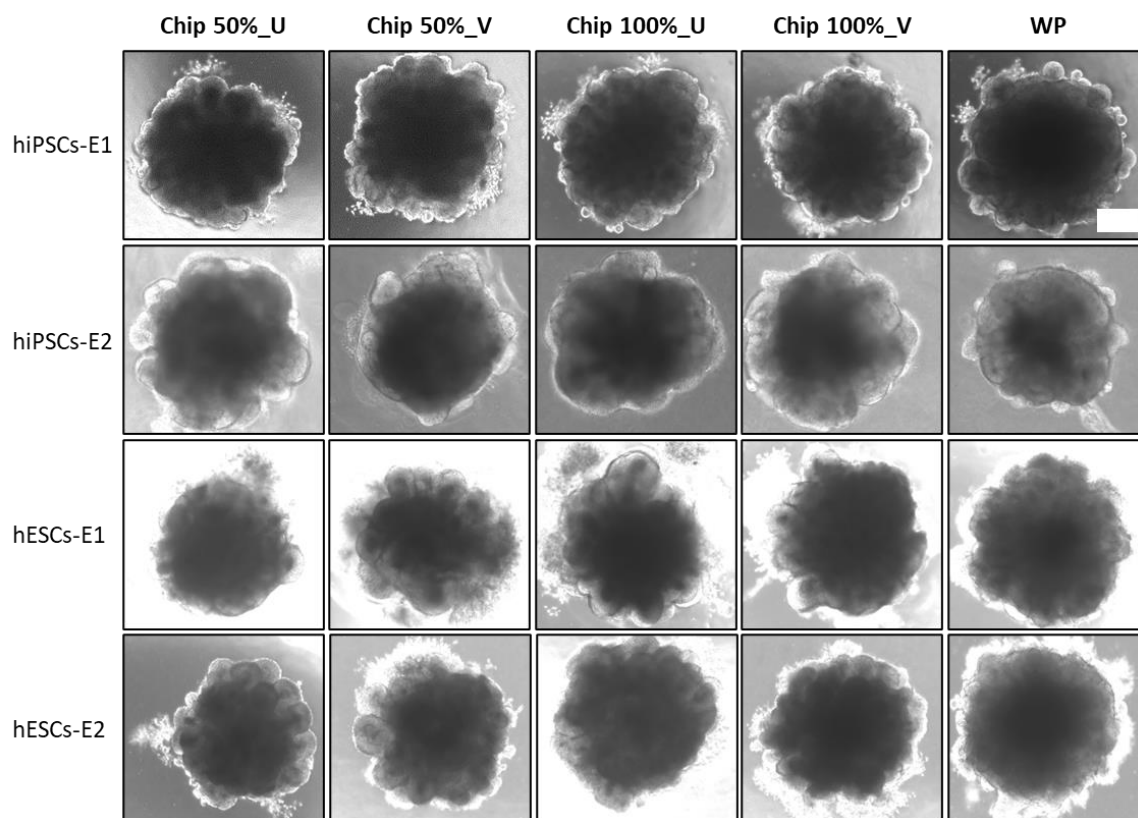


Fig. 58: Brightfield microscopy analysis of cerebral organoids

To further examine the histological composition of the developing organoids, we performed hematoxylin and eosin staining. All analyzed organoids exhibited increased complexity, forming neural rosettes and ventricle-like lumens (Fig. 59). A subsequent IHC analysis demonstrated well-developed ventricle-like cavities, with N-cadherin localized at the apical side of these structures. No substantial differences were noticed between the four chip conditions, suggesting that all tested conditions supported the neural differentiation to a similar extent (Fig. 59).

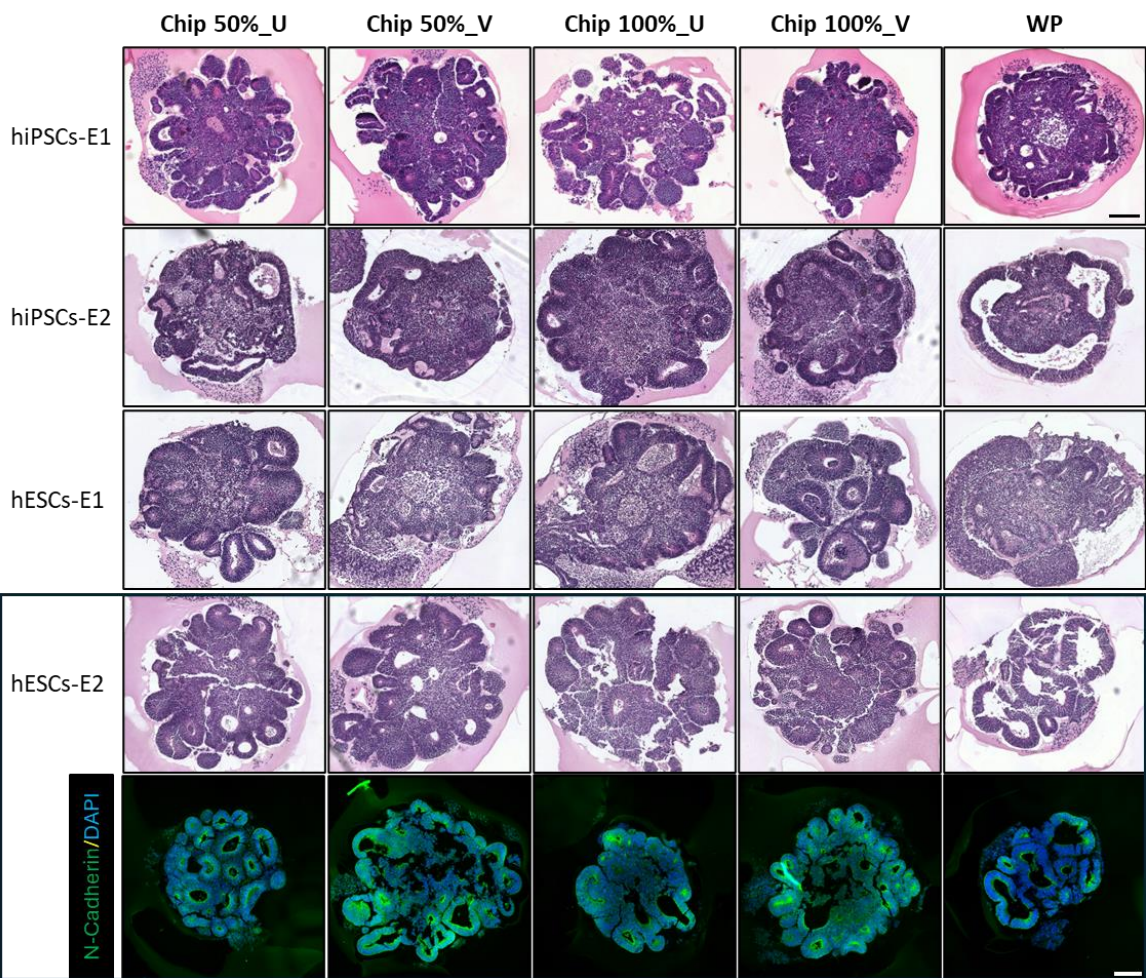


Fig. 59: Histological composition and Immunofluorescence analysis of ventricle-like structures

Interestingly, the analysis revealed that organoids cultured in the microfluidic device exhibited a higher abundance of smaller rosettes compared to those in the WP format. Specifically, the average size of rosettes in organoids cultured in the microfluidic chip ranged from 1000 to 3000 μm^2 , with rosette counts varying from 10 to 17. In contrast, the WP exhibits fewer rosettes, ranging from 5 to 10, with sizes ranging from 1000 to 8000 μm^2 (Fig. 60).

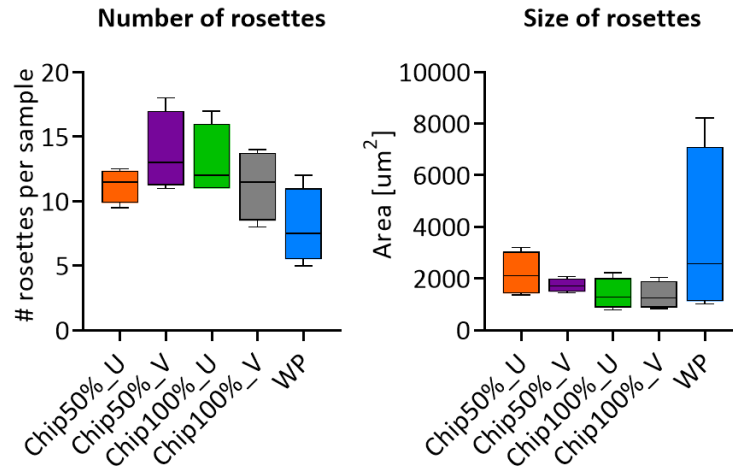


Fig. 60: Statistical analysis of rosettes

To further examine the extent of neural differentiation of organoids taken out of the chip and WP and embedded into Geltrex droplets, we aimed to quantify two mentioned morphological features that are consistent with developing neuroepithelium *in vitro* – the appearance of bright edges and the development of neuroepithelial buds. The brightness of organoid edges was analysed as the proportional brightness of organoids by measuring intensity in four crossing lines¹²⁸ as schematized in Fig. 61.

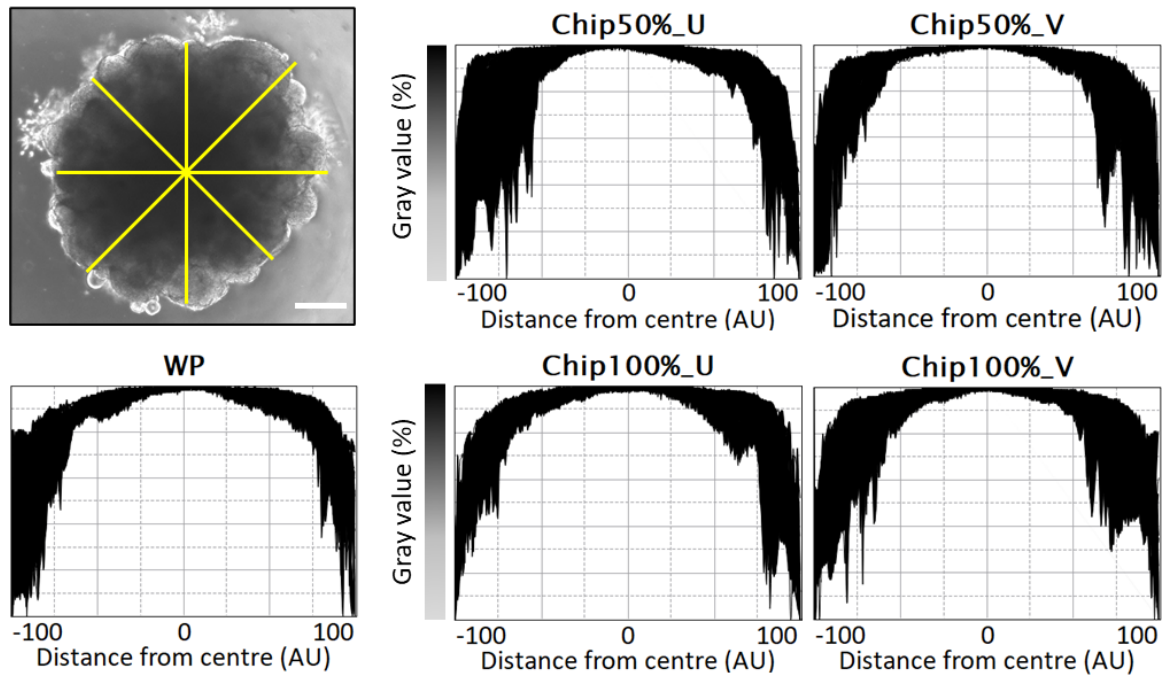


Fig. 61: Brightness of organoid edges

The wrinkling of organoids (i.e., the appearance of neuroepithelial buds) was quantified using the wrinkling index as previously described by Karzbrun et al.¹²⁹ and calculated as the ratio of the contour length (L_G) of the organoid to the outer convex length (L_F) (Fig. 62).

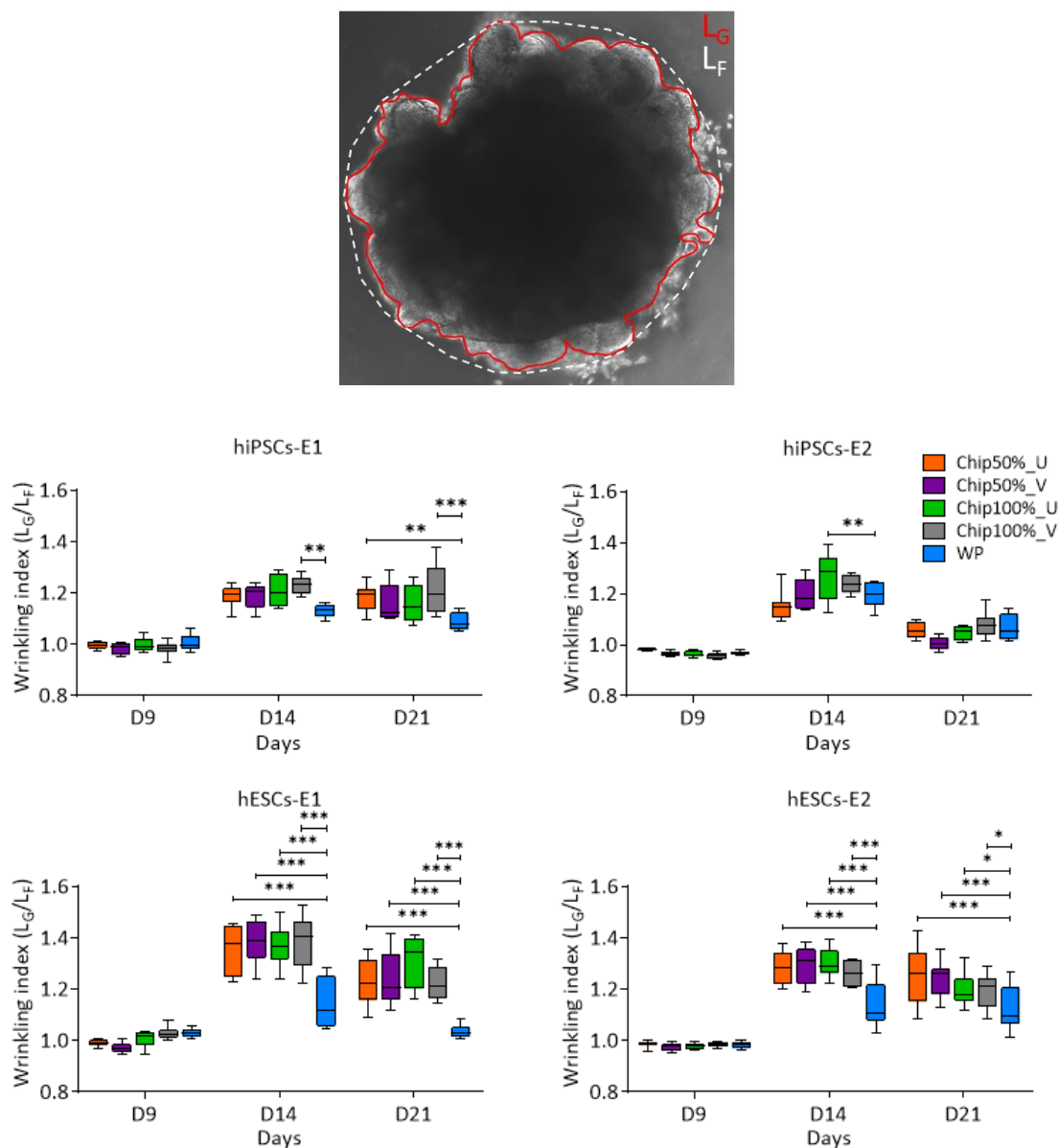


Fig. 62: Organoids wrinkling analysis

Both analyses confirmed that organoids cultured in the chip did not significantly differ between the four tested chip conditions in either of the tested parameters, confirming ongoing neural differentiation *in vitro* (Fig. 61, Fig. 62). However, we noticed several differences between organoids grown on a chip and in a standard WP. Specifically, the growth on the chip seemed to promote the formation of a larger bright area

on their edges compared to those cultured in the WP, although the quantification was not statistically significant (data not shown). Importantly, the analysis of the growth of neuroepithelial buds showed that at day 14 (4 days after embedding to Geltrex), organoids grown in chip had consistently higher wrinkling index than those grown in WP, with the differences being significant in more chip conditions with ongoing cell culture (i.e., at day 21, 11 days after embedding to Geltrex, Fig. 62). The reason behind these observations remains to be defined.

Lastly, we aimed to assess the impact of various chip parameters on the development of mature cerebral organoids and cortical neurogenesis. The evaluation focused on multiple aspects: i) overall organoid morphology, size, and patterning; ii) expression of neurodifferentiation-related markers; and iii) thickness of MAP2/NeuN positive neuronal layers in 55-day-old organoids. The results demonstrated that cerebral organoids cultured in the four different chip conditions, as well as in the standard 96WP, exhibited comparable outcomes within each batch. Specifically, when analyzing the tissue morphology and size, organoids from all tested conditions within each batch displayed similar sizes regardless of the initial cell culture conditions (Fig. 63).

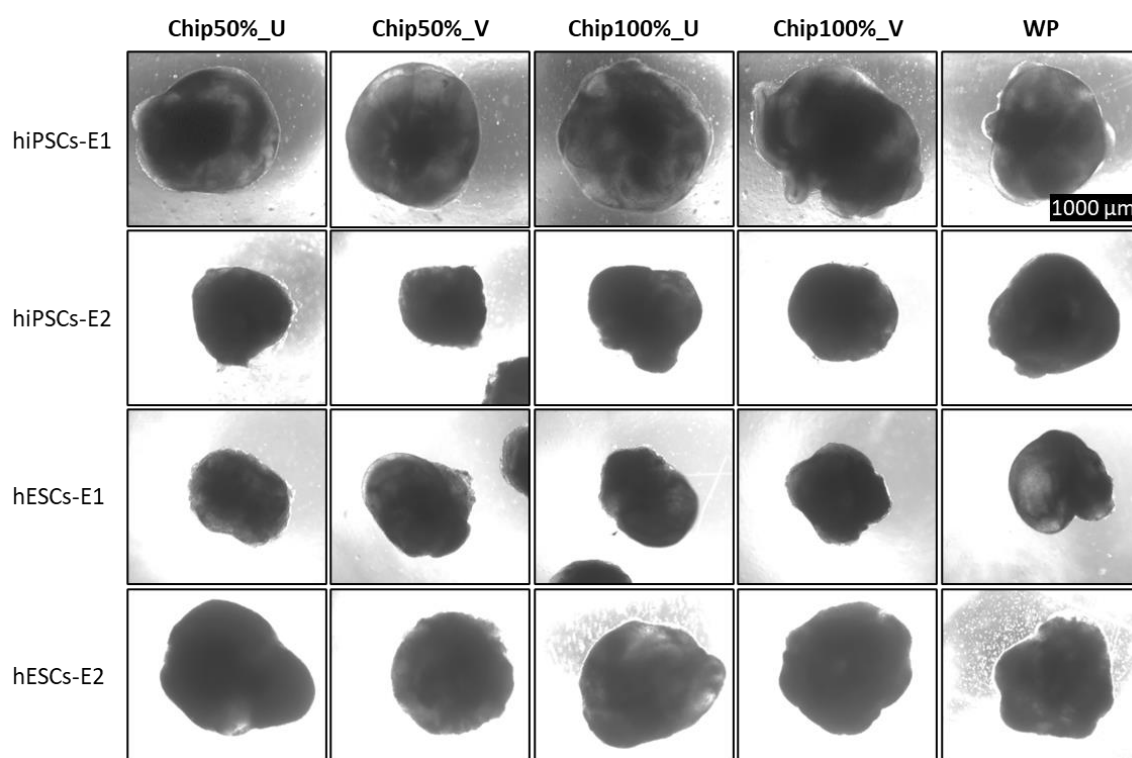


Fig. 63: Brightfield microscopy analysis of mature organoids

Patterning of cerebral organoids was evaluated by DAPI staining in representative images from the experiment with hiPSCs-E1. We also observed presence of PAX6/TUJ-positive luminal ventricle-like formations and neural rosettes (Fig. 64).

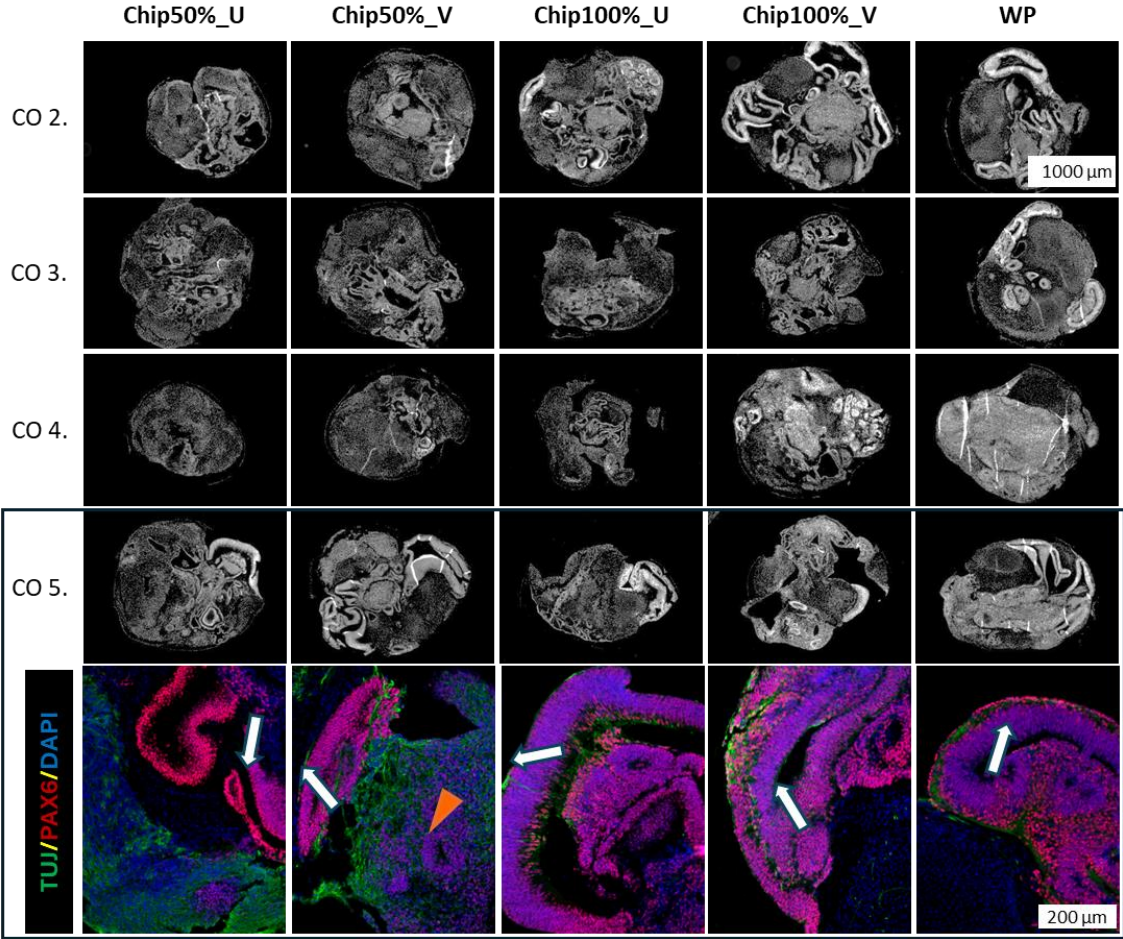


Fig. 64: Morphology and patterning of cerebral organoids

Additionally, the expression levels of selected neurodifferentiation markers were assessed using qPCR. Specifically, we analyzed the expression of early neuronal markers (*PAX6*, *SOX2*), immature neuronal markers (*DCX*, *TUBB3/TUJ*), and mature neuronal markers (*MAP2*, *RBFOX3/NeuN*). Variability in gene expression was observed between batches. However, within each batch, there was no trend in the expression of the analyzed markers among the tested conditions; therefore, significant differences were not detected for these analyzed biological replicates by qPCR (Fig. 65).

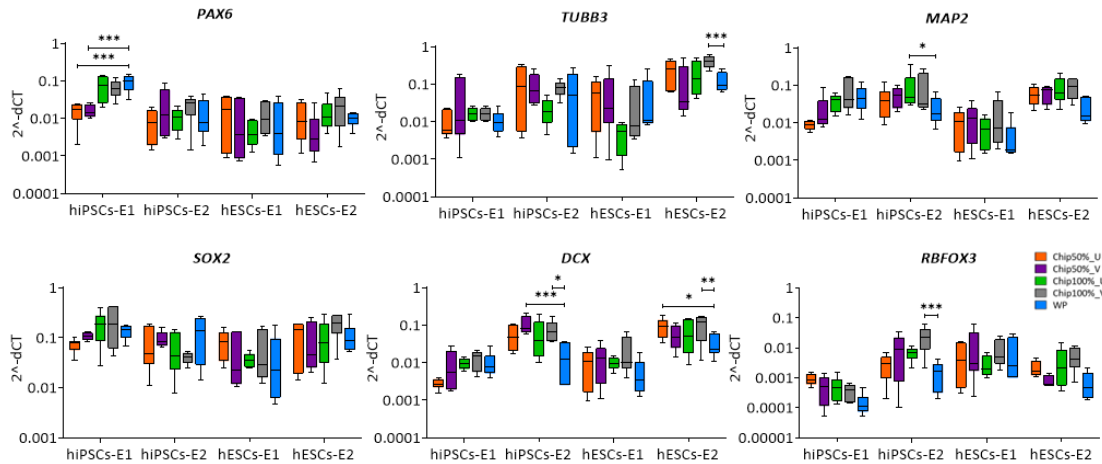


Fig. 65: The gene expression analysis of cerebral organoids at day 55 by qPCR

Finally, to evaluate organoid maturation, we examined the expression of the neuron-specific cytoskeletal protein MAP2 and the nuclear protein NeuN and measured the thickness of MAP2/NeuN-positive layers. The results showed that both MAP2- and NeuN-positive neurons formed similarly thick layers, and there were no significant differences between organoids cultured using different approaches (Fig. 66). Overall, these findings suggest that the tested chip conditions had comparable effects on the development of mature cerebral organoids and cortical neurogenesis when compared with each other and also to the standard WP method.

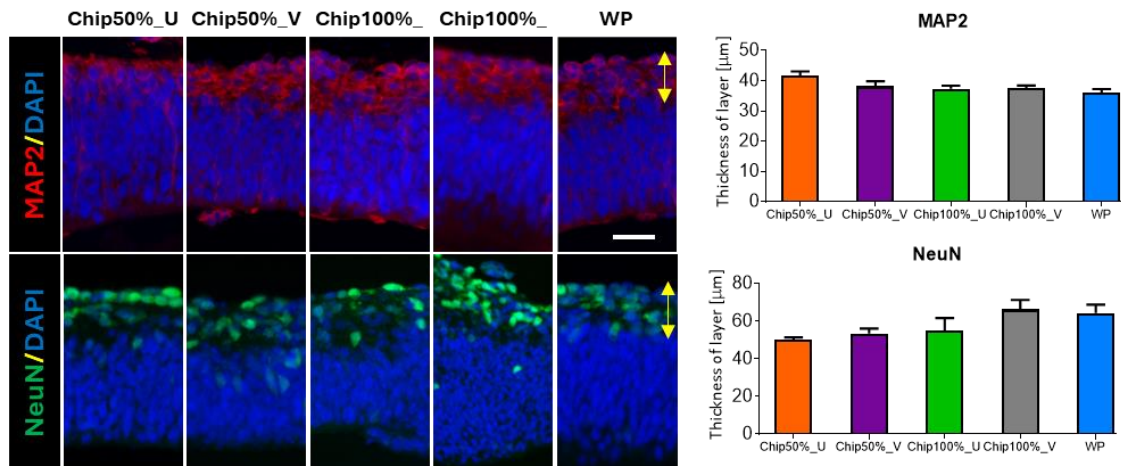


Fig. 66: The thickness of the layer for MAP2 and NeuN-positive cells

4.5 Retinal organoids cultivation

The initial objective was to design a microfluidic chip as a versatile platform for cultivating diverse cell structures and organoids. Therefore, after successfully optimizing the microfluidic system and cultivating cerebral organoids, retinal organoids were also cultivated in the chip as a proof-of-concept experiment and compared with the standard protocol. The cultivation process was designed according to the standard retinal differentiation protocol (Fig. 67) as published before¹³⁰.

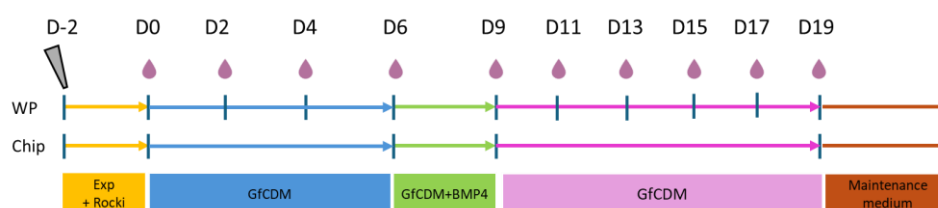


Fig. 67: Retinal organoids cultivation process

After four days of cultivation (day 2), organoids were observed to have similar sizes and spherical shapes (Fig. 68). In U-shaped wells, there were few cells around the aggregates, even after centrifugation. In the WP, where no centrifugation was used, microscopy revealed the presence of multiple aggregates in each well. After BMP4 treatment on day 8, the number of cells around the organoids increased (Fig. 68). The highest number of cells was found around the organoids in the Chip50%_V condition. The retinal organoids exhibited a faint, bright outer rim, indicating developing neuroepithelium¹³¹. Around day 12, the brighter regions of the retinal domains grew, and the organoids became less uniform in shape and size. In V-shaped wells, a stronger neuroepithelial rim was observed on one side of the organoids, while in U-shaped wells, the organoids grew in multiple directions, resulting in a more heterogeneous population.

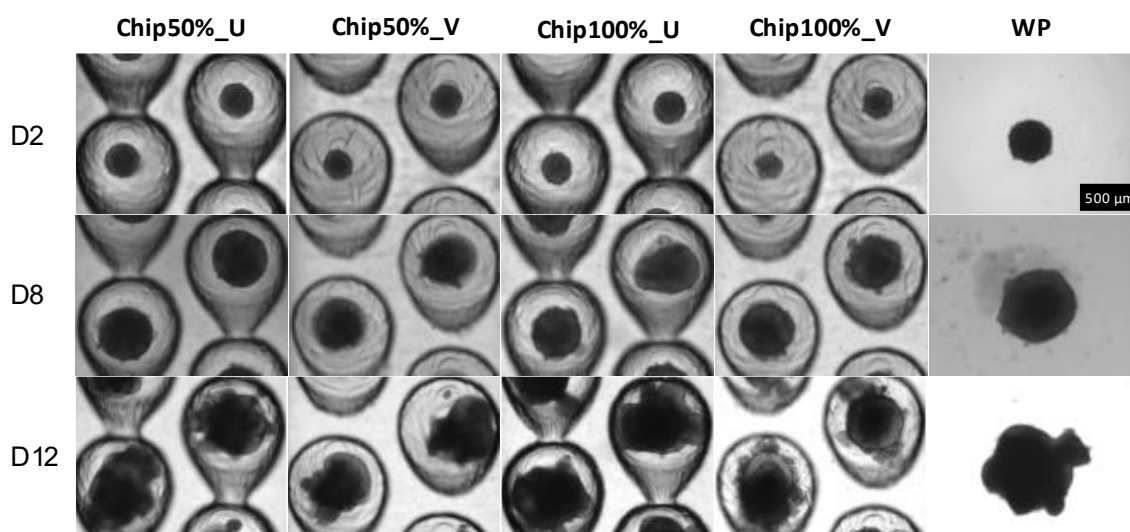


Fig. 68: Morphology of retinal organoids in a microfluidic chip

On day 21, early-stage retinal development was analyzed by examining the markers PAX6 and VSX2. PAX6, an early retinal marker, was expressed in both the chip and WP approaches (Fig. 69). In the Chip50%_V condition, the expression was weak and localized to single cells. In contrast, the Chip100% and Chip50%_U conditions showed comparable expression levels to those in the WP. VSX2, a marker for retinal progenitor cells, was only present in Chip100% and the WP (Fig. 70). A low signal was detected in the Chip50% condition, which did not confirm VSX2 expression.

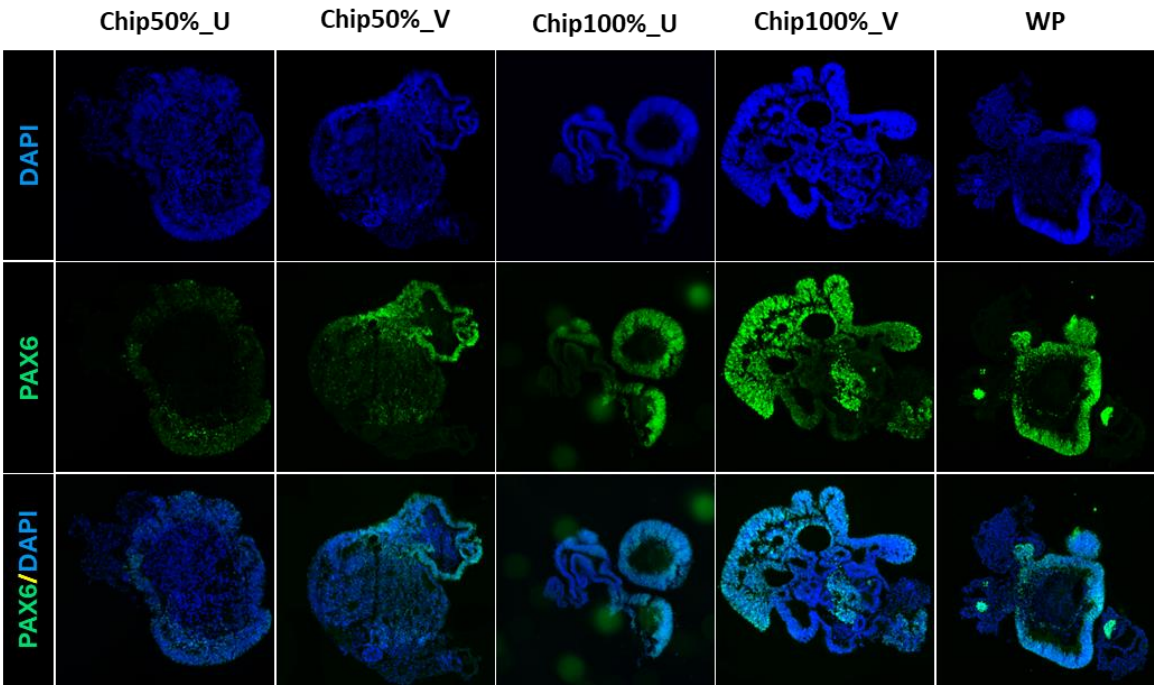


Fig. 69: Immunofluorescence analysis of retinal neurogenesis

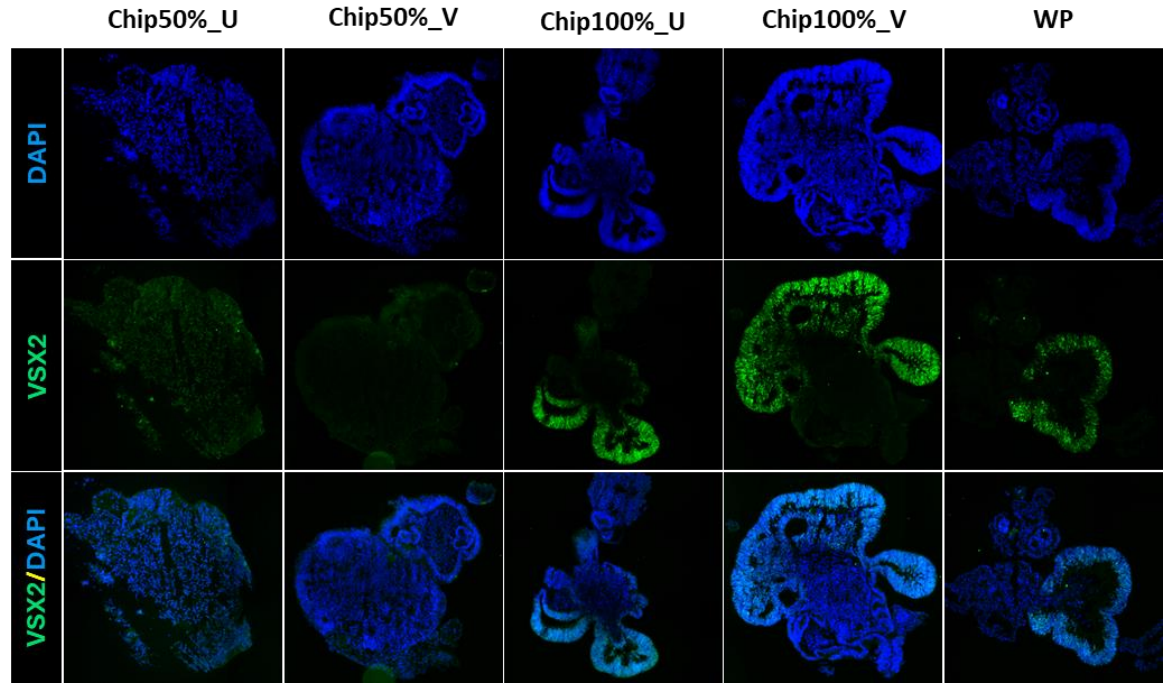


Fig. 70: Immunofluorescence analysis of photoreceptor differentiation

Next, the expression levels of selected retinal development markers were analyzed using qPCR (Fig. 71). Specifically, we analyzed the expression of *PAX6*, *VSX2* and *RAX*. Expression of *PAX6* was comparable for each cultivation method. For *VSX2* and *RAX* markers, there was similar lower expression in organoids from Chip50%. For Chip100% expression was comparable to WP. In the case of expression uniformity, there was not significant difference between cultured using different methods. The chip was tested for different and longer cultivation periods without transferring the organoids, and it had a comparable impact on the differentiation of retinal organoids compared to the standard WP method.

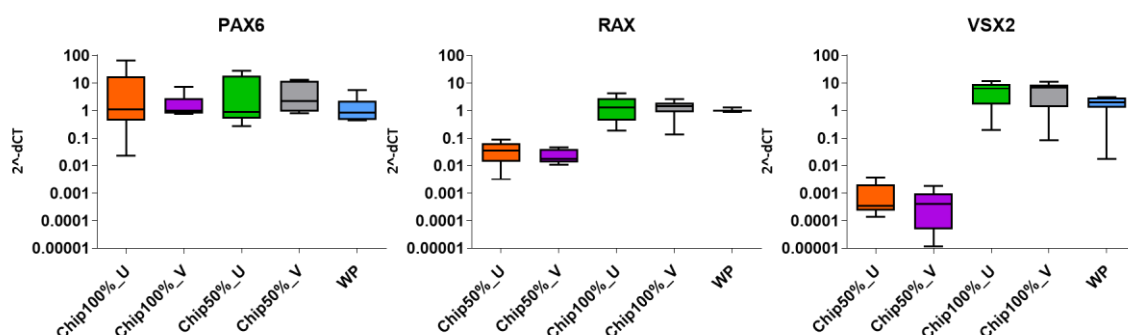


Fig. 71: qPCR analysis of retinal organoids

4.6 Microfluidic droplet generator for cell isolation/separation

In addition to working on the differentiation of hPSC-derived organoids, I contributed to a project focused on developing a microfluidic device for cell isolation. During my six-month internship at OTH Regensburg in the Biofluid Mechanics and Microsystems Engineering laboratories, I developed a rapid fabrication protocol for a droplet generator. This effort was part of a broader project to create a reusable microfluidic tool for single-cell multi-omics analysis.

4.6.1 Chip fabrication for single droplet generation

The design of the single droplet generator includes buffer and cell inlets as the dispersed phase, oil as the continuous phase, and an outlet (Fig. 72). Cells from liquid biopsies will be introduced into the chip, where they can mix with the buffer or specific medium. In the T-junction, the cells will be separated and encapsulated in droplets. The entire chip has a depth of 60 μm , and the channel width in the T-junction is also 60 μm for both phases. The chip was fabricated from a silicon semiconductor substrate using laser ablation (Quantel, Evergreen EVG00200). The laser was used to create the chip design, including the holes for inputs and outputs, and to cut the chip from the substrate.

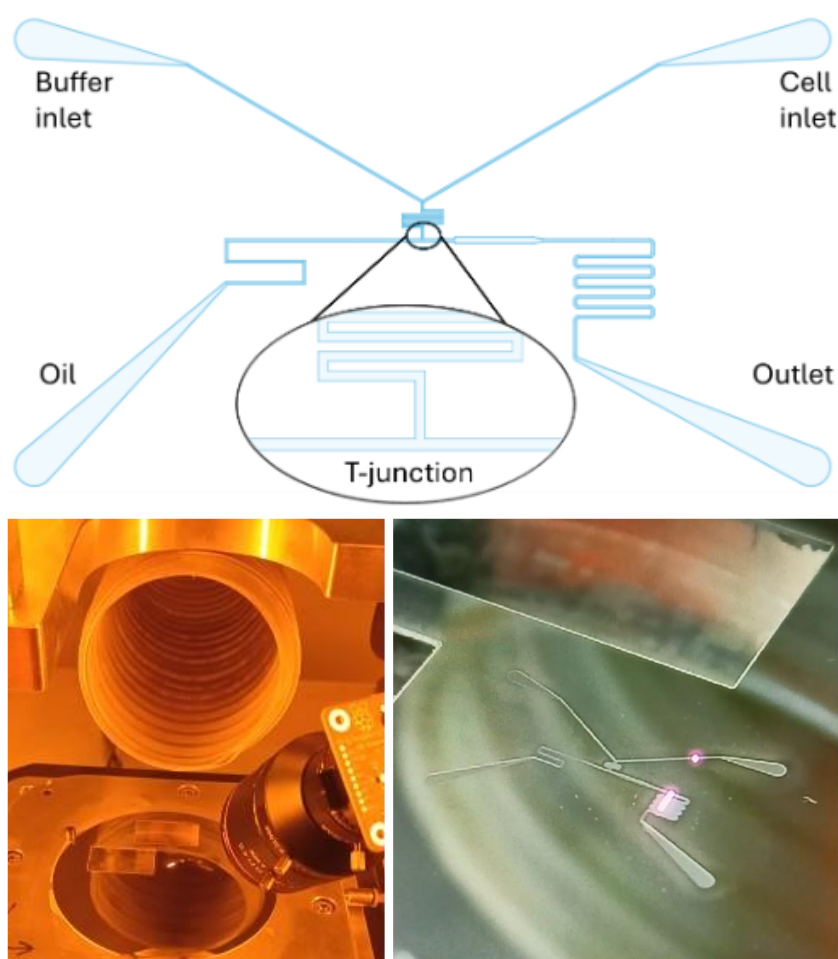


Fig. 72: Design and laser ablation of chip

4.6.2 Surface smoothing and coating

After the laser ablation of the chip surface, there was residual molten silicon around the ablated channels (Fig. 73). First, silicon dioxide was removed using hydrogen fluoride (HF), and then molten silicon was etched away with TMAH. The etching process was optimized alongside laser ablation to achieve the desired depth and smoothness of the surface. Next, the chips were permanently bonded to glass using anodic bonding (Fig. 74). This procedure made the walls of the chips hydrophilic. Thus, to create a hydrophobic surface for water-oil droplets, the chip was coated with Aquapel.

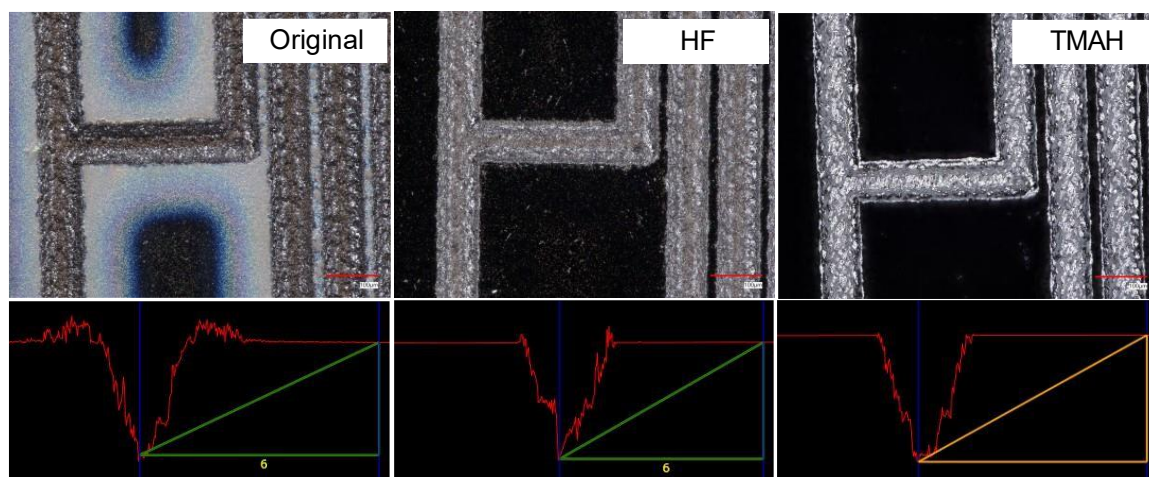


Fig. 73: Cleaning and etching of melted silicon

4.6.3 Droplet generator

The chip was fixed to a chip holder using screws and plexiglass (Fig. 74). Tubing was connected to the holder via fittings, and this tubing was linked to syringes. Three pumps were used for the experiment: one for oil (the dispersed phase) and two for water (the continuous phase), which were introduced to the cell and buffer inputs.

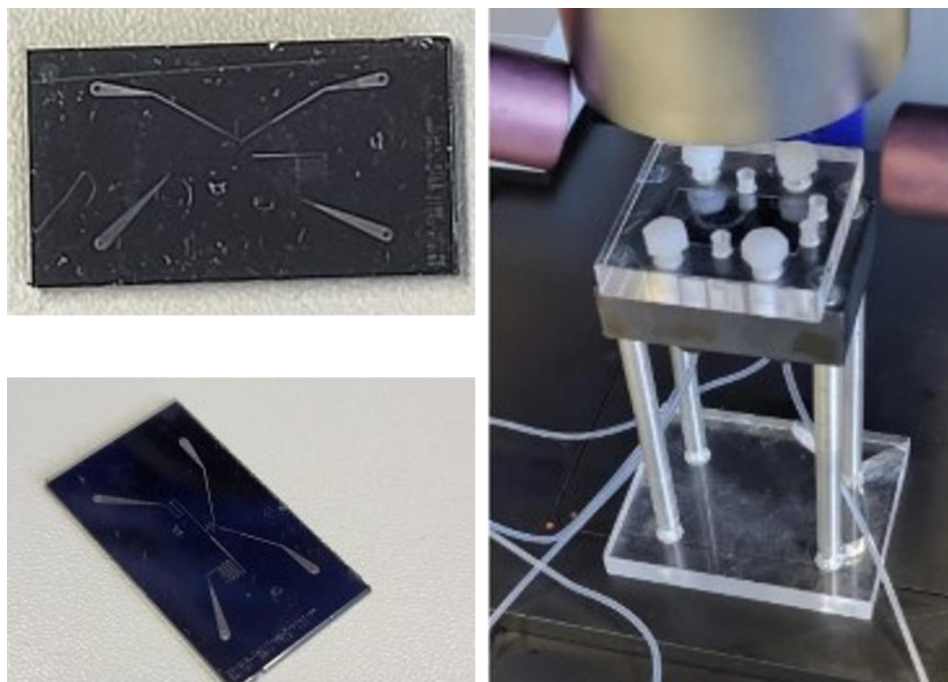


Fig. 74: Bonded chip and platform with chip connected to tubes

The flow rate of water was set to 2.5 $\mu\text{l}/\text{h}$. For initial water droplet generation, the flow ratio between the continuous and dispersed phases was set to 0.25. Water

droplets were formed in the T-junction (Fig. 75) and moved continuously across the channel without disruption. The meandering channel was used to verify droplet homogeneity and measure their size. Videos and images showed successful generation of water droplets, with sizes of $65\text{ }\mu\text{m}$ at a 0.25 ratio. Changing the ratio to 0.5 produced droplets that were $105\text{ }\mu\text{m}$ in size.

Overall, this initial task was aimed at verifying the fabrication process for droplet generation. These findings serve as initial data for the project, and individual steps are currently being examined in more detail OTH University in Regensburg.

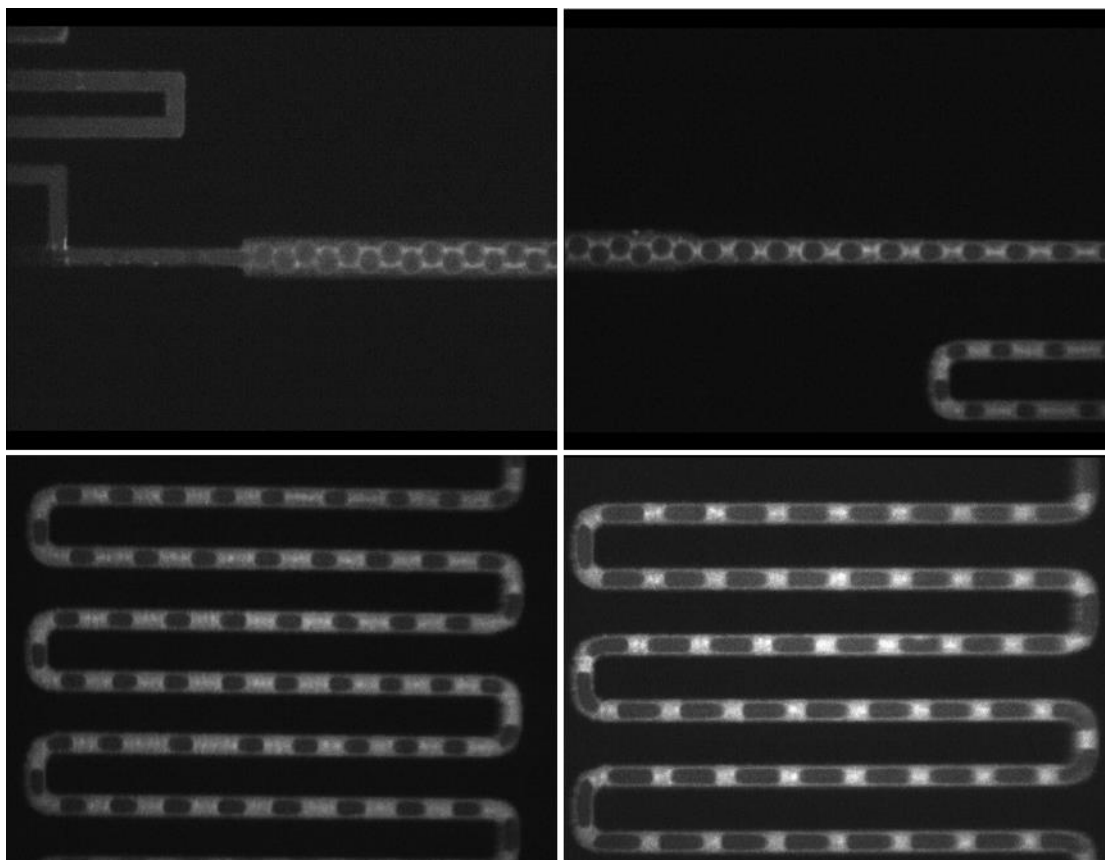


Fig. 75: Water droplets formed in oil

5 Discussion

The use of microfluidics for cell cultivation and differentiation is still not widespread. Conventional tools with well-established protocols and standardized materials remain the preferred choice. However, in the field of microfluidics, there is ongoing development of new fabrication methods and materials. Due to the closed nature of microfluidic systems, protocols and workflows must be adapted, and a unified approach for cultivation has yet to be established. This dissertation thesis explored the usability of the microfluidic method and the versatility that it offers for the cultivation of cerebral organoids. This work also aimed to simplify workflow with microfluidics, minimizing the time and material requirements for the cultivation of cerebral organoids. The versatility was highlighted during an internship, where microfluidics was used for droplet generation and fabricated with different method and material.

5.1 Fabrication

Microfluidic chips are currently fabricated using various techniques, including soft lithography, 3D printing, laser ablation, and injection molding, among others¹³². These methods typically rely on 2D designs, where the depth of the microfluidic system is specified during the production process. Each technique presents distinct advantages and trade-offs regarding cost, scalability, fabrication complexity, and material compatibility. As a result, the choice of fabrication method depends on factors such as the desired chip design, material requirements, production volume, and available resources.

Today, 3D printing has become the most widely used technology for prototyping. With the development of new materials, it offers the advantages of rapid prototyping and the ability to create complex geometries with high precision¹³³. Stereolithography (SLA) is particularly favoured for its ability to quickly and easily produce 3D objects with customized designs¹³⁴. However, the quality of the fabricated structures depends on the 3D design. The resolution of SLA printing is primarily constrained by the available technology and its associated costs¹³⁵.

For this project, the Form 2 printer by Formlabs was utilized, offering an XY resolution of 140 microns. It is important to note that advancements in 3D printing have led to more common resolutions of around 50 microns, as seen with newer models like the Form 4 (Formlabs). Despite the limitations of Form 2, these were reduced by adjusting the fabrication settings and applying post-processing techniques to the printed mold.

Firstly, finding the optimal printing angle was crucial for minimizing the distance between structures and achieving maximum surface smoothness. Several studies have discussed the impact of part orientation on printing accuracy^{136–139}. In one study using the same printer, a 45-degree angle was identified as ideal for achieving optimal print

quality¹³⁷. In our project, findings show that the best results were also 45 degrees. Taken into consideration different 3D model and conditions, results were comparable. We thus used this 45 degree angle for all our future fabrication.

After that, we identified that the surface roughness of the 3D-printed mold's details was crucial in achieving better cultivation results. The type of resin used plays a significant role in determining the surface characteristics¹⁴⁰. Different resins offer varying degrees of smoothness, depending on their specific properties and intended applications. For this project, we used clear resin (FLGPCL03) and white resin (FLGPWH02), both of which are known for capturing fine details. The clear resin produced superior results, likely due to its transparency, which allows for more uniform post-curing under UV light. When surface smoothness is insufficient, several techniques can be employed to improve it, such as sanding, solvent vapor smoothing, polishing, or applying polyethylene wax¹⁴¹. However, most of these methods were either ineffective for the fine details required in this project or were too complex and time-consuming. Applying a commercially available varnish provided a quick and effective method to achieve the desired smoothness. The drawback of this technique is that it is operator-dependent, and achieving consistent results is challenging, with limited repeatability.

The next step involved PDMS molding, a process whose effectiveness depends on the post-curing of SLA-printed molds and the removal of uncured residues¹⁴². While the use of IPA and UV curing effectively removes surface residues, studies have shown that uncured materials often remain embedded within the 3D-printed structure¹⁴². When PDMS was molded, exposure to higher temperatures caused these internal residues to migrate to the mold's surface, where they reacted with the PDMS—a phenomenon confirmed by another study¹⁴³. Various studies have combined UV curing, IPA washing, and baking to optimize the preparation of different resins^{142–145}, with some also applying salinization to seal the surface¹⁴³. Our findings revealed that the most critical step was baking, with 60°C for 24 hours being sufficient to eliminate internal residues effectively. Finally, to bond the two PDMS layers, fresh, uncured liquid PDMS was used. The most common method for bonding PDMS to glass or PDMS to PDMS is plasma treatment^{146,147}, which creates a stable, permanent bond through surface activation. Other alternatives, such as PDMS mortar (a mixture of PDMS and hexane)¹⁴⁸, have been previously used to achieve an even closure between two PDMS layers. Chemical bonding methods were also explored by other studies¹⁴⁷. While these methods are precise and effective, they are usually time-consuming and relatively complex. In our setup, we initially tested plasma treatment; however, it proved unsuccessful due to the imperfect smoothness of the surface. Ultimately, we opted for bonding with liquid, uncured PDMS, which provided an optimal solution regarding of both time efficiency and bonding quality.

5.2 Cell aggregation and cultivation

Before the cultivation process, the microfluidic chip required preparation to prevent issues with cell adhesion and the formation of bubbles during cultivation. Cell adhesion to the chip surface can significantly reduce the quality of cell aggregation. There are several methods for surface treatment. For long-term cultures, coatings like mPEG or Lipidure are commonly used, as they create covalent bonds with PDMS⁷⁴. Other studies have used Pluronic F-68 to create an anti-fouling layer¹²³, to maintain a steady protein level with low serum protein adsorption for a PDMS microbioreactor in a culture system. However, there is limited research on coating closed systems with mPEG or Lipidure, and we were unable to successfully coat our chip using these methods. Therefore, for our purposes, based on previously published study¹²³, we chose bovine serum albumin (BSA), a readily available and easy-to-use coating that is suitable for short-term cell culture. By utilizing BSA's charge-blocking properties, our system effectively promoted the aggregation of cells into a single cellular cluster.

For initial cultivation, the shape of microwells is an important parameter for cell aggregation⁷⁹. There are commercially available cultivation tools that offer various well shapes, with U-shaped and V-shaped wells being the most commonly used [Greiner, Corning, etc.]. Some companies provide wells specifically designed for analysis [e.g., InSphero]. Most studies utilize common U and V-shaped wells or some variation of this design [62,71,149,150]. Individual studies have also explored pyramidal shapes or different curvatures to enhance aggregation⁷³. In our study, we initially focused on the widely used U and V shapes. During our preliminary experiments, we investigated additional shapes that could improve aggregation or cultivation. We found that the optimal shape should be selected based on the specific needs of the experiment. It is also important to note that centrifugation after cell seeding improved aggregation, which we included as a necessary step in subsequent cultivation experiments.

For further investigations, we selected U and V-shaped wells. U-shaped microwells generally promote the formation of single aggregates. However, when seeding a low number of cells (e.g., hundreds) into wells with a diameter exceeding several hundred micrometers, cell dispersion occurs, similar to what is observed in cylindrical microwells with a flat bottom. In contrast, V-shaped microwells demonstrate enhanced capabilities for cell aggregation, even with low cell numbers. While V-shaped microwells facilitate aggregation, it is essential to consider their impact on the final shape of the organoids (^{71,74,151} and our unpublished observations) and the accessibility of nutrients to the cells at the bottom of the well as aggregates grow. Our simulations of lactate production and diffusion indicated a markedly increased concentration of metabolites at the bottom of V-shaped wells. However, this property did not significantly affect the overall quality of the organoids, as neural differentiation was comparable across all analyzed chip conditions. Overall, our results demonstrated that seeding low

cell numbers into both U- and V-shaped microwells led to early aggregation into spherical organoids.

The next step was to achieve a homogeneous distribution of cells within the cultivation channel. Cell seeding depends largely on the chip design. Previously described methods often seed cells through an inlet located above the cultivation area, which is then closed after seeding¹⁵². Another approach divides the cells into two groups, gradually seeding them from both sides of the perfusion channel¹⁵³.

In our setup, to achieve uniform cell distribution, we utilized a single straight channel and injected the cells from one side. Seeding from both sides proved ineffective with the small number of cells we were working with. The uniformity of cell distribution depended significantly on the operator technique and the quality of the pipette, introducing the potential for error.

After seeding, cell aggregation is enhanced using ROCK inhibitor (ROCKi). The standard protocol for cultivating cerebral organoids typically utilizes a concentration of 50 μ M ROCKi^{62,73}, while other protocols have successfully used 20 μ M to promote cell aggregation^{125,154,155}. From our initial tests, we observed that a certain amount of ROCKi is essential for effective aggregation. However, we discovered that, for one particular cell line, the combination of ROCKi concentration and cell number had a noticeable impact on the final spheroid shape. Further observations indicated that the cell line itself also plays a significant role, suggesting that the concentration of ROCKi should be optimized for each specific experiment based on the cell line and experimental conditions. However, it is worth noting that the optimal cell numbers and the efficiency of cell aggregation can vary depending on the specific characteristics of the cell line under investigation^{81,156}.

Cultivation in a closed microfluidic system depends on the continuous exchange of nutrients. Microfluidic flow mediates more efficient nutrient transport⁸⁸ and reduces lactate concentration⁹⁶. For cultivation within microfluidic channels, two main approaches are typically used: active pumping^{150,157} and passive pumping^{79,88}. Active pumping, which utilizes syringe pumps, allows for precise control of flow rates but requires more complex maintenance¹⁵⁸. In contrast, passive pumping, which often uses a rocking shaker driven by gravity, is easier to manage but offers less control over the flow¹⁵⁹. In our study, we chose a pump-driven system to maintain better control over the microenvironment inside the microfluidic chip. This setup enabled unidirectional flow through a single channel containing 48 wells.

Subsequently, to determine the appropriate flow rate, we referred to a commonly used protocol for cultivation in 96WP^{81,156}. From this protocol, we extracted the volume of media used per day for a single aggregate and calculated the flow rate accordingly. Our goal was also to demonstrate that using a microfluidic system could reduce the volume of medium required for cultivation. To test this, we added a chip where aggregates were cultivated using only 50% of the originally calculated medium. Before proceeding with the 50% medium cultivation, we conducted simulations to

theoretically validate this approach. Our experimental data demonstrated and confirmed simulation data that chip using 50% of the original medium can be used in this designed setup for cell clusters cultivation.

Lastly, bubble formation during chip preparation (BSA coating) or cultivation is a common issue that can negatively impact cell seeding and cultivation. Several commercial companies, such as Elveflow, uFluidix, and Darwin Microfluidics, offer components and guidance on preventing bubble formation. Significant research has also been conducted to address this issue, employing methods such as bubble trapping¹⁶⁰, membrane integration¹⁶¹, lateral degassing¹⁶², and designing microfluidic systems specifically to counteract bubble formation¹⁶³. In our work, to prevent creating bubbles, we first degassed the medium used for cultivation. To eliminate bubbles from the chip, we leveraged the natural gas permeability of PDMS to design a vacuum channel system. By generating negative pressure within the flow channel, this system efficiently eliminated air bubbles. To simplify the design, we implemented a single vacuum channel running across the entire chip. This degassing system not only effectively removes bubbles from the flow channel but also enhances ease of use for end-users, making chip handling more straightforward.

5.3 Simulations

To support our approach prior to cultivation, we conducted numerical simulations. Previous studies have utilized simulations to calculate basic flow rates¹⁶⁴, nutrient distribution¹⁶⁵, and oxygen diffusion¹⁶⁶. The proper simulation setup is crucial for accurately predicting environmental behaviour. Additionally, comparing simulation results with laboratory experiments is essential to validate the simulation methods. For validation, we applied a methodology adapted from a study that simulated a gradient patterning device and the dynamic diffusion transfer of specific molecules¹⁶⁷.

In our study, we first compared the diffusion of dextran and fluorescein in an agarose block over time. This comparison provided a straightforward verification of our simulation settings and methods. For more complex simulations, we then added medium above the agarose block which decreased concentration of dextran and fluorescence. Using these validated settings, we could predict the behaviour of bioprinting structures and molecule diffusion, which aided in calculating the appropriate concentrations of specific molecules.

After that, we simulated the diffusion of lactate in dynamic cultivation conditions. Simulation of lactate production can be used to monitor several aspects, e.g., flow rate, diffusion of lactate from aggregates into the wells and channel, and exchange of lactate with fresh medium. In contrast to previous models of lactate production, where lactate was produced by a thin layer of cells¹⁶⁸, in this study, lactate was produced by the spheroids inside the microwells, offering an illustrative view of the chip's dynamic

environment and validating its design. First, we examined the overall generation, diffusion, and washout of lactate from the chip. We also compared the dynamic and static systems, estimating the time required to reach stable conditions in each of them. Then, we closely analysed the microenvironments within the wells, focusing on lactate concentrations immediately surrounding the spheroids. A lactate concentration of 40–80 mM was previously determined to decrease cell viability^{169,170}. Our simulations showed that lactate concentration in the wells was below 40 mM. This finding confirms that our system with flow rates of 50 and 100 $\mu\text{L}/\text{h}$ is suitable for sufficient nutrient exchange and can reduce the amount of medium required for static cell culture conditions.

Additionally, we closely examined how the maximum flow rate impacts the conditions within the wells, specifically whether it increases shear stress to levels that could compromise cell survival. Previous studies have used similar simulations to determine shear stress caused by flow rate^{151,171}, with 1 Pa identified as a threshold detrimental to cell viability^{126,127,172}. However, since these studies used different geometries and simulation parameters, we did not have direct validation for our setup. Nevertheless, in our simulation, even at a maximum flow rate of 100 $\mu\text{L}/\text{h}$, we did not reach the 1 Pa threshold, allowing us to proceed with this setting for *in vitro* cell cultivation.

5.4 Cerebral organoids

To date, several studies have explored different approaches for culturing hPSC-derived brain organoids on microfluidic devices. These technologically diverse systems enable the long-term culture and maturation of brain organoids while allowing the perfusion of cell culture media around them^{88,96,129,157}. However, these methods present several drawbacks, such as the high cost of microfluidic devices, complex fabrication and setup processes, limited scalability, and the use of hydrostatic pressure for media exchange. Importantly, none of these microfluidic devices are designed as closed perfusion systems for cultivating cerebral organoids from the single-cell suspension stage (Day 0); instead, they are typically designed to differentiate and culture embryoid bodies previously developed in standard 96WP. In contrast, our microfluidic device offers a unique, fully closed system with microwells and a continuous supply of fresh medium, allowing single-cell suspensions to be loaded from Day 0. It is also easily scalable, can be produced using widely accessible 3D printers and PDMS materials, and features a relatively simple design, making it accessible to a broad scientific community.

Cerebral organoid differentiation has become widely used for modelling early human brain development and neurological diseases *in vitro*. The “Lancaster’s protocol” is one of the most frequently used protocols today and was originally published by Lancaster et al. in¹⁷³. Human PSCs are first dissociated into single cells, allowed to re-aggregate to form EBs, and then subjected to neural induction in a minimal medium

that allows the neuroectoderm to develop. This phase is represented by the formation of an organized translucent neuroectoderm on the borders of the forming aggregates, which are then embedded into a floating droplet of a surrogate extracellular matrix, promoting the outgrowth of neuroepithelial buds. Finally, the tissues are transferred to an orbital shaking plate, which promotes nutrient and oxygen exchange to allow more extensive growth and further development into defined brain regions⁶².

Importantly, the initial culture steps are guided by specific time points, based on the appropriate size of the aggregates and the morphology characteristic of cerebral organoids⁶¹. In addition to the previously described optimization steps, we have also refined these culture stages. Since the publication of the Lancaster protocol, several modified protocols have been developed, varying in cell type¹⁷⁴, cell number¹⁵⁶, medium composition¹⁷⁵, medium volume⁸¹, and critical time points¹⁷⁶. These protocols were further optimized according to the cultivation tools and techniques used. In collaboration with other members of Dáša Boháčiková's group, we optimized the commonly used Lancaster cultivation protocol for 96WP and simplified it for use in microfluidic systems, taking advantage of their unique properties. In this study, we switched different differentiation media when forming aggregates that reached specific sizes or acquired specific morphological features. While in the 96WP format all media changes must be done manually, our data show that the chip automation during the initial 9–11 days reduces labour intensity and minimizes the risk of organoid damage and contamination.

Additionally, during the optimization of the cultivation protocol, we observed that the successful cultivation and differentiation of cerebral organoids is cell line-dependent, as also noted in other studies^{81,156}. In our study, we tested various cell lines and found issues with initial aggregation, early differentiation, or the maturation of cerebral organoids in some cases. Following this screening process, we selected two cell lines for the validation of our microfluidic system.

Subsequently, we started with the cultivation of cerebral organoids. In cultivation, we confirmed that the chip allows the proper formation and growth of cell aggregates, with similar sizes achieved during the neural induction phase in all experimental conditions. The expression of neurodifferentiation-related markers, including PAX6 and N-cadherin, was successfully observed in both the chip and plate conditions. It is of note that our IHC analysis showed that in two independent experimental batches, the N-cadherin and PAX6 expression showed a stronger signal in Chip100%_U and Chip100%_V conditions in comparison to the 96WP. This increased expression could be related to the increased abundance of small rosettes observed in microfluidic conditions, where a continuous flow of medium may enhance the accessibility of trophic factors and nutrients, thereby promoting the proliferation of neural precursors. Although our qPCR analysis did not confirm this finding, it is worth noting that enhancement in PAX6 expression and higher rosette numbers were observed in vascularized cerebral organoids¹⁷⁷. In addition, examination of other biological parameters

indicated favourable cell growth, minimal DNA damage, and proper glucose metabolism in all chip cultures. All data thus confirmed that the initial differentiation phase of cerebral organoids can be successfully transferred to the microfluidic device.

Lastly, after embedding the organoids in a surrogate extracellular matrix, they further followed neuroepithelial expansion, forming neural tube-like buds and exhibiting increased complexity with the formation of neural rosettes and ventricle-like lumens as described in Giandomenico et al.⁸¹ The immunofluorescence analysis confirmed well-developed ventricle-like cavities and N-cadherin expression in all tested conditions, suggesting proper neural differentiation. However, differences were observed between organoids grown in the chip and those grown in the 96WP, including a larger bright area on the edges and a higher wrinkling index in chip-grown organoids. Similar observations of organoids growing better in microfluidic conditions were reported⁸⁸, but the significance of these differences and their underlying mechanisms require further investigation. Finally, the analysis of mature tissue morphology, size, and patterning at day 55 showed similar results across all tested conditions within each batch. The expression levels of neurodifferentiation markers exhibited variability between batches but showed consistent trends among the tested chip conditions. Furthermore, the thickness of MAP2/NeuN-positive neuronal layers⁷⁴, indicative of organoid maturation, was comparable between organoids cultured using different approaches. These findings indicate that the chip conditions had similar effects on the development of mature cerebral organoids and cortical neurogenesis, showing a clear potential of the tested microfluidic chip as an alternative method to the standard 96WP for organoid culture.

5.5 Retinal organoids

The successful cultivation of cerebral organoids within the microfluidic chip confirmed our assumption that microfluidic systems can serve as effective tools for organoid cultivation. The next step was to determine whether this system could function as a universal platform for organoid cultivation and support longer-term culture. In collaboration with the Tomáš Bárta group, we adapted the cultivation protocol to our system and successfully cultivated retinal organoids without the need for transferring them to other cultivation tools.

During cultivation, successful cell aggregation and growth were observed, followed by the appearance of small bright areas along the edges. According to the literature, this is a characteristic morphological feature indicating the development of neuroepithelium^{131,178,179}. These bright areas became more pronounced over time, particularly in the V-shaped wells. Early retinal development was confirmed by the expression of PAX6 and VSX2 markers in both the Chip100% and WP conditions. However, results from the Chip50% condition were not comparable to the others, likely due to

insufficient nutrient and chemical factor supply for differentiation. Overall, this initial experiment confirmed the versatility of the microfluidic system for cultivating different types of organoids and protocols. Optimization of the decreased flow rate will be necessary for future experiments.

5.6 Droplet generator

In recent years, multi-omics technologies have emerged as a promising tool to integrate multiple analyses into a single process, yielding comprehensive results for a given research question^{180,181}. However, these technologies are still in the developmental phase, with protocols being refined. One of the critical factors for ensuring a successful and reproducible process is cell isolation and preparation¹⁸². The literature indicates that microfluidic tools are reliable and frequently employed for single-cell analysis¹⁸³. Yet, these tools are still primarily optimized and prototyped for research purposes or specific experimental approaches.

In collaboration with OTH Regensburg, we proposed using a microfluidic system for clinical applications, aiming to create a reusable tool rather than a single-use product. In our initial steps, we successfully validated the fabrication process, materials, and workflow for preparing a microfluidic separator from a silicon wafer. Microfluidic chips made from silicon are typically fabricated using photolithography-dry etching¹⁸⁴, wet etching¹⁸⁵, or laser ablation combined with wet etching¹⁸⁶. While photolithography and etching processes are time-consuming and complex, laser ablation with wet etching was our preferred choice for quick and high-quality prototyping.

After fabrication, the chip surface remained hydrophilic. For hydrophobic coating, we were inspired by studies using HMDS¹⁸⁷ and APTES¹⁸⁸; however, these protocols were ineffective for our closed microfluidic chip. Instead, we utilized Aquapel, a ready-to-use solution commonly employed for hydrophobic coatings on PDMS and glass, which we injected into the chip.

In summary, we have developed a protocol for the rapid fabrication of microfluidic chips. This method enables reproducible and cost-effective mass production of chips with consistent quality. Moreover, the surface of this reusable tool can be customized for specific applications, making it highly versatile. This approach holds significant potential for further optimization and advancements in single-cell multi-omics analysis.

6 Conclusion

This dissertation focused on designing and applying a microfluidic system for brain component formation, particularly cerebral organoids. Microfluidic technology, known for its ability to simulate complex systems and integrate multiple functions, was shown to address key challenges in organoid cultivation effectively. Specifically, we established an efficient fabrication protocol for microfluidic chips. Using stereolithography and soft lithography, a streamlined process was developed, resulting in high-quality PDMS chips with optimized properties for cell cultivation. The protocol emphasized simplicity and reproducibility, enabling rapid prototyping. We subsequently optimized the chip for cell aggregation and cultivation. Various well shapes and dimensions were evaluated, along with cell seeding density and ROCK inhibitor concentrations, to ensure uniform aggregate formation. CFD simulations were used to model fluid flow, metabolite diffusion, and shear stress, demonstrating that continuous flow reduced media consumption by half while maintaining safe and efficient cultivation conditions. Finally, we applied the microfluidic system for cerebral organoid formation. Two cell lines were successfully cultivated, and organoids produced in the microfluidic chip were comparable in quality and homogeneity to those generated using standard protocols, with reduced costs and time. Additionally, the system was adapted for retinal organoid cultivation as proof of concept. Lastly, during an internship at OTH Regensburg, fabrication methods for reusable microfluidic systems were explored. A droplet generator chip was fabricated using laser ablation and wet etching, demonstrating a simple and effective process for single-cell applications. In conclusion, this work highlights the potential of microfluidic systems to streamline organoid cultivation, reduce resource usage, and expand applications in tissue engineering and single-cell analysis.

Summary of publications and research activities

List of publications related to the doctoral thesis

1. **KANDRA, Mário**, Tereza VÁŇOVÁ, Vincent Alexander JONGEN, Jakub POSPÍŠIL, Josef NOVAK, Václav CHOCHOLA, Tomas BURYSKA, Zbyněk PROKOP, Zdenek HODNY, Aleš HAMPL, Dáša BOHAČIAKOVÁ a Josef JAROŠ. A closed 3D printed microfluidic device for automated growth and differentiation of cerebral organoids from single-cell suspension. *Biotechnology Journal*. WEINHEIM: WILEY-V C H VERLAG GMBH, 2024, roč. 19, č. 8, s. 1-17. ISSN 1860-6768.
DOI: <https://dx.doi.org/10.1002/biot.202400240>.
Journal category according to WoS: Biotechnology & Applied Microbiology, Q2
Impact factor: 4.7
Number of citations: 0
Contributions: Development and fabrication of microfluidic chip, Cultivation of cells, *In silico* simulations, Analysis (Microscopy, Image analysis, PCR), Data analysis, Preparation of Figures, Manuscript writing
2. SEDLÁKOVÁ, Veronika, Michaela KLOUČKOVÁ, Zuzana GARLÍKOVÁ, Kateřina VAŠÍČKOVÁ, Josef JAROŠ, **Mário KANDRA**, Hana KOTASOVÁ a Aleš HAMPL. Options for modeling the respiratory system: inserts, scaffolds and microfluidic chips. *Drug Discovery Today*. Oxford (England): Elsevier Sci Ltd., 2019, roč. 24, č. 4, s. 971-982. ISSN 1359-6446.
DOI: <https://dx.doi.org/10.1016/j.drudis.2019.03.006>.
Journal category according to WoS: Pharmacology & Pharmacy
Impact factor: 7.321
Number of citations: 9
Contributions: Wrote a section about microfluidics

Research activities related to the thesis

Internship

September 2023 - January 2024

- Internship at **Biofluid Mechanics** and **Microsystems Engineering** Laboratories, University of Applied Sciences, Regensburg.
- Supported by **Erasmus+**

List of conference abstracts for oral presentations

1. **KANDRA, Mário**, Tereza VÁŇOVÁ, Vincent Alexander JONGEN, Aleš HAMPL, Dáša BOHAČIAKOVÁ a Josef JAROŠ. Microfluidic systems for easy and uniform generation of cerebral organoids. In The Biomania Student Scientific Meeting 2022. 2022.
2. **KANDRA, Mário**, Josef JAROŠ a Aleš HAMPL. Microfluidic device – little helper for big science. In PhD conference of Biomedical Sciences 13.- 14. 10. 2020. 2020.
3. **KANDRA, Mário**, Jakub POSPÍŠIL, Václav CHOCHOLA, Aleš HAMPL a Josef JAROŠ. Microfluidic chip as a tool for uniform generation of spheroids. In Organ-on-a-Chip & Tissue-on-a-Chip Europe, 18-19.6.2019, Rotterdam, NL. 2019.
4. **KANDRA, Mário**, Eva MATUROVÁ, Aleš HAMPL a Josef JAROŠ. Microfluidic designs for 3D cell culture. In *8th Annual meeting on stem cells, DNA damage and apoptosis, Karlov*, 31.1.-3.2.2018. 2018.
5. CHOCHOLA, Václav, Karolína SPUSTOVÁ, Richard MACKOVIČ, **Mário KANDRA**, Jakub POSPÍŠIL, Vladimír PROKS, Aleš HAMPL a Josef JAROŠ. 3D-bioprinting and cell culture with modified alginate bioinks. In Biofabrication & Biomanufacturing Europe, 20-21.6.2019, Rotterdam, NL. 2019.
6. JAROŠ, Josef, Jakub POSPÍŠIL, Karolína SPUSTOVÁ, Josef LAVICKÝ, Václav CHOCHOLA, **Mário KANDRA**, Veronika VALOUCHOVÁ, Richard MACKOVIČ, Vladimír PROKS a Aleš HAMPL. 3D bioprinting of stem cells and their microenvironment. In V4 Society for Developmental Biology. 2018.

List of conference abstracts for poster presentations

1. **KANDRA, Mário**, Tereza VÁŇOVÁ, Vincent Alexander JONGEN, Václav CHOCHOLA, Jakub POSPÍŠIL, Aleš HAMPL, Dáša BOHAČIAKOVÁ a Josef JAROŠ. Microfluidic system for easy and uniform generation of 3D cerebral organoids. In PhD conference of Biomedical Sciences 3.- 5. 11. 2021. 2021.
2. **KANDRA, Mário**, Jakub POSPÍŠIL, Aleš HAMPL a Josef JAROŠ. Microfluidic chip as a tool for uniform spheroid generation. In XIX. Interdisciplinary meeting of young life scientists, Milovy, 20-23.5.2019. 2019.

3. JAROŠ, Josef, **Mário KANDRA**, Václav CHOCHOLA, Tereza VÁŇOVÁ, Dáša BOHAČIAKOVÁ, Ivana AĆIMOVIĆ a Aleš HAMPL. PRODUCTION OF UNIFORM ORGANOIDS IN MICROFLUIDIC CHIPS AND AND THEINTERACTION WITH CAPILLARIES. In TERMIS EU 2022. 2022. ISSN 1937-3341. Dostupné z: <https://dx.doi.org/10.1089/ten.tea.2023.29046.abstracts>.
4. JONGEN, Vincent Alexander, **Mário KANDRA**, Tomáš BÁRTA, Aleš HAMPL a Josef JAROŠ. FORMATION AND LONG-TERM CULTIVATION OF RETINAL ORGANOIDS IN MICROFLUIDIC SYSTEMS. In TERMIS EU 2022. 2022. Dostupné z: <https://dx.doi.org/10.1089/ten.tea.2023.29046.abstracts>.
5. CHOCHOLA, Václav, Karolína SPUSTOVÁ, Richard MACKOVIČ, Josef LAVICKÝ, **Mário KANDRA**, Jakub POSPÍŠIL, Vladimír PROKS, Aleš HAMPL a Josef JAROŠ. Modification of alginate-based bioinks for 3D printing and stem cell cultivation. In XIX. Interdisciplinary meeting of young life scientists, Milovy, 20.5-23.5.2019, 2019. 2019. ISSN 2336-7210.
6. JAROŠ, Josef, Václav CHOCHOLA, Karolína SPUSTOVÁ, **Mário KANDRA**, Jakub POSPÍŠIL, Vladimír PROKS a Aleš HAMPL. 3D bioprinting of stem cells for creating tissue models. In 10th International Conference Analytical Cytometry. 2019.

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Appendix A List of primary antibodies

Antibody	Host	Cat. No	Dilution	Company
anti-8-OHG	Mouse monoclonal	200-301-A99	1:50	Rockland
anti-cCasp3	Rabbit polyclonal	9661	1:100	Cell Signaling Technology
anti-N-Cadherin	Rabbit monoclonal	13116s	1:100	Cell Signaling Technology
anti-GFAP	Rabbit monoclonal	12389P	1:100	Cell Signaling Technology
anti-GLUT1	Rabbit monoclonal	07-1401	1:100	Merck
anti-Ki67	Rabbit monoclonal	RBK027	1:100	Cell Signaling Technology
anti-MAP2	Rabbit monoclonal	8707	1:200	Cell Signaling Technology
anti-NeuN	Rabbit monoclonal	24307	1:100	Cell Signaling Technology
anti-PAX6	Rabbit monoclonal	60433S	1:100	Cell Signaling Technology
anti-TUJ	Mouse monoclonal	5568	1:200	Cell Signaling Technology
anti-RAX	Mouse monoclonal	271889	1:200	Santa Cruz
anti-VSX2	Mouse monoclonal	365513	1:200	Santa Cruz

Appendix B Sequence of primers used for qPCR

Gene name	Forward Sequence 5'-3'	Reverse Sequence 5'-3'
<i>CDH2</i>	AGTATCCGGTCCGATCTGC	CTGTGGGGTCATTGTCAGC
<i>DCX</i>	GCAGTCTCCCATCTCTACGC	GGACAGAGGCAGGTACAGGT
<i>MAP2</i>	TTGGTGCCGAGTGAGAAGA	GTCTGGCAGTGGTTGGTTAA
<i>PAX6</i>	GCCGGCACACACACATTA	GAGCAATTCTCAGATTCTATGC
<i>RBFOX3</i>	CCAAGCGGCTACACGTCT	GCTCGGTCAGCATCTGAG
<i>RPL13A</i>	ATGACAAGAAAAAGCGGATG	CTTTTCTGCCTGTTTCCGTA
<i>SOX1</i>	GCCACAGTTTGGTCCAGAG	TTCATCCATGGCCTGGTC
<i>SOX2</i>	ATGGGTTCGGTGGTCAAGT	CTGATCATGTCCCGGAGGT
<i>TUBB3</i>	GCAACTACGTGGGCGACT	CGAGGCACGTACTTGTGAGA
<i>RAX</i>	GGCAAGGTCAACCTACCAGA	CTTCATGGAGGACACTTCCAG
<i>VSX2</i>	GGCGACACAGGACAATCTTTA	TTCCGGCAGCTCCGTTTTTC