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Development and fabrication by 3D printing of an openable microfluidic system instrumented with a bioimpedance biosensor for *in vitro* culture of 3D biological tissues

Développement et fabrication par impression 3D d'un système microfluidique ouvrable et instrumenté avec un capteur de bioimpédance pour la culture *in vitro* de tissus biologiques 3D

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“Never forget, the one who believes they know can no longer learn”

Pierre Bottero

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Abstract

Development and fabrication by 3D printing of an openable microfluidic system instrumented with a bioimpedance biosensor for *in vitro* culture of 3D biological tissues

Cell culture is an indispensable tool in biology. It has led to a better understanding of fundamental biological mechanisms and has been at the heart of progress in biology and medicine. However, conventional culture models (static *in vitro* 2D and 3D and *in vivo*) have limitations such as simplified tissue representation and interspecies incompatibility. Microfluidic systems better reproduce the dynamic environment of biological tissues by incorporating features such as interstitial flow. In this way, they facilitate the discovery of new drugs and the development of personalised medicine, in line with the 3Rs rule. Assessing the efficacy of these therapeutic candidates is generally based on measuring post-treatment cell viability. However, these tests are destructive and endpoint, providing no dynamic information about the tissues being studied. Bioimpedance sensors, based on the low-frequency measurement of the opposition of the cell membrane to the passage of an alternating current, provide a non-destructive, real-time measurement of cell density and therefore viability. In this context, the first objective of my thesis was to develop an openable and parallelised microfluidic system integrating a solid 3D tissue model. We defined its geometry by numerical modelling, taking into account the *in vivo* data available in the literature. We then developed a manufacturing protocol based on 3D printing. We studied its culture capacity by carrying out dynamic cultures of pancreatic cancer cells in 2D monolayers and 3D cultures. The second objective of my thesis was to integrate and characterise an impedance biosensor in the chambers of the microfluidic system. We defined its geometry using numerical modelling and studied its sensitivity. It was manufactured using photolithography and lift-off. Its characterisation enabled us to quantify the impact of geometry and temperature on the electrical measurement. Initial preliminary results validated that the instrumented microsystem could be used to monitor changes in cell density in dynamic 2D and 3D cultures of pancreatic cancer cells.

Résumé

Développement et fabrication par impression 3D d'un système microfluidique ouvrable et instrumenté avec un capteur de bioimpédance pour la culture *in vitro* de tissus biologiques 3D

La culture cellulaire constitue un outil indispensable en biologie. Elle a permis de mieux comprendre des mécanismes biologiques fondamentaux et a été au cœur de progrès en biologie et médecine. Cependant, les modèles de culture classiques (statiques *in vitro* 2D et 3D et *in vivo*) présentent des limitations telles que la représentation simplifiée du tissu ou l'incompatibilité inter-espèces. Les systèmes microfluidiques reproduisent mieux l'environnement dynamique d'un tissu biologique en intégrant des caractéristiques comme le flux interstitiel. Ils favorisent ainsi la découverte de nouveaux médicaments et le développement de la médecine personnalisée, tout en s'inscrivant dans la lignée de la règle des 3R. L'évaluation de l'efficacité de ces candidats thérapeutiques repose généralement sur la mesure de la viabilité cellulaire post traitement. Ces tests sont cependant destructifs et réalisés en point final, ne fournissant pas d'information dynamique sur les tissus étudiés. Les capteurs de bioimpédance, basés sur la mesure à basse fréquence de l'opposition de la membrane des cellules au passage du courant alternatif, permettent une mesure en temps réel, non destructive, de la densité cellulaire, et donc de la viabilité. Dans ce contexte, le premier objectif de ma thèse a été le développement d'un système microfluidique ouvrable et parallélisé intégrant un modèle tissulaire 3D solide. Nous avons défini sa géométrie par modélisation numérique en prenant en compte les données *in vivo* disponible dans la littérature. Nous avons ensuite développé un protocole de fabrication reposant sur l'impression 3D. Nous avons étudié sa capacité de culture en réalisant des cultures dynamiques de cellules cancéreuses pancréatiques d'une part en monocouche 2D et d'autre part en culture 3D. Le deuxième objectif de ma thèse consistait à intégrer et caractériser un biocapteur d'impédance dans les chambres du système microfluidique. Nous avons défini sa géométrie par modélisation numérique en étudiant sa sensibilité. Sa fabrication a été réalisée par photolithographie et lift-off. Sa caractérisation a permis de quantifier l'impact de la géométrie et de la température sur la mesure électrique. Des premiers résultats préliminaires ont permis de valider que le microsystème instrumenté permettait le suivi de l'évolution de la densité cellulaire au sein de cultures 2D et 3D dynamique de cellules pancréatiques cancéreuses.

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Abbreviations

AC	Alternating current
BMC	Bio-Med Clear
CP	Characterisation Platform
CC	Current carrying
ECM	Extracellular matrix
IF	Interstitial Flow
IFP	Interstitial fluid pressure
SLA	Laser stereolithography
LOD	Limit of detection
LCD	Liquid crystal display
MEA	Microelectrode array
OoCs	Organ-on-chips
PDAC	Pancreatic ductal adenocarcinoma
PBS	Phosphate-Buffered Saline
PU	Pick up
Pt	Platinum
PDMS	Polydimethylsiloxane
PVC	Polyvinyl Chloride
PCB	Printed circuit board
SBS	Society for Biomolecular Screening
SBB	Sudan Black B
Ti	Titanium
TOCs	Tumoroid-on-chips

Introduction

Chapter 1: Solid tumours: structure, models and viability assays

In this chapter, we discussed the significant and global health challenge posed by cancer, and more specifically solid tumours, which represent around 90 % of cancer cases. Solid tumours are complex and heterogeneous tissues. This complex biological entity generates intrinsic and extrinsic mechanical strains which are distinct from the inherent mechanical properties of the healthy tissue, such as its stiffness, that facilitate tumour progression and hinder its response to treatments. We then described the various models used to represent solid tumours and their specific advantages and drawbacks. Finally, we established an exhaustive list of cell viability assays to highlight their strengths and weaknesses based on various parameters such as their biological principle and compatible culture models.

1.1. Solid tumours

Cancer represents a major societal, public health and economical challenge of the 21st century. It is a critical obstacle that must be overcome to increase life expectancy and survival rates worldwide. It is the leading cause of death worldwide and represents almost one in six (16.8 %) deaths and one in four (22.8 %) of deaths due to non-transmissible diseases. In 2022, 20 new million cases of cancers were declared across the globe, while it was responsible for almost 10 million deaths. It is projected that by 2050, the number of new cases will increase to 35 million.¹

The same observation can be made in France, where cancer is the 1st cause of death in men and the 2nd in women. Historically, cancer incidence has seen a 100 % increase since 1990, with 433,136 new cases in 2023.² This increase can be attributed to environmental factors as well as to an aging population. However, thanks to new therapies and better diagnostic tools, cancer mortality rate has seen a steady 2 % decrease per annum for men (90,900) and 0.6 % decrease for women (71,500), which is comparable to the global trend with in average a 2.2 % annual decrease in mortality rate since 2009.³

The most common types of cancers are solid cancers with nearly 90 % of new cancers originating from solid tissue organs such as breast, lung, colon or pancreas.⁴ Not only are they the most common cancers, but they also account for 85 % of cancer related mortality,⁵ making them a major cause of morbidity and mortality throughout the world.

1.1.1. A complex and heterogeneous tissue

Solid tumours are defined as an abnormal and heterogeneous mass of tissue that result from the uncontrolled proliferation of cells (**Figure 1**). They can be either benign or malignant.⁶ Among them, carcinomas, which are the most common form of cancer, originate from healthy epithelial cells. A solid tumour's structure has two distinct but interdependent compartments: the parenchyma and the stroma.⁷ The parenchyma is composed of the cancer cells which originate from healthy cells. However, due to a dysregulation of growth and multiplication mechanisms, cancer cells proliferate uncontrollably until they invade the surrounding tissues.⁸

The stroma surrounds the cancer cells and is necessary for all solid tumours to survive and grow beyond a few mm³ as it provides the cancer cells with nutritional support and waste removal through the vascular and lymphatic systems.⁹ While emphasis has been historically placed on the blood vessels contained in the stroma, mainly to study angiogenesis, the tumour microenvironment is a highly heterogeneous structure with both acellular and cellular components.¹⁰ Cellular components are specialised connective-tissue (fibroblasts) and immune cells, but also endothelial cells for both blood and lymphatic vessels, while the acellular component of the stroma is mainly composed of extracellular matrix (ECM) proteins such as collagen fibres and hyaluronic acid.

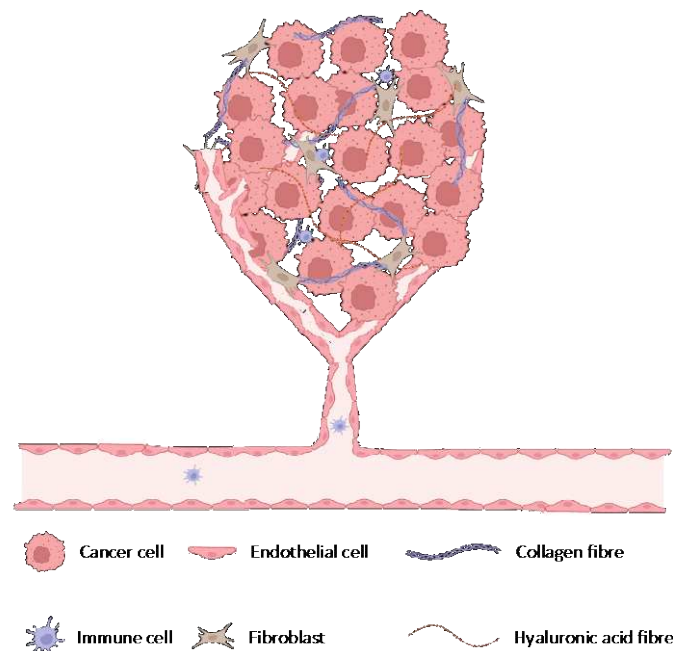


Figure 1. Solid tumour and microenvironment. Solid tumours are complex and heterogeneous tissues composed of both cellular (cancer cells, fibroblasts, immune cells) and acellular components.

This hyperproliferation of cancer cells results in the generation of mechanical forces that are anomalous and have an impact on both the cells and the tumour microenvironment through mechanosensitive developmental processes.¹¹

1.1.2. The mechanics of solid tumours

Tumour development involves the generation of intrinsic and extrinsic mechanical strains which are distinct from the inherent mechanical properties of the tissue, such as its stiffness, that facilitate tumour progression and hinder its response to treatments. These mechanical cues can be decomposed in two types: solid stress, which results from the mechanical forces exerted by non-fluid components, and fluid stress, which results from the mechanical forces exerted by fluid components.¹²

1.1.2.1. Solid mechanics of solid tumours

Solid stress accumulates during tumour growth and has two components. The first one is growth-induced stress, which is solely held within the tumour and represents roughly 30 % of total solid stress. It has cellular origin from the cancer cell proliferation as well as their contraction against the ECM.^{13,14} It has also an acellular origin from the increased deposition of ECM proteins, with some being resistant to tensile (collagen) and others resistant to compressive forces (hyaluronic acid).¹⁵ The second component is externally applied stress, which is generated as the tumour pushes against the surrounding healthy tissue.¹⁶ Both are additive, and their sum is known as the total solid stress. This stress is direction-dependent, as it is compressive both radially and circumferentially at the tumour's core,¹⁷ while it is compressive radially and tensile circumferentially at the tumour periphery (**Figure 2.a**).¹⁶

The generation of solid stress affects tumour cells through mechanosensitive oncogenic signalling pathways, and upregulates the deposition of ECM components, but also increases the chemoresistance of cancer cells.¹⁸ Solid stress also compresses and collapses intratumoral blood and lymphatic vessels, and deforms peritumoral lymphatic vessels (**Figure 2.b**). This disturbs both the blood and lymphatic flows, generating fluid stresses that further affect cancer cells and the tumour's microenvironment.

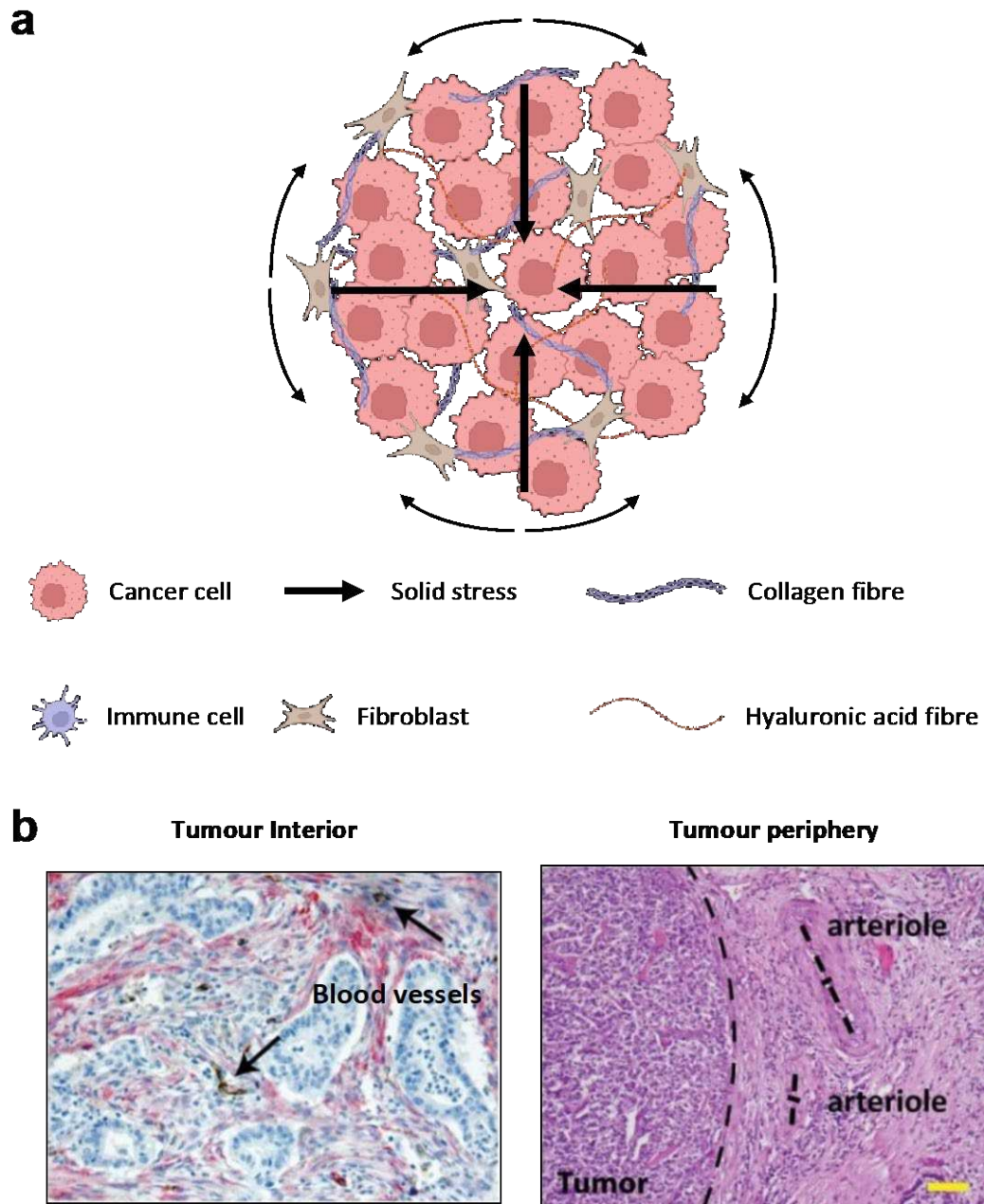


Figure 2. Solid stress profile in solid tumours. (a) At the tumour's core, solid stress is radially and circumferentially compressive, while it is compressive radially and tensile circumferentially at the tumour's periphery. (b) This stress profile causes intratumoral vessels to collapse (histologic section of human pancreatic tumour, arrows show position of collapsed vessels) [17] while peripheral vessels have elliptical shapes instead of their normal circular shape (dashed lines show tumour margin and two main axes of the arterioles) [16].

1.1.2.2. Fluid mechanics of solid tumours

In healthy tissues, the blood and lymphatic systems work together to regulate fluid homeostasis, nutrient exchange, and immune surveillance (**Figure 3.a**). Blood capillaries supply oxygen and nutrients while facilitating waste removal, whereas lymphatic vessels drain excess interstitial fluid and macromolecules, maintaining both interstitial fluid pressure (IFP) and composition.¹⁹ Interstitial fluid is the fluid contained within the stroma, or interstitial space. Its movement, known as the interstitial flow (IF), is driven by both hydrostatic and osmotic pressure gradients between the vascular and interstitial space in a laminar and pulsatile manner.²⁰

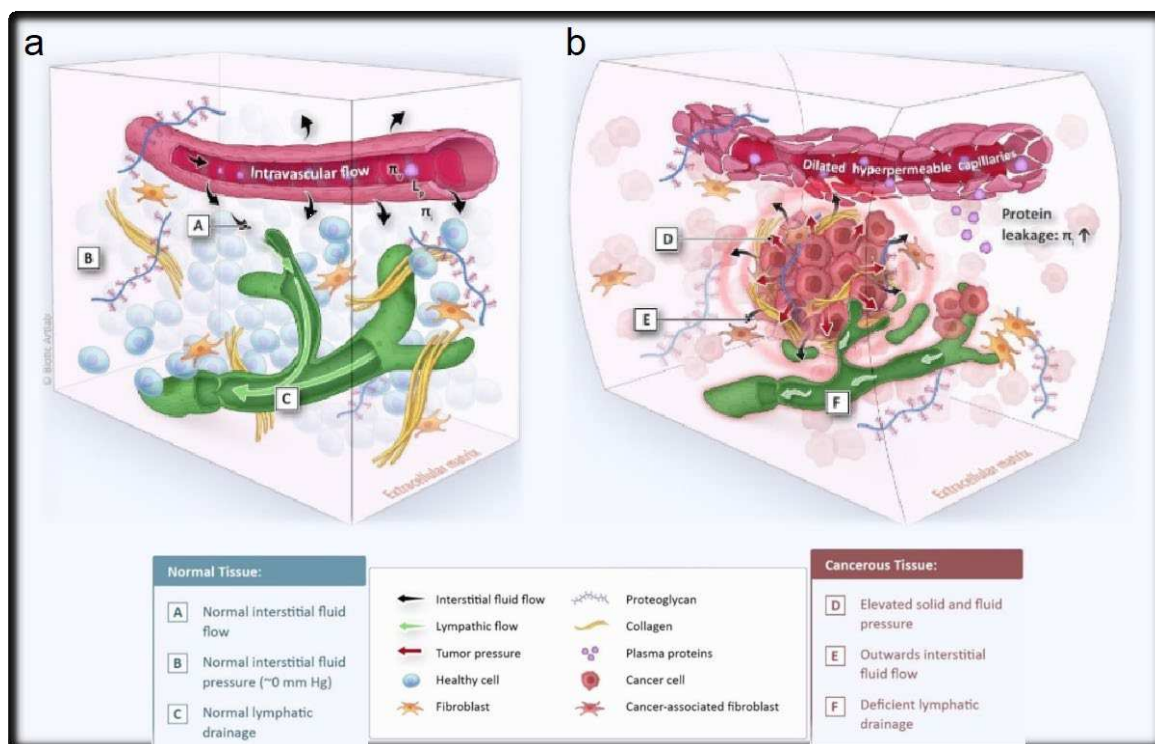


Figure 3. Differences in architecture and biophysics between normal tissue and solid tumour tissue. (a) In normal tissue, fluid homeostasis is preserved through balancing fluid inflow (vascular) and outflow (lymphatic). This results in a low or even negative interstitial fluid pressure. (b) In solid tumours, blood vessels are often immature resulting in increased leakage of fluid in the interstitial space. On the other hand, lymphangiogenesis occurs at the tumour periphery. These changes result in increased interstitial fluid pressure leading to a radially outward leakage of interstitial fluid. This in turn reduces the convective flux of drugs into the tumour core. From [23].

However, as we have previously described, solid stress collapses intratumoral vessels.²¹ Moreover, tumour blood vessels are often immature, with significantly larger pores (~ μm) compared to mature vessels (~ nm),²² leading to excessive fluid leakage into the interstitial space. Extravasation of blood plasma and proteins into the tumour interstitial space, combined with poor lymphatic drainage results in an increase of IFP within the tumour (**Figure 3.b**).²³

As for peritumoral lymphatic vessels, the increased secretion of vascular endothelial growth factor induces tumour associated lymphangiogenesis, resulting in lymphatic drainage occurring only at the tumour periphery.²⁴ The ensuing pressure difference between the tumour and surrounding tissue leads to an IFP gradient which decreases from the tumour centre towards its periphery. This creates an outward directed IF, which is estimated to be of a magnitude higher than the velocity of IF in healthy tissues (~ 1 to $4 \mu\text{m.s}^{-1}$ in solid tumours vs ~ 0.1 to $1 \mu\text{m.s}^{-1}$ in healthy tissues).²⁵ Consequently, this outward-directed IF reduces the net convective flux of drugs into the tumour core, reducing therapeutic efficacy.²⁶

To summarise, solid tumours are complex abnormal masses of tissue containing a variety of cellular and acellular components. Furthermore, as a solid tumour develops, it generates anomalous mechanical cues that protects it from both the immune system and targeted therapies. Incorporating these mechanisms in tumour models is therefore essential to understand and counter act them when developing new therapeutic strategies.

1.2. Classical models of solid tumours

2D cell culture has been a method for studying cell biology, tissue morphology and mechanisms of diseases for more than a century. It was first pioneered out by Ross Granville Harrison in 1907, when he studied embryonic neurons from a frog.²⁷ Since then, cell culture has been at the foundation of scientific advances in biology and medicine such as the discovery of the polio vaccine.²⁸ Naturally, cell culture is heavily utilised in cancer research, and is a tool to study cancer biology and drug resistance.²⁹ For example, HeLa cells, the first “immortal” human cell line immortalised from a human cervical cancer tissue in 1952,³⁰ were used to study a variety of cancer-related questions such as the effect of X-rays on cells³¹ or drug sensitivity.³²

Although the 2D culture of cells as a monolayer is the oldest and simplest method for cell culture, alternative culture methods have been developed, with three approaches commonly used to study cancer: the static 2D model, the static 3D model and the animal, or *in vivo*, model. Despite advances in these techniques, developing new, effective treatments for cancer remains difficult. Indeed, as these models fail to fully represent the complex characteristics of the disease, they are inherently incapable of precisely predicting *in vivo* the drug response.

1.2.1. Static 2D *in vitro* models

As mentioned, 2D *in vitro* models, or “in glass” models, were the first cell culture models to be developed. They rely on the culture of adherent cells in a single 2D monolayer on a plastic layer (Petri dish, culture flask).³³ It is the simplest type of cell culture, and has been extensively used for drug discovery as it has a low-cost and maintenance.^{34,35}

2D models have also been used to study the effects of mechanical cues on cells. Zhaodong et al. isolated and characterised rat portal fibroblasts and subsequently demonstrated that cells cultured on polyacrylamide supports of variable stiffness had an increasingly myofibroblastic phenotype proportional to stiffness increase.³⁶ Similarly, compression has also been studied in 2D cell culture models. Using MCF-7 cells (mammary carcinoma), and by pushing them against a membrane surface with a weighted piston, Tse et al. studied the effects of compression.³⁷ They showed that compression remodelled the cell's cytoskeleton, inducing a leader-cell phenotype to appear which was correlated with increased migration and adhesion.

However, 2D cell culture is sometimes considered as over simplistic. As cells are cultured in a monolayer, they cannot recapitulate the complex and heterogeneous aspect of solid tumours and their microenvironment, prohibiting the generation of meaningful results.³⁸ Moreover, a homogenous amount of nutrients and oxygen is available for all cells, which is not representative of intratumoral gradients. Finally, 2D models also have limitations such as lack of tissue specificity and cell-cell and cell-matrix interactions.³⁵ Collectively, these limitations decrease the likelihood of results being predictive of *in vivo* tests, encouraging researchers to develop more relevant tools, leading to the advent of 3D models.

1.2.2. Static 3D *in vitro* models

In 1977, Anne W. Hamburger and Sydney E. Salmon developed an *in vitro* assay that permitted colony formation of human myeloma stem cells in soft agar.³⁹ With this method, they were able to grow tumour cells in 3D which formed cell aggregates called spheroids. Interestingly, they found that different cell types appeared during the cell culture, proof that this method could reproduce the tumour's *in vivo* cell population heterogeneity. They believed that, because many biophysical aspects of tumour stem cell could be characterised with this method, it could be applied for predictive trials of anti-cancer drugs.

Nowadays, static 3D cell culture methods can be categorised in two major types: scaffold-based 3D cell culture, and scaffold-free 3D cell culture.⁴⁰ Scaffold-based cultures utilise a scaffold to mimic the native ECM, with the scaffold being either of natural origin, such as Collagen 1 or MatrigelTM (derived from Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells),⁴¹ or of synthetic origin.⁴² Scaffold-free assays, on the other hand, rely on cell-cell interactions and on cells depositing their own ECM to form 3D structures. Scaffold-based assays better mimic cell-to-ECM interactions while scaffold-free spheroids allow for more accurate cell-cell interactions.⁴³ Both methods allow cells to grow in a more physiological 3D environment and to interact with other cell types and ECM, resulting with physical and spatial organisations that more closely mimic *in vivo* conditions.

When compared to 2D cell culture, 3D models exhibit more phenotypically heterogeneous cell colonies that are organised in layers, with proliferating cells at the outer layers, and necrotic cells located towards the spheroid core.³⁵ This is due to the 3D spatial organisation resulting in the formation of nutrients and oxygen gradients across the spheroid, with the core cells being deprived compared to the outer cells.⁴⁴ This was shown to have a direct impact on drug sensitivity, with Tung et al. demonstrating that because a spheroid had less proliferative cells than a 2D layer, it had a lower response to 5-fluorouracil,⁴⁵ as it is a chemotherapy that triggers apoptosis in cancer cells by disrupting DNA and RNA synthesis. This was also demonstrated in colorectal cancer HCT-116 spheroids by Karlsson et al. with a survival index of 100 % for 6 days old 3D spheroids and 0 % for the monolayer when exposed to 100 μ M of 5-fluorouracil.⁴⁶ More recently, Nsaif et al. performed a similar study on five different colorectal cancer cell lines. They cultured them in both 2D monolayers and 3D spheroids, and characterised viabilities when exposed to a variety of anti-cancer drugs.⁴⁷ For all cell lines, 3D structures had a lower drug sensitivity compared to 2D monolayer. This highlights that 3D models are being found to be better predictor of *in vivo* drug response than 2D models.⁴⁸

Similar to 2D models, 3D models have been used to study the effects of mechanical cues on cancer cells and their resistance to drugs. Rizzuti et al., through the confined compression of a pancreatic cancer cell line in agarose gel, showed that compression led to a less sensitive phenotype, with unconfined spheroids decreasing in size by roughly 30-40 % and confined spheroids by less than 10 % when exposed to gemcitabine, a chemotherapy that induces apoptosis by halting DNA replication.¹⁸ However, although promising and more

physiologically relevant than 2D *in vitro* models, 3D *in vitro* models also suffer from drawbacks. Most notably, these models have been described as expensive, time consuming and lacking reproducibility.^{49,50} Furthermore, while permitting the incorporation of certain mechanical cues, its static aspect prevents the reproduction of fluid stress and its effects on tumour growth and development.

1.2.3. In vivo animal models

In vivo means “within the living” and involves studying cells within a living organism. It has long been considered the golden standard for understanding complex tissue interactions. There are many animal models used for cancer research, such as genetically modified mouse models,^{51,52} zebra fish⁵³ or pigs.⁵⁴ However, these models have often faced increasing criticism in recent years.⁵⁵ Each animal cancer model has its specific limitations. For example, zebrafish and drosophila models present difficulty in the examination of fixed tissues, while pig cancer models require larger housing equipments.⁵⁶ Despite their specificities, animal models also share common limitations, such as their time-consuming and technically complicated nature. Furthermore, they present a different immune response to humans, reducing the predictivity of animal models for drug response assays. In addition, even though these models summarise the mechanical forces experienced by tumours, it remains difficult to apply controlled mechanical strains and to differentiate their effects from other experimentally induced factors.⁵⁷

Recent governmental regulations aiming to push alternative methods to animal experimentation, in accordance with the 3R principles, are being passed. The 3R principles, developed in 1959 by William Russell and Rex Burch, have become synonymous with measures to improve the welfare of animals used in research.⁵⁸ They are defined as Replace (to avoid animal experiments altogether), Reduce (limit the numbers of animals used for experimentation) and Refine (limit the animal’s suffering to a maximum) and are more and more required for new studies.⁵⁹ Furthermore, as of December 2022 and the signature of the FDA Modernization Act 2.0, animal testing is not mandated for every new drug development protocol,⁶⁰ paving the way for the development of alternative models.

In conclusion, *in vivo* animal models, 2D *in vitro*, and 3D *in vitro* static models offer distinct advantages and limitations in cancer research, reflecting at various degrees the complexity of solid tumours (**Table 1**). *In vivo* animal models remain the gold standard for capturing the interactions within living organisms but face growing ethical and practical

criticisms. Simple and cost-effective, static 2D *in vitro* models have contributed significantly to drug discovery but fail to replicate the microenvironments and nutrient gradients of solid tumours. Meanwhile, static 3D *in vitro* models offer a more physiologically relevant system that better mimics *in vivo* conditions but still lack parameters such as fluid flow. While these models exhibit greater resemblance to tumour heterogeneity and drug response dynamics, they suffer from complexity and reproducibility challenges. This underlines the need for continued development of more physiologically relevant models, to better understand cancer mechanisms and improve therapeutic outcomes.

Table 1. Comparison of static *in vitro* 2D, 3D and *in vivo* cell culture models. Inspired from [37] and [58].

Type of culture	Static 2D	Static 3D	<i>In vivo</i>
Time of culture formation	Minutes to hours	Hours to days	Days to months
Morphology	Sheet-like flat and stretched monolayer	Spheroid/aggregate 3D structure	<i>In vivo</i> cellular morphology
Cell interactions	Limited cell-cell and cell-ECM interactions	More relevant cell-cell and cell-ECM interactions	Physiological interactions
Access to essential compounds	Equal access to nutrients/oxygen/drug compounds	Molecule gradients formation	Physiological metabolism of drugs
Mechanical cues incorporation	Stiffness, solid stress	Stiffness, solid stress	Stiffness, solid and fluid stresses
Relevance	+	++	+++
Costs	\$	\$\$	\$\$\$

1.3. Viability assays of solid tumours

As part of developing new anti-cancer therapies, it is not sufficient to only develop biological models, but it is also crucial to properly assess the efficacy of the drugs on such models. For most anti-cancer drugs, the aim is to induce cancer cell death through varying mechanisms (apoptosis, necrosis, autophagy).⁶¹ Efficacy is therefore measured as a product of both reduced cell viability and proliferation⁶² and important analytical considerations such as reproducibility and sensitivity need to be taken into account. While there are many cell viability assays such as real-time imaging (Incucyte) or electrical-based viability assays, the most commonly assays used for drug sensitivity testing rely on two working principles: metabolic activity and cell membrane integrity.⁶³

1.3.1. Metabolic activity-based cell viability assays

Metabolic activity is a widely used indicator for assessing cell health, as it reflects the functional state of living cells. These assays operate on the principle that viable cells enzymatically reduce added substrates, leading to the formation of detectable products (fluorescent or colorimetric) that can be quantified. A key example is the reduction of mono-tetrazolium salts by active mitochondria, yielding a violet-blue, water-insoluble molecule known as formazan.⁶⁴ This reaction forms the basis of the MTT assay, which has long been considered a gold standard for evaluating cell viability in cancer research.⁶⁵ A major application of this technique lies in determining the half-maximal inhibitory concentration (IC₅₀) of a drug defines the concentration required to reduce the targeted cell population by 50 % (**Figure 4**).

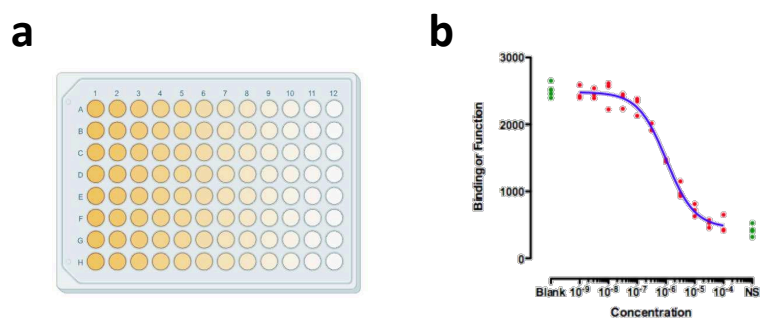


Figure 4. Cell viability assessment post-drug exposure. (a) Top view of a 96-well plate for MTT colorimetric assay. As the cell viability decreases, so does the opacity of the MTT solution (left to right). From Biorender. (b) IC₅₀ curve extracted from the colorimetric assay reading. From GraphPad.

However, metabolic activity-based assays have inherent flaws. First, they rely on live cells reducing a chemical substrate. The reduced molecule is then released from the cells by cell lysis as it is not permeable. Consequently, metabolic activity-based assays do not allow for real-time measurements. This is not the case for the resazurin viability assay as resorufin (reduced form of resazurin) is permeable to cell membrane.⁶⁶ Secondly, although simple, the experimental protocols associated with these assays can introduce unwanted variability in viability measurements.⁶⁷ Factors such as incubation time, reagent stability, and cell density can significantly influence the final readout. Finally, studies have demonstrated that the measured absorbance does not solely correlate with the number of cells but is also influenced by their metabolic activity. Indeed, for a fixed cell count, a higher metabolic rate results in greater substrate reduction, leading to ambiguous cell survival results.⁶⁸

1.3.2. Membrane integrity-based viability assays

Membrane integrity-based assays are another widely used method to assess cell viability after drug administration. They rely on the differential uptake or expression of fluorescent markers between live and dead cells. This can be performed for example through propidium iodide staining. As the membrane of live cells is intact, and because propidium iodide is membrane-impermeant, it will only stain dead cells, allowing visual differentiation between both live and dead cells.⁶⁹ Other dyes, such as Trypan Blue, can be used to stain dead cells and is used for manual or automated cell counting.⁷⁰ However, such staining methods do not reveal the cell death mechanism and data reproducibility relies on microscope/camera/software settings.⁷¹ Although 2D cell cultures are easily imaged, that is not the case for 3D cell cultures as their thickness commonly exceeds the depth of focus of a conventional microscope.⁷² This results in out-of-focus images superposed to the focal plane, inducing a decrease in both image quality and contrast ensuing in a blurry image (**Figure 5.a**). This phenomenon, known as light scattering, can be reduced by imaging individual samples of the plane. This is achieved by more complex imaging techniques such as confocal laser microscopy or light sheet fluorescence microscopy, but these techniques often require more complex sample preparation such as sample clearing for light sheet fluorescence microscopy (**Figure 5.b and c**).

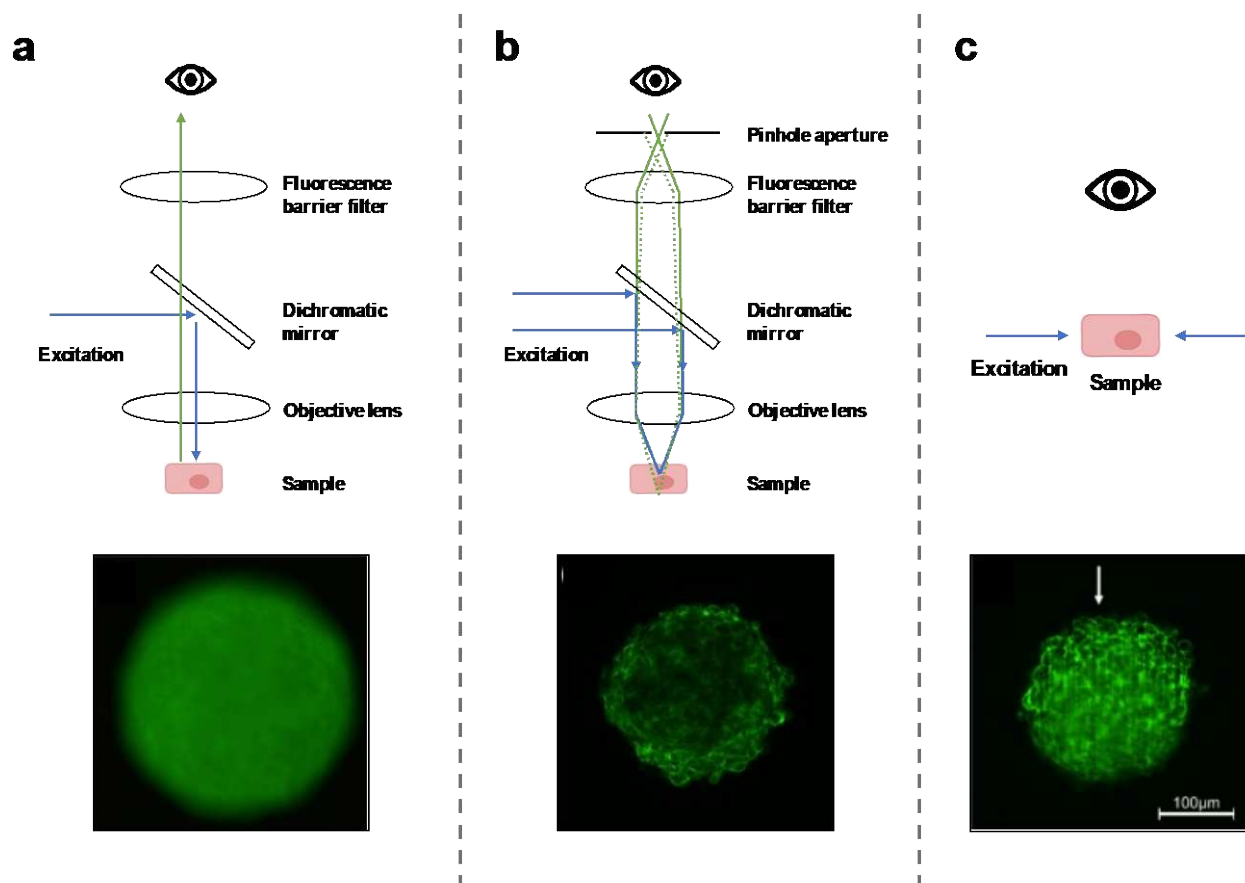


Figure 5. Different fluorescence microscopy techniques and principles. (a) Wide field microscopy. The entirety of the sample is exposed to a light source and subsequently emits fluorescence. As out-of-focus images are not filtered, this results in a blurry image. (b) To counteract this, an aperture is utilised in confocal imaging to focus the resulting fluorescence. (c) Light sheet laser microscopy illuminates the sample from the side to excite only the desired plane which is always in focus with the objective lens. All images have an excitation wavelength of 488 nm and fluorescence detection at 505 nm. Reproduced in parts from [72].

A more reproducible membrane integrity-based assay involves staining cells with Annexin V followed by analysis via flow cytometry. In the early stages of apoptosis, although cells maintain overall membrane permeability and integrity, their membrane composition undergoes distinct changes with phospholipids becoming loosely packed and phosphatidylserine translocating to the outer leaflet. Fluorescently labelled Annexin V reliably binds to these exposed phosphatidylserine residues. This method enables researchers to precisely distinguish between early apoptotic, late apoptotic, and necrotic cells.⁷³ However, it remains an endpoint assay, offering only a snapshot of cell death without real-time monitoring of dynamic changes during drug administration. Flow cytometry can also be directly used as a measure of cell viability via membrane integrity without the need for fluorescent markers. It

enables the quantitative measure of cell viability based on both cell size and membrane granularity.⁷⁴

1.3.3. Hybrid cell viability assays

Hybrid viability assays combine principles from both metabolic activity and membrane integrity tests, offering a more comprehensive evaluation of cell fate. For instance, Calcein-AM requires intracellular esterases to convert it into a fluorescent product to reflect metabolic activity. However only cells with intact membranes retain the dye, indicating membrane integrity.⁷⁵ LDH release assay detects lactate dehydrogenase in the extracellular medium, an enzyme produced in cells undergoing apoptosis, necrosis or other form of cellular damage, that leaks out upon membrane rupture.⁷⁶ Although these approaches provide a more nuanced assessment of cell viability, they are typically endpoint and often destructive. Collecting supernatant for LDH measurements can disrupt the culture, and repeated washing or staining steps in calcein-based assays may introduce variability.

To summarise, a wide variety of cell viability assays that provide valuable insights into drug sensitivity testing exist (**Table 2**). Yet, each assay has distinct advantages and limitations. Metabolic-based assays remain a gold standard due to their simplicity and reliability but lack real-time monitoring and can introduce variability.^{77–80} Membrane integrity-based assays offer more precise differentiation between live and dead cells enabling deeper mechanistic insights. However, their applicability to 3D cultures remains challenging due to imaging constraints such as light scattering and depth-of-focus limitations. Other live imaging assays such as colony formation assay and Incucyte are non-toxic but often only applicable to 2D cell monolayers.^{81,82} Hybrid viability assays bridge the gaps between both metabolic and membrane integrity-based assays but are still destructive and endpoints assays. These drawbacks can be circumvented by utilising electrical-based impedance assays, as we will see in later.⁸³

Table 2. Principles and applications of cell viability assays.

Assay	Biological principle	Measurement method	Temporal readout	Impact on cells	Compatible culture models	References
Trypan blue	Membrane integrity	Microscopy, automatic cell counter	Endpoint	Toxic	Cell suspension	[70]
MTT	Metabolic activity	Colorimetric	Endpoint	Toxic	Cell suspension, 2D	[65]
MTS	Metabolic activity	Colorimetric	Endpoint	Toxic	Cell suspension, 2D	[77]
Resazurin	Metabolic activity	Fluorometric	Endpoint, can be adapted to real-time	Non-toxic	Cell suspension, 2D, 3D	[78]
ATP quantity	Metabolic activity (ATP production)	Luminometric	Endpoint	Toxic	Cell suspension, 2D, 3D	[79]
Flow cytometry	Membrane integrity	Cell size, granularity	Endpoint	Destructive	Singles cells derived from previous 2D or 3D culture	[74]
Annexin V	Membrane integrity	Fluorescence flow cytometry	Endpoint	Toxic	Single cells	[73]
Calcein-AM	Metabolic activity, membrane integrity	Fluorescence microscopy	Endpoint, can be adapted to real-time	Non-toxic based on used concentration	Cell suspension, 2D, 3D	[75]
Propidium iodide	Membrane integrity	Fluorescence microscopy	Endpoint	Destructive	Single cells, cell suspension, 2D, 3D	[69]
MitoTracker	Mitochondria mass	Fluorescence microscopy	Real-time	Non-toxic based on used concentration	Cell suspension, 2D, 3D	[80]
Colony formation	Proliferation	Microscopy	Endpoint	Destructive	2D	[81]
LDH	Lactate dehydrogenase production	Colorimetric	Endpoint	Non-toxic	Cell suspension, 2D, 3D	[76]
Incucyte	Proliferation	Phase-contrast microscopy	Real-time	Non-toxic	2D	[82]
Electrical impedance	Electrical properties	Bioimpedance	Real-time	Non-toxic	Single cells, cell suspension, 2D, 3D	[83]

1.4. Conclusion

In conclusion, cancer, and in particular solid tumours, represents a significant public health challenge. Although it has been the focus of research, the number of cases is projected to keep increasing to 35 million by 2050. Solid tumours are complex and heterogeneous mass of tissue, with mechanical cues such as IF playing a crucial role in their progression and resistance to treatment. While traditional models such as *in vivo* animal models, 2D *in vitro* models, and 3D *in vitro* models have significantly advanced our understanding of cancer biology, each has its inherent limitations in fully recapitulating the complexity of solid tumours. Animal models, despite being the gold standard for *in vivo* interactions, face ethical and practical challenges, while 2D cell culture remains a simplistic tool that fails to reproduce the tumour microenvironment accurately. 3D models, although more physiologically relevant, still struggle with issues such as static conditions and lack of fluid flow, which are crucial for understanding tumour mechanics and treatment responses.

Furthermore, current viability assays lack the capability to provide viability measurements in a real-time, non-destructive manner when measuring responses to therapy, limiting their utility in studying tumour progression and drug efficacy. Addressing these limitations through the development of more representative models and the incorporation of non-destructive viability assessment methods will enable more precise investigation of solid tumour therapeutic responses. This highlights the ongoing need for improved analytical methods to enhance reproducibility and sensitivity in drug efficacy assessment, particularly in physiologically relevant 3D models.

Chapter 2: Microfluidic systems

In this chapter, we present the relevance of microfluidic technologies, particularly Organ-on-Chips (OoCs), in developing more accurate *in vitro* models to study physiological processes and disease mechanisms. We discuss how these systems leverage microfluidic principles to generate well-controlled laminar flows ensuring precise manipulation of fluids and well-defined concentration gradients. Their micrometric dimensions are particularly suited for recreating biological phenomena such as IF, allowing for the reproduction of key physical aspects of tissues. This led to the emergence of Tumoroid-on-Chips (ToCs), dedicated to studying solid tumours and their microenvironments, providing invaluable insights into cancer progression, drug resistance, and therapeutic efficacy. We then discuss fabrication methods for ToCs, with the emergence of 3D printing as an alternative fabrication method.

2.1. Microfluidic systems as alternative biological models

To address the afore mentioned limitations in the development of more accurate and relevant solid tumour models for studying diseases and drug response/toxicity, OoCs, also known as microphysiological systems, have attracted substantial interest.⁸⁴ OoCs are platforms that serve as an alternative culture system to traditional culture methods, as their versatility enables *in vitro* models for studying physiological processes.⁸⁵

Their working principle relies on the adaptation of microfluidic technologies to cell culture.⁸⁶ Microfluidics is defined as the science and technology of systems that process and manipulate small (10^{-9} to 10^{-18} L) amounts of fluids using channels with dimensions of tens to hundreds of μm .⁸⁷ The use of such dimensions presents the advantage of enabling the generation of laminar flows with no turbulences. Laminar flow (**Figure 6.a**) occurs when a fluid flows in infinitesimal parallel layers with no disruption between them, as opposed to a turbulent flow (**Figure 6.b**), and was first studied and theorised by Osborne Reynolds in the 19th century.⁸⁸

He later proposed a mathematical parameter, *Reynolds number*, that describes the global behaviour of flow:

$$Re = \frac{\rho U_0 L_0}{\eta} \quad (1)$$

with ρ the fluid density, U_0 its velocity, L_0 the characteristic length and η the fluid's dynamic viscosity.

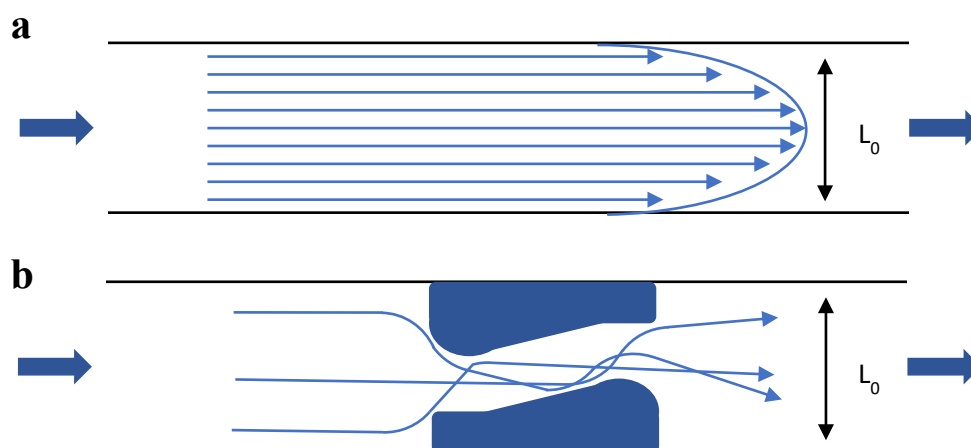


Figure 6. Velocity profile of a fluid in a pipe. (a) Velocity profile of a laminar flow in a pipe with characteristic length L_0 . (b) Velocity profile of a turbulent flow in a pipe due to obstacles.

In microfluidic systems, the Reynolds number is typically very low (< 100), ensuring laminar flow conditions where viscous forces dominate over inertial forces.⁸⁹ This laminar regime allows for well-defined concentration gradients, as mixing occurs predominantly via molecular diffusion rather than convective turbulence.⁹⁰ Such diffusion-limited transport enables the generation of sharp step gradients across microchannels, which is particularly advantageous for microfluidic platforms requiring precise spatiotemporal control of reactions. These properties facilitate reduced reagent consumption and enhanced reaction efficiency through precise dimension control.⁹¹

2.1.1. Historical development of microfluidic systems for cell culture

In 1979, S.C. Terry developed what can be considered as the first microfluidic system: a miniature gas analysis system, or chromatograph, that enabled the separation of gaseous hydrocarbon mixtures (**Figure 7.a**).⁹² He achieved this by utilising microfabrication techniques to create a miniaturised silicon microchannel. Although this work represented a significant breakthrough, it did not resonate in the scientific community. It was only in the 1990s that the development of miniaturised systems had a second wind. Indeed, both the development of DNA microarray (**Figure 7.b**) to monitor the expression of genes in parallel,⁹³ and funding from the military, especially from DARPA (Defence Advanced Research Projects Agency), resulted in a boost in the development of microfluidic systems. Another reason is the publication in the early 1990s of the first theorised Lab-on chip, or Micro Total Analysis System by Manz et al.,⁹⁴ where they presented the key points and requirements for such a

platform, with potential techniques and the advantages presented by such a device. The purpose of such devices is to enable the precise manipulation of fluid samples, reduced consumption of samples and reagents, shorter analysis times, greater sensitivity and facilitating commonplace biological approaches such as microscopy.⁹⁵

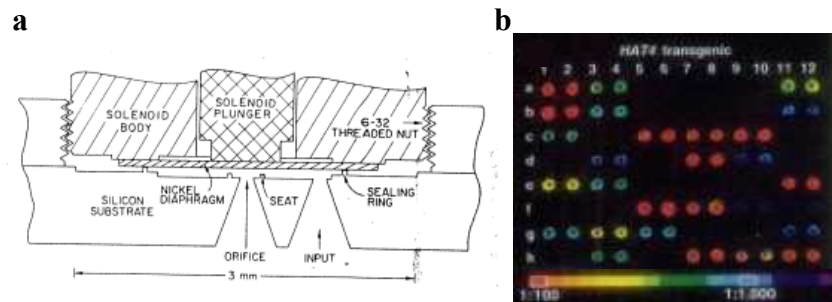


Figure 7. First microfluidic systems. (a) Cross section of miniature valve of the developed chromatograph by S.C. Terry. Adapted from [92]. (b) Single DNA array developed by Schena et al. Adapted from [93].

Thanks to their micrometric dimensions which are particularly suited for cell manipulation, and to the advent of new fabrication materials such as polydimethylsiloxane (PDMS) in the 1990s,⁹⁶ microfluidic systems have since been a focal point in cell culture. Due to the application of a dynamic laminar flow, they enable the reproduction of key physical aspects of biological tissues such as the IF. One of the first studies focusing on the effects of flow on cells was performed by Tilles et al., whom cultured 2D rat primary hepatocytes in a microfluidic system.⁹⁷ They showed that while capable of maintaining a viable cell culture over a 10-day period, higher levels of shear stress (5 to 20 dyn.cm⁻²) negatively affected hepatic function, with a reduction in both albumin and urea secretion.

However, similar to static *in vitro* 2D models, 2D microfluidic models cannot recapitulate tissue specificity and cell-cell and cell-matrix interactions. To overcome these limitations, researchers have developed more sophisticated models, with the aim of recapitulating tissue and organ specific functions, giving rise to the name Organ-on-chips. The first OoC was developed in 2010 by Huh et al., who presented a multifunctional microdevice reproducing the structural, functional, and mechanical properties of the human alveolar-capillary interface.⁹⁸ The device featured human alveolar epithelial cells and pulmonary microvascular endothelial cells separated by a porous, flexible PDMS membrane, with vacuum side chambers to simulate breathing motions through membrane stretching. Despite key differences when compared to the *in vivo* alveolar-capillary barrier, the device demonstrated

the potential of OoCs to mimic physiological functions. It successfully stimulated surfactant production, strengthened barrier integrity, and replicated key lung-specific responses, including inflammatory processes and nanoparticle uptake. OoCs have since being utilised to model a wide variety of tissues such as the heart,^{99,100} liver,¹⁰¹ gut,¹⁰² neurons,¹⁰³ and many others.

These works show that microfluidic systems, and more specifically OoCs, offer a high degree of versatility in replicating physiological interactions. Over the past decades (**Figure 8**), microfluidics systems have therefore emerged as powerful tools across various biological applications, showcasing its potential to unravel complex processes and refine experimental approaches to enhance the physiological relevance of *in vitro* studies.

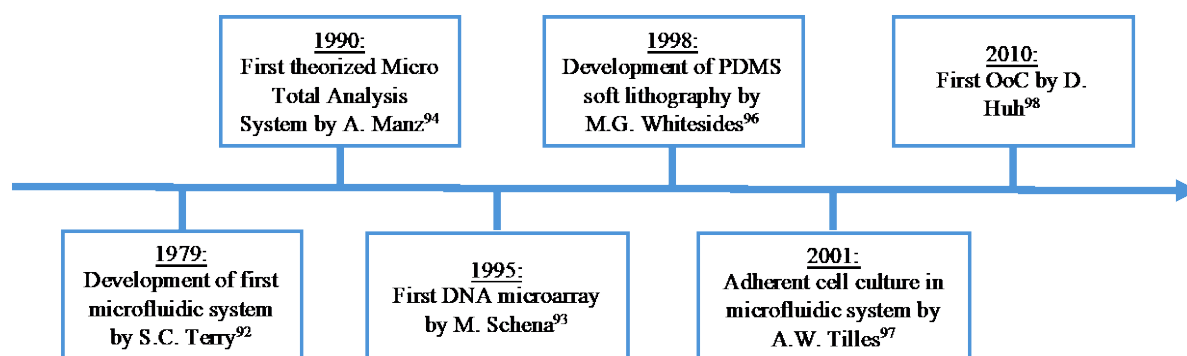


Figure 8. Historical timeline of microfluidic system development for cell culture.

2.1.2. Contribution of microfluidics to cancer research

The capacity of OoCs to recreate tissue-level and organ-level structures and functions was also leveraged to model solid tumours and their microenvironments, giving rise to a sub category of OoCs known as ToCs.¹⁰⁴ They have been utilised to study tumour characteristics and to shed light on key aspects of tumour growth and development such as angiogenesis,^{105,106} epithelial-mesenchymal transition¹⁰⁷ or metastasis.¹⁰⁸ Furthermore, ToCs have been used to impose dynamical flow to study the effects of shear stress on tumour behaviours and development,¹⁰⁹ as well as studying the effects of solid stress on cancer metastasis and aggressiveness.¹¹⁰ Onal et al. for example studied the effects of cyclic compression on ovarian cancer cells.¹¹¹ They observed that cells responded differently when faced with varying levels of compression, giving insight into the mechanics of cancer cells through the observation of morphological, cytoskeletal and nuclear differences.

Another aspect of ToCs is the study of resistance to treatment by recreating more physiologically relevant culture conditions. Such studies investigating lung cancer, revealed the impact of chemical gradient on the tumour's drug response. More precisely, Chen et al. developed a microfluidic system capable of generating oxygen gradient and demonstrated that lung cancer cells were less sensitive to tirapazamine (chemotherapy that forms toxic radicals in hypoxic conditions) in lower oxygen conditions.¹¹² Another study performed by Beer et al in 2017 investigated the drug response of pancreatic ductal adenocarcinoma (PDAC) cells in different culture models. They cultured Panc-1 PDAC cells and assessed treatment response to cisplatin (platinum-based chemotherapy that blocks replication and transcription) in 2D, 3D static, 3D microfluidic (commercial HEPA chip), and *in vivo* models.¹¹³ The measured IC₅₀ values were $3.3 \pm 0.2 \mu\text{M}$ and $14.6 \pm 1.6 \mu\text{M}$ in 2D and 3D static models, respectively. Notably, the microfluidic chip demonstrated a closer approximation to *in vivo* (animal) drug response, with a concentration of 100 μM required to achieve a 30 % reduction in cell viability, compared to the estimated *in vivo* plasma concentration of 240 μM needed to inhibit tumour growth. However, neither the 3D culture (scaffold-free based culture) nor the microfluidic culture incorporated an ECM. The incorporation of a matrix component could therefore accentuate the differences in IC₅₀ and bring the *in vitro* values closer to those measured *in vivo*.

As ToCs enable the incorporation of IF, researchers have utilised them to investigate its effects on drug resistance. Kramer et al. developed a microfluidic model for PDAC distinguishing “perfusion flow,” running parallel to the ECM, from “interstitial flow,” passing directly through it.¹¹⁴ Under “interstitial flow”, the cells exhibited reduced proliferation yet demonstrated significantly increased resistance to gemcitabine, a phenomenon largely attributed to higher expression levels of multiple MRP-family transporters such as ABCC1 and ABCC5. These transporters are known to actively efflux the drug, hereby reducing its cytotoxic effect on cells. Meanwhile, Kobayashi et al. investigated the impact of flow velocity on drug penetration in feline mammary tumour organoids.¹¹⁵ They used a branched microfluidic device to create three conditions: non-perfusion, pre-branching and post-branching. Due to the branching, post-branching chambers were perfused with a slower flow velocity than pre-branching chambers (**Figure 9**).

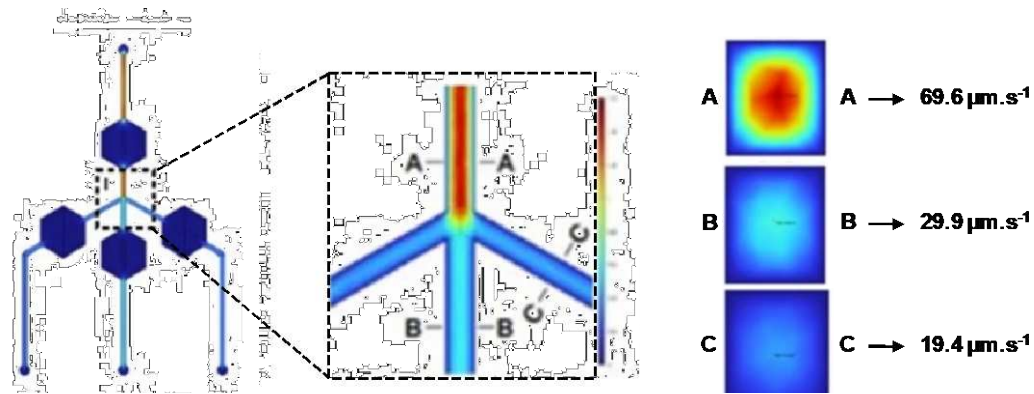


Figure 9. Fluid velocity in the microfluidic system developed by Kobayashi et al. [115] The branching after the first culture chamber enables the division in flow velocity. This results in lower flow velocities in post-branching chambers (B and C) compared to pre-branching chamber (A).

After 48 hours, the authors observed two times as much cell death in the pre-branching chamber compared to non-perfusion controls when treated with two drugs (toceranib, tyrosine kinase inhibitor interfering with growth factor signalling, and doxorubicin, a chemotherapy that causes DNA damage). In contrast, less cell death was observed in the post-branching chambers. This difference in drug response was attributed to the difference in flow velocity, with less drug penetration occurring at slower flow velocity. Indeed, they performed additional numerical simulations and observed an increased transport and distribution of drugs in pre-branching chambers due to the higher flow velocity generating a thinner boundary layer and consequently generating higher diffusion gradients. However, they could not solely attribute this difference in viability to the flow velocity as faster flow velocity could deteriorate the ECM, subsequently causing cell death.

These findings aligned with prior research,¹¹⁶ reinforcing the relevance of microfluidic systems as physiologically representative platforms for evaluating solid tumour treatment responses. In summary, ToCs are advanced tools for cancer research. By closely replicating *in vivo* conditions, they provide a robust framework for studying tumour biology and evaluating drug response and efficacy. A key aspect of these systems lies in their fabrication, as tight control over their dimensions is essential for enabling precise control over parameters such as gradients generation.

2.2. Microfluidic system fabrication methods

2.2.1. SU-8-based fabrication methods

Microfluidic platforms utilise microfluidic technologies to host and study biological models. As a result, their initial fabrication method originates from microelectronics, and more

specifically from the derivation of photolithography into soft lithography to pattern soft polymers.¹¹⁷ The most commonly used polymer for microfluidic platform fabrication is PDMS^{118,119} since its first use in 1998 by Whitesides et al.⁹⁶ Its predominant use in microfluidics is due to its advantageous physical properties (optical transparency, gas permeability, chemical inertness and biocompatibility¹²⁰) that make it particularly suitable for μ TAS systems.¹²¹ Furthermore, it is a material that is both non-expensive and easy to work with.

Soft lithography utilises a master mould to pattern soft polymers into the desired shape (**Figure 10**). Briefly, a layer of photosensitive resin (most commonly SU-8) is deposited on a silicon substrate, or wafer, with a height of less than 200 μm . A mask is then placed over the silicon substrate to expose the resin to ultraviolet rays through an opening of the shape of the desired pattern. The wafer is then bathed in a solvent to remove the unexposed resin, leaving only the desired 3D pattern on the silicon substrate. An additional CHF₃ coating of the SU-8 mould is usually needed to prolong the usability of the mould by rendering it hydrophobic and reducing PDMS attachment to the wafer. A mixture of PDMS monomer base and curing agent is then poured over onto wafer, cross-linked in an oven and peeled off. The PDMS then undergoes an O₂ plasma to bond it to a glass substrate. The fluidic inlets and outlets are then punched to complete the microfluidic system. This fabrication protocol enables the fabrication of devices with a sub-micron accuracy, which makes it particularly suited for biological applications.¹²²

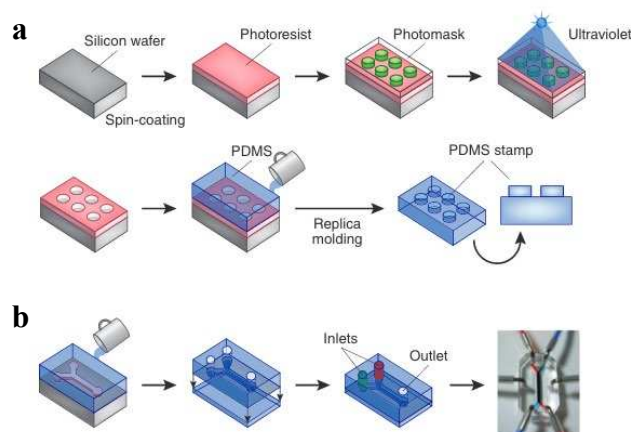


Figure 10. Fabrication steps of a polydimethylsiloxane (PDMS) microfluidic system. (a) A thin film of photoresist is deposited onto the silicon wafer and exposed to UV lights through a mask. The resulting pattern is obtained after development and removal of exposed photoresist. PDMS is then poured onto the mould, cured and demoulded to obtain the PDMS stamp. **(b)** Single channel microfluidic system fabrication with two inlets and one outlet bonded onto a glass substrate. Photograph of a two-chamber microfluidic system. From [38].

However, despite its advantages, SU-8 lithography has often faced criticism for its complexity, its time-consuming and costly nature, and the necessity of accessing specialised equipment such as a clean room.¹²³ Furthermore, as patterned features are transferred in a single plane, SU-8 is limited to 2.5D structures, preventing the fabrication of complex structures and although the XY precision of SU-8 photolithography is very satisfactory, the production of Z patterns is very complicated, if not impossible beyond 200 μm . Furthermore, PDMS has previously been shown to be able to absorb small molecules such as drugs.¹²⁴ This results in PDMS based devices difficult to disseminate outside of research labs,¹²⁵ and the subsequent investigation of alternative fabrication methods.

2.2.2. Alternative fabrication methods

Alternative methods have been explored for microfluidic system fabrication. Aluminium milling has been explored for PDMS moulds^{126,127} as it is capable of creating precise patterns in a wear-resistant material. Other techniques such as plastic laser ablation were developed to directly fabricate constitutive components of TMOCs, by cutting plastics such as Poly(methyl methacrylate) to the desired shapes.¹²⁸

However, these techniques have their limitations: aluminium milling is constrained by limited geometries, while plastic laser ablation faces challenges with resolution and reproducibility. Although microfluidic systems have become a highly important tool in various scientific and engineering fields,^{38,129} they suffer from limitations related to their fabrication, highlighting the need for substitute fabrication methods.

2.2.3. 3D printing fabrication methods

To resolve these issues, and thanks to its affordability and ease of use, 3D printing represents a pivotal factor in the mainstream adoption of microfluidic platforms. 3D printing can be divided in six main categories: Vat Photopolymerisation, Material Extrusion, Powder Bed Fusion, Material Jetting, Binder Jetting and Sheet Lamination. Only vat photopolymerisation will be explored in this manuscript.¹²⁵ Vat polymerisation 3D printing's working principle is rather simple. Briefly, a building plate descends into photosensitive ink filled VAT where a UV light solidifies the resin within a defined thickness to form a layer. The process is then repeated until the desired object is obtained. Three applications of this technology in particular are used in microfluidic system manufacturing: Laser stereolithography (SLA), Liquid-Crystal Display (LCD) and Digital Light Processing

(DLP).¹³⁰ Although DLP 3D printing has seen its use increase for lab-on-chip fabrication, the cost of common research-grade microfluidic DLP 3D printers constitutes a significant entry barrier for many potential users. Furthermore, as DLP 3D printers utilise an array of micro-mirrors to redirect the light source to cure the resin, only small objects can be printed due to the reduced size of the projector to ensure high precision. Hence why DLP will not be further mentioned in this manuscript.

Thanks to its high-resolution prototyping, SLA has gained popularity since its creation in the 1980s.¹³¹ Its working principle relies on the use of a sweeping UV laser to cure a photopolymer resin layer voxel by voxel, layer by layer (**Figure 11.a**), enabling the creation of microchannels with dimensions typically ranging from 200–500 μm .¹³² Additionally, the materials used in SLA are often branded biocompatible, making this technology suitable for biomedical applications.¹³³ However, SLA's reliance on point-by-point photopolymerisation using single or dual lasers can lead to longer build times, particularly for microfluidic devices that require substantial solid structures with embedded channels. Furthermore, the use of dedicated lasers significantly increases both the operating complexity, as well as the initial purchase price (~ few thousand dollars).

LCD-based 3D printers have an initial purchase price of a few hundred dollars, mainly due to the use of an array of discrete light-emitting diodes. Their light is then either collimated by a lens and filtered by the LCD screen, or directly transmitted through the screen, to expose entire layers of the resin with the desired pattern (**Figure 11.b**). This method avoids the optical distortions found in DLP 3D printing caused by projecting an image, and was shown to achieve resolutions of 30–50 μm depending on the LCD's pixel density and building plate area.¹³⁴ Entry level LCD 3D printers will often have approximately 2 million pixels. However, pixel number has seen a constant increase in recent years and can now reach up to 68,157,440 pixels (Anycubic Photon Mono M7 Pro, Anycubic), increasing printing resolution while reducing printing time,¹³⁵ therefore outperforming both DLP and SLA 3D printers in terms of pixel number and affordability. Studies have also shown that entry level LCD-based 3D printers are still close enough to SLA printers in accuracy in regards of biomedical applications such as orthodontics clinical threshold.¹³⁶

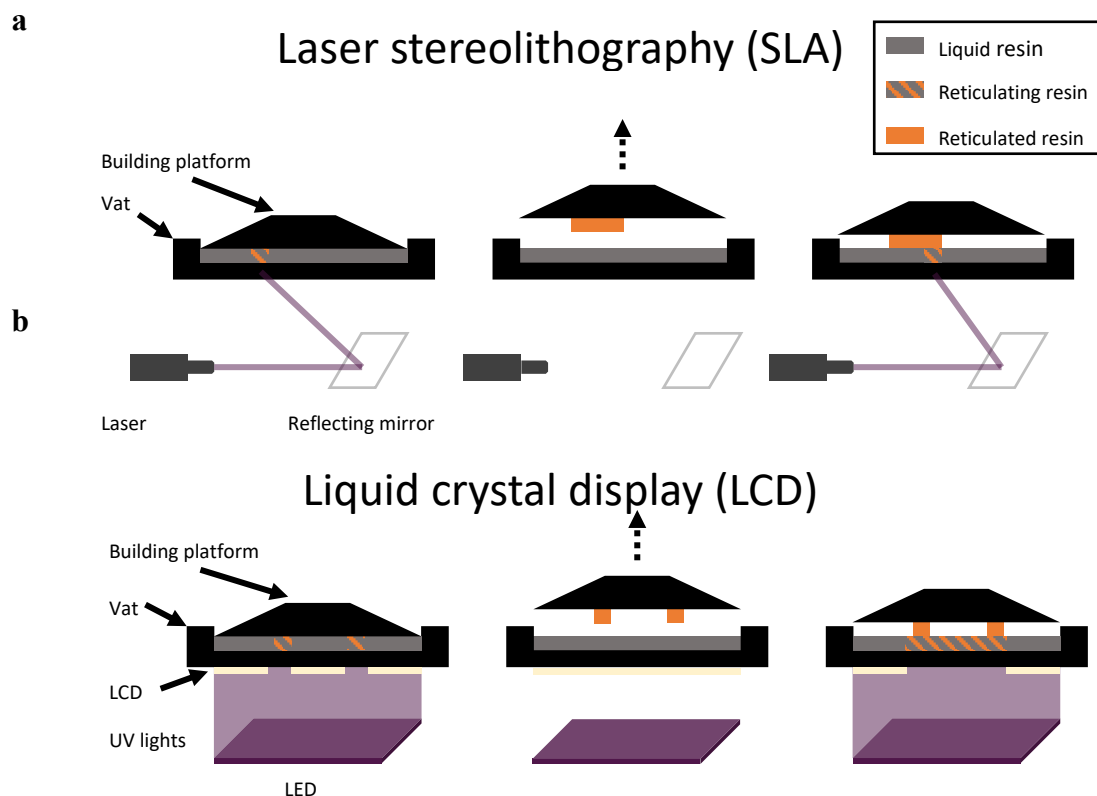


Figure 11. Laser stereolithography and liquid crystal display 3D printing techniques. The two printers consist of a building platform, a resin vat with fluorinated ethylene propylene (FEP) film at the bottom, filled with resin and a translation axis connected to a motor that controls the Z position of the platform. **(a)** Steps involved in SLA 3D printing. The laser is switched on and polymerises a resin layer voxel by voxel as per the desired pattern. It is switched off and the building platform is raised. The laser is then switched back on to polymerise the next layer of resin. **(b)** Steps involved in LCD 3D printing. The LEDs are switched on and the LCD screen is switched on to form a mask with the pixels that allow the UVs to pass through and polymerise the entire desired layer. The LEDs are switched off and the printing platform is raised, allowing the resin to disperse in the reservoir. For both printers, the process is repeated until the component is fully polymerised.

Another crucial parameter of a successful printing is the used resin and the printing parameters.¹³⁷ Mechanical, optical and biochemical (biocompatibility) properties of printing are important characteristics that may differ from one resin to another and must therefore be evaluated. Indeed, to be a constitutive component of culture systems, or at least part of it, the biocompatibility of 3D printed parts needs to be investigated and established. This is often performed by following standards set by the International Standards Organisation (ISO) and more specifically following the ISO 10993.¹³⁸ However, ISO family defines biocompatible materials as materials resulting in at least 80 % cell viability after 24 h and does not specify the test to be carried out. Furthermore, little details is given by the manufacturers on the performed

tests.¹³⁹ It is therefore critical to investigate the resin's biocompatibility of a resin before incorporation in a microfluidic system.

2.3. 3D printing for Tumour-on-Chips fabrication

3D printing has in the recent years gained in importance for microfluidic device fabrication. Its ease of use and ability to create sub millimetre features faster than traditional fabrication methods seems optimal for rapid production of microfluidic systems. This was demonstrated by Mohamed et al. who leveraged the printing resolution of LCD 3D printer to fabricate PDMS moulds for microfluidic applications, demonstrating its potential for creating high-resolution features.¹⁴⁰ They first evaluated the printer's feature resolution by comparing the printed dimensions to the designed specifications. Their findings revealed a minimum achievable pattern size of 100 μm in the horizontal direction and 50 μm in the vertical dimension. Notably, the printed width was slightly larger than the designed dimensions, while the height closely matched the intended measurements, but still theoretically allowed the fabrication of micrometric channels. However, although this fabrication precision has been leveraged and applied to cell culture,^{123,125,132} the use of 3D printing to fabricate ToCs is still limited.

The simplest way of utilising 3D printing for ToCs fabrication is to fabricate PDMS moulds. Feng et al. investigated the use of a three-layered PDMS microfluidic device, fabricated with 3D-printed moulds, for liver tumour spheroid culture under flow conditions.¹⁴¹ They applied an alcohol polishing technique to reduce surface roughness, improving the hydrophilicity of the moulds. Unlike previous studies, they incorporated direct flow for drug delivery (**Figure 12.a and b**). However, spheroids were still developed externally and introduced into the device without ECM, potentially limiting physiological relevance. Additionally, the study did not assess the potential cytotoxicity of residual resin from the 3D-printed moulds. The system was not parallelised, making high-throughput experimentation difficult, and the lack of an openable design restricted access to biological samples.

Poskus et al. examined the cell-cell interactions of breast cancer cells and mammary fibroblasts when co-cultured in a microfluidic system. They fabricated a three channel microfluidic system, with the central channel filled with collagen I hydrogel and side channels seeded with cancer cells after having validated cell viability when in contact with PDMS mould from 3D printed components as well as 3D printing precision (**Figure 12.c and d**).¹⁴² However,

their characterisation did not investigate the effects of printing orientation nor surface roughness of PDMS moulds. Additionally, PDMS moulding using 3D printed moulds has previously been shown to inhibit PDMS curing due to remaining photo-initiators leaching out of the moulds into the PDMS.¹⁴³ Furthermore, the demoulding process must be considered when defining channel geometry, thereby restricting the range of possible design.

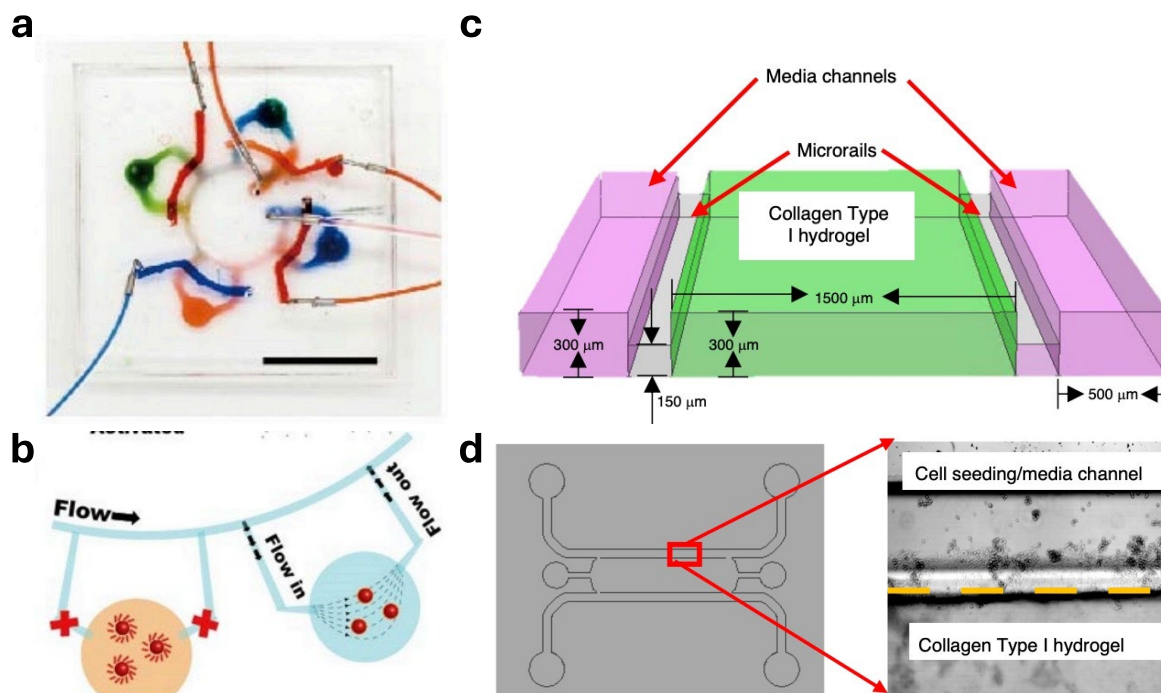


Figure 12. PDMS-based microfluidic devices fabricated using 3D printed moulds. (a) Optical image of the device developed by Feng et al. and (b) illustration of valve-actuated flow across the culture chambers. From [141]. (c) Three-channel device developed by Poskus et al. (d) Cells are seeded in collagen I hydrogel within the central channel while culture medium is perfused in both lateral channels. From [142].

To circumvent this issue, other teams directly fabricated microfluidic devices using 3D printing, thereby eliminating the need for PDMS moulding. Labonia et al. investigated the use of a 3D-printed microfluidic system to study drug responses in colorectal cancer spheroids.¹⁴⁴ They developed a 3D-printed microfluidic system designed to fit a 24-well plate, featuring six independent channels (**Figure 13.a**). They then placed spheroids into the device by placing them within porous membrane inserts and subsequently exposed them to irinotecan treatment, which causes DNA strand break leading to cell apoptosis. The inserts could then be extracted from the device, allowing easy access to the biological sample to perform further analysis. However, due to the porous membrane of the inserts, only drug diffusion occurred with no advection. Additionally, the 3D printing precision was not characterised, introducing possible flow inconsistencies between channels. Additionally, resin cytotoxicity was not assessed

before cell culture, and drug delivery relied solely on diffusion, limiting its physiological accuracy.

Markoski et al. investigated the optimisation of 3D printing precision for microfluidic applications and used their optimised parameters to fabricate a microfluidic system.¹⁴⁵ They reported height and width deviations of $9.2 \pm 8.2 \mu\text{m}$ and $22.0 \pm 10.0 \mu\text{m}$, respectively, but did not examine the impact of printing orientation on these errors. Their device featured a single rectangular perfusion channel with a trap to retain spheroids and enabled direct perfusion, leading to a 70 % fluorescence reduction under static conditions and a 30 % reduction under flow (**Figure 13.b**). However, they did not assess resin cytotoxicity, leaving the possibility that micro-particles released during culture contributed to fluorescence changes. Additionally, the system was not parallelised, and the closed-channel design limited biological sample access for further analysis.

Another device developed by Gallegos-Martinez et al. featured three channels, with two for culture medium and one central channel for hydrogel-embedded cell encapsulation (**Figure 13.c**).¹⁴⁶ Before cell culture in the device, they investigated the impact of 3D-printed materials on breast cancer cell viability by comparing cell responses in a 3D-printed 96-well plate replica, showing that while Live/Dead assay suggested that viability at 24 h was comparable to controls, cell confluence was reduced, suggesting an effect on adhesion. Furthermore, the 24-hour viability test was also insufficient to assess long-term material effects, raising concerns about prolonged culture compatibility. Additionally, although capable of long-term cell culture (9 days), their device was non-openable, restricting sample retrieval, and it was not parallelised, limiting throughput. Furthermore, the system operated with a syringe pump rather than a closed circuit, making long-term experiments less practical.

Shafique et al. developed a novel 3D printing ink (PLInk) specifically tailored for LCD-based printing, enabling the fabrication of microfluidic devices without relying on PDMS moulding.¹⁴⁷ They formulated and mechanically characterised the ink, validating it for microfluidic applications. Importantly, the cytocompatibility of the resin was established through cell viability assays involving HUVECs co-cultured with 3D printed PLInk rings. Cell viability remained above 80 % across various time points, meeting the ISO 10993-5:2009 standard and demonstrating its suitability for microfluidic device fabrication in cell culture applications. However, although cell viability surpassed the 80 % threshold required by ISO

standards, this level may still be insufficient for longer-term cell cultures or drug assays, where higher viability is often critical. Additionally, they did not investigate the fabrication precision, preventing them from fine-tuning the flow velocity profiles within their microfluidic device. They then developed a ToC model with three channels: the side channels were seeded with a lung spheroid and a breast cancer spheroid, while the central channel contained endothelial cells (**Figure 13.d**). Over five days of culture, endothelial cells reorganised and tightened into a vascular structure, with initial sprouting observed towards the spheroids. Breast cancer cells were seen migrating from the spheroid towards the vascular barrier, illustrating the potential for cancer-related studies within LCD 3D-printed microchannels. However, their platform has inherent constraints. Indeed, the device design lacks direct flow, which could limit its capacity to mimic the dynamic microenvironment typically experienced by spheroids *in vivo*. Furthermore, as the device constitutes a single, enclosed component, accessing the cell cultures for interventions or downstream analyses remains challenging.

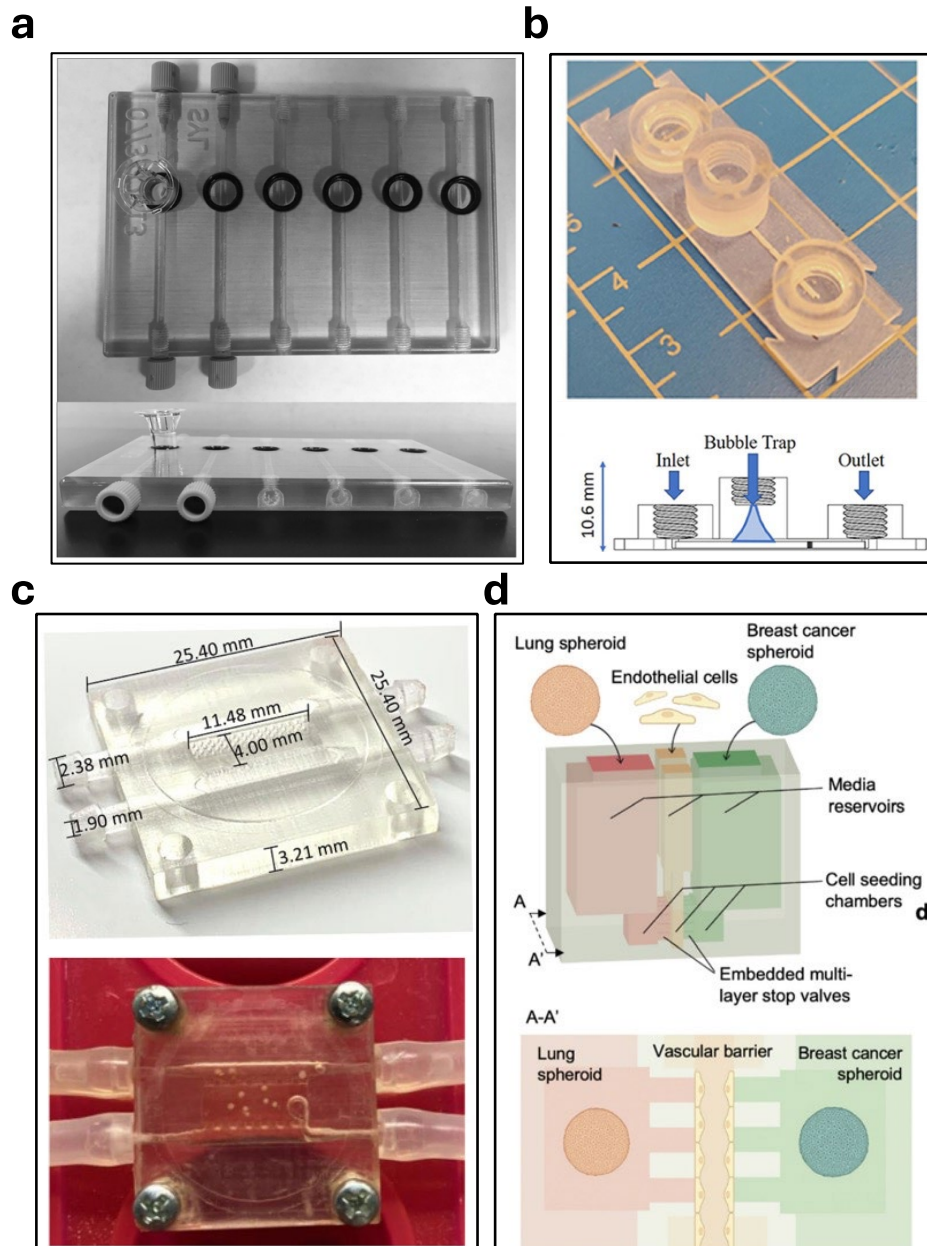


Figure 13. Examples of 3D printed microfluidic devices. (a) Device developed by Labonia et al. The device has six channels, each of which have a circular opening to place a membrane insert with the solid tumour model. From [144]. (b) Isometric and cross-sectional views of the device developed by Markoski et al. From [145]. (c) Technical and actual image of the chip developed by Gallegos-Martínez et al. From [146]. (d) Design of the tumour-on-chip developed by Shafique et al. with two culture chambers separated by a channel filled with endothelial cells. From [147].

2.4. Conclusion

In conclusion, microfluidic systems, through the precise control of their dimensions, enable a better reproduction of some solid tumour characteristics such as IF flow and solid stress than traditional culture methods. They have since been more and more utilised to study key aspects of both tumour growth and development, and response to treatment. However, traditional fabrication methods such as SU-8 PDMS soft lithography have often faced criticism for their complexity, their time-consuming and costly nature, and the necessity of accessing specialised equipment such as a clean room. Furthermore, SU-8 is limited to 2.5D structures, preventing the fabrication of complex structures and although the XY precision of SU-8 photolithography is very satisfactory, the production of Z patterns is very complicated, limiting the real of possibilities.

Thanks to its affordability and ease of use, 3D printing represents a pivotal factor in the mainstream adoption of microfluidic platforms. It enables researchers to create more complex geometries, while allowing for faster and cheaper prototyping. It has since then been more and more utilised to fabricate ToCs. However, the investigation of key aspects such as resin biocompatibility and printing precision remain to be fully investigated. Indeed, more often than not, resin biocompatibility testing is performed with manufacturers not disclosing details about the performed tests. Additionally printing precision is not yet often characterised including parameters such as printing orientation.

Chapter 3: Bioimpedance

In this chapter, we discuss the theoretical concepts of electrical impedance. We then discuss the instrumentation utilised to perform electrical impedance measurements and how electrode configuration can be modulated to affect the electrical measurement. We then discuss how biological tissues, and more specifically the extracellular fluid, the ECM and the cells, have the ability to oppose the flow of electrical current and therefore can be modelled as an equivalent electrical circuit. Finally, we present how this has been leveraged to quantify the viability and drug response of *in vitro* static and dynamic 3D solid tumour models.

3.1. Theoretical concepts

In the 1820s, Georg Simon Ohm established a mathematical relationship between voltage (or tension), current and resistance in direct current systems.¹⁴⁸ This relationship, also known as Ohm's law, is the foundation of electrical circuit analysis, and while it served as a cornerstone for analysing electrical circuits, the rise of alternating current (AC) systems in the mid-19th century led to the establishment of a more sophisticated framework. Michel Faraday, who worked on inductance and capacitance,¹⁴⁹ and James Clerk Maxwell, whose equations described the dynamic behaviour of electromagnetic fields,¹⁵⁰ laid the grounds for AC circuit theory. This was further explored by Oliver Heaviside in 1887, whom through his work on AC circuits, developed terms such as resistance and reactance (which are developed in the following chapter), which he combined in a single complex quantity known as Impedance ($\mathbf{Z}$).¹⁵¹ His contributions formed the foundation of modern AC circuit theory and practical impedance measurements, and provided a mathematical tool for engineers to analyse signal behaviour in electrical systems.¹⁵²

3.1.1. Electrical impedance

The electrical impedance $\mathbf{Z}$ of an electrical circuit refers to the measure of its total opposition (*to impede*) to the passage of an AC generated by applying a voltage or current to it.¹⁵¹ When applying constant voltage or constant current (direct current electrical circuit), we refer to this opposition as electrical resistance, whereas in the case of an AC, we refer to it as electrical impedance.

Electrical impedance can be represented by a generalisation of Ohm's law to an AC circuit. It therefore corresponds to the ratio between the voltage of a sample and the current

passing through it. As both voltage and current are alternating, impedance also depends on the frequency of the signal:

$$Z(\omega) = \frac{V(\omega)}{I(\omega)} \text{ Ohm } [\Omega] \quad (2)$$

with $V(\omega)$ the voltage, $I(\omega)$ the current and ω the angular frequency of the signal ($\omega = 2\pi f$, f being the electrical signal's frequency).

As both voltage and current are alternating, they both have a characteristic amplitude and oscillate with a phase shift. They can therefore both be represented in complex form:

$$V(\omega) = |V|e^{j(\omega t + \theta_V)} \quad (4)$$

$$I(\omega) = |I|e^{j(\omega t + \theta_I)} \quad (3)$$

with $|V|$ and $|I|$ the respective amplitudes of voltage and current, j the imaginary unit (such that $j^2 = -1$) and θ_V and θ_I the respective phases of voltage and current. Complex impedance can subsequently be represented as:

$$Z(\omega) = \frac{V(\omega)}{I(\omega)} = \frac{|V|}{|I|} e^{j(\theta_V - \theta_I)} = |Z|e^{j\varphi} \quad (5)$$

with $|Z|$ the impedance amplitude and the phase shift between voltage and current.

In cartesian form, complex impedance can be written as:

$$Z(\omega) = R(\omega) + jX(\omega) \quad (6)$$

with $R(\omega)$ the impedance's real part representing its resistance, and $X(\omega)$ the impedance's imaginary part representing its reactance.¹⁵³ Reactance represents an electrical circuit's ability to oppose to a change in tension or current. We talk about capacitive reactance when $X(\omega) < 0$ and inductive reactance when $X(\omega) > 0$.¹⁵⁴ As inductive reactance does not appear in biological tissues, we will only consider capacitive reactance. We can therefore write $X(\omega)$ as:

$$X(\omega) = -\frac{1}{C\omega} \quad (7)$$

with $C(\omega)$ the complex capacitance.

In general, resistance R represents the opposition to the flow of conduction currents, while reactance X represents the opposition to displacement currents. Resistance quantifies the ability of a material to dissipate electrical energy, whereas reactance characterises its ability to store electrical energy.

3.1.2. Complex permittivity and conductivity

An ideal conductor is a material with infinite conductivity, meaning it can sustain a steady current with no energy dissipation. An ideal capacitor on the other hand, is a dielectric material with zero electrical conductivity, and only supports displacement current.¹⁵⁵ However, the majority of materials are not ideal conductors nor capacitors, and experience additional phenomena, either through displacement currents (conductors) or conduction currents (capacitors). To account for these non-ideal behaviours, materials are often characterised using either a complex permittivity ε^* , if the material is considered as a dielectric with losses, or a complex conductivity σ^* , if the material is considered as a conductor with capacitive properties:¹⁵⁶

$$\varepsilon_D^* = \varepsilon_D' - j\varepsilon_D'' \quad (8)$$

with ε' and ε'' representing energy storage and dielectric losses mechanisms, respectively.

$$\sigma_C^* = \sigma_C' + j\sigma_C'' \quad (9)$$

with σ' and σ'' the imaginary parts representing energy dissipation and energy storage mechanisms, respectively.

If the material is considered as a dielectric with losses, we have $\varepsilon_D'' = \frac{\sigma_D}{\omega}$, with σ_D being the conductivity of the non-ideal dielectric, then we can rewrite equation 8 as:

$$\varepsilon_D^* = \varepsilon_D' - j\frac{\sigma_D}{\omega} \quad (10)$$

To note, with ε_r the relative permittivity of the material and ε_0 the permittivity of vacuum.

If the material is considered as a conductor with capacitive properties, then we have $\sigma_C'' = \omega\varepsilon_C'$, with ε_C' being the permittivity of the material.

When a conductive material is considered non-ideal, conduction current occurs in parallel with displacement current. This physical behaviour can be represented with an analogue circuit model corresponding to a capacitor in parallel with a resistance (**Figure 14**).

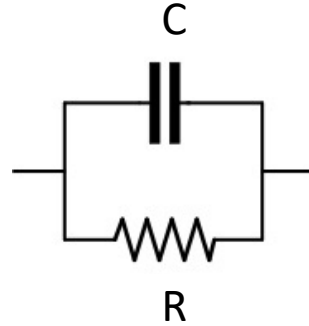


Figure 14. Non-ideal conductive material equivalent electrical circuit.

This case of a non-ideal conductor is of particular interest as it is the one representative of biological tissues.

Since both components are in parallel, the impedance Z of the circuit is given by:

$$\frac{1}{Z} = \frac{1}{R} + j\omega C \quad (11)$$

We therefore have:

$$Z = \frac{R}{1 + j\omega RC} \quad (12)$$

From equation 12, we can obtain both impedance magnitude and phase angle:

$$|Z| = \frac{R}{\sqrt{1 + (\omega RC)^2}} \quad (13)$$

and

$$\arg(Z) = -\tan^{-1}(\omega RC) \quad (14)$$

Both quantities can be represented in a bode diagram such as the one shown below to illustrate their variation with respect to frequency (**Figure 15**):¹⁵⁷

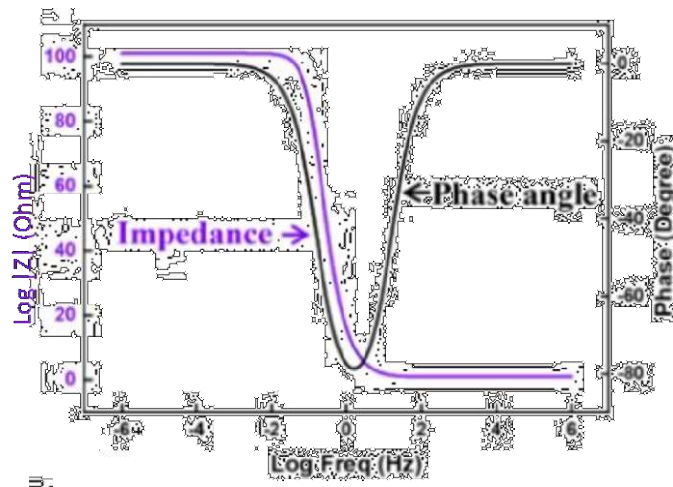


Figure 15. Bode diagram representing both impedance magnitude (left y-axis) and impedance phase angle (right y-axis). From [157].

3.2. Impedance measurement instrumentation

An impedance measurement system is required to perform impedance measurements on a sample. Conceptually, such a system can be decomposed in three parts: a signal generator (voltage or current) that produces the electrical signal applied to the sample, an electrode interface to the sample, that allows to apply the electrical signal to the sample, and a voltage or current analyser that measures the voltage drop across the sample or the current flowing through it. Modern day technologies allow for the simplification of the circuitry, by combining both the signal generator and analyser into one dedicated back end, also known as an impedance analyser.

3.2.1. Impedance analyser

Thanks to its broad measurement range, large-frequency spans, and high-resolution impedance spectroscopy, impedance analysers are the current most popular hardware instrumentation used for the back-end of impedance measurement systems.^{156,158} The basis of impedance analysers relies on the use of a dedicated lock-in amplifier, as it enables the detection of signals buried in background noise.¹⁵⁹

A lock-in amplifier functions by measuring the voltage output resulting after the analyser has generated a reference current, through the sample of interest. The output voltage is then multiplied by in-phase (0°) and quadrature (90°) components and filtered by a low pass-

filter to extract an estimated value of impedance. This technique is known as the *multiply and average technique*, and is particularly powerful as it allows for the extraction of both the real and imaginary parts of the sample's impedance independently,¹⁶⁰ hence the current use of a lock-in amplifier in most impedance analysers.

To note:

Lock-in amplifiers can be of different types: Digital, Analog and Microelectronic (Complementary metal-oxide-semiconductor). Digital lock-in amplifiers are more flexible and precise than analogue lock-in amplifiers. However, they consume more power and operate at lower frequencies. Complementary metal-oxide-semiconductor based lock-in amplifiers focus on low-voltage and low-frequency and are particularly well suited for medical devices.

The impedance analyser used in this project is the '*compact multichannel impedance analyser*' or CSX-64 manufactured by the German company *Sciospec Scientific Instruments GmbH*.¹⁶¹ It is capable of hosting 64 individual electrodes divided into 8 banks. This allows to perform measurements on 8 individual samples simultaneously while measurements on one bank can be performed sequentially. The signal frequency ranges between DC to 1 MHz, with measurable impedances ranging between 0 and 10 GΩ.

3.2.2. Electrodes

The electrode serves as the interface between the sample and the instrumentation electronics and is, therefore, the building block of the impedance measurement system. Electrodes in an impedance measurement system can be divided in two categories: current carrying (CC) and pick up (PU) electrodes. CC electrodes inject the current into the sample, while the PU electrodes measure the potential difference between them. The simplest impedance measurement setup is therefore composed of two individual electrodes, with both electrodes utilised for current injection and voltage drop measurement (**Figure 16**).

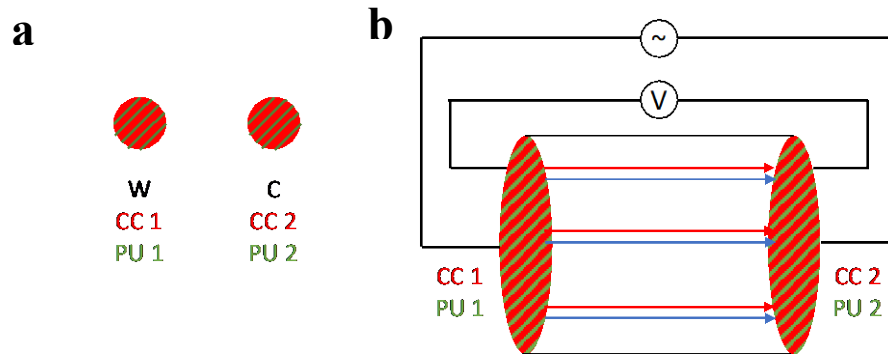


Figure 16. Schematic of a 2-point electrode configuration. (a) 2-point electrode configuration. W is the first current carrying (CC) and pick up (PU) electrode; C is second CC and PU electrode. (b) Use in measuring a generic cylindrical conductor. Red arrows represent the current density lines, blue arrows represent the electric field. Inspired from [163].

However, as the CC electrodes inject the current, the electrical charges accumulate at the electrode/sample interface. This is known as interfacial polarisation, and is composed of two layers, hence it is named electrical double layer.⁸³ The first layer consists of electrons accumulated within the electrode, while the second layer is composed of ions of the opposite charge that are adsorbed onto the electrode surface attracted to the surface charge to ensure electroneutrality (**Figure 17**).

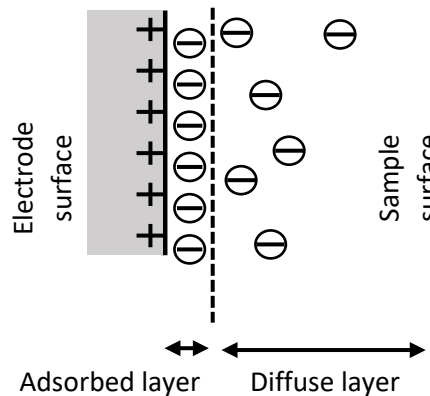


Figure 17. Illustration of electrical double layer due to interfacial polarisation.

This double layer behaves like a capacitor, and its capacitance (C_{EDL}) generates a polarisation impedance of the electrode. This capacitance, which is not a characteristic of the sample itself, adds to the measured sample impedance and is often undesirable (**Figure 18.a**). Additionally, due to the extremely small distance between the positive and negative charges (less than 1 nm), the capacitance is very high and thus affects measurements at low frequencies ($f < 1$ kHz). At low frequencies, this parasitic capacitance masks the phenomena occurring in the bulk of the sample (**Figure 18.b**).

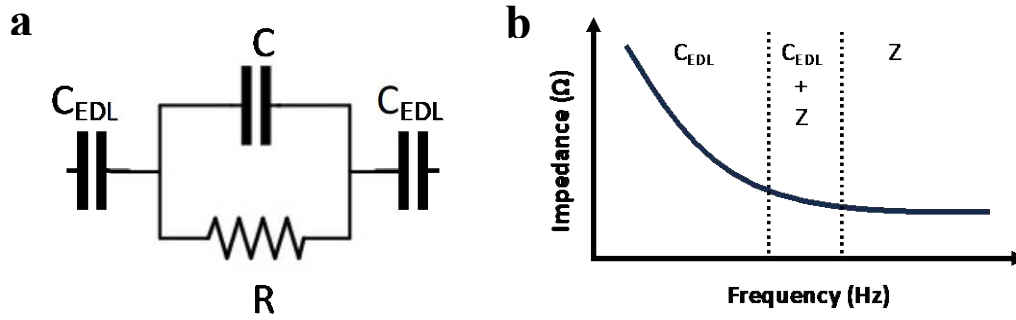


Figure 18. Effects of parasitic capacitance on impedance measurement. (a) Non-ideal conductive material equivalent electrical with added electrode polarisation capacitance C_{EDL} . **(b)** Magnitude of the impedance spectra of 2-point impedance measurement. The impedance measurement is dominated by the electrode double layer at low frequencies, consequently masking the sample's impedance Z .

To minimise the impact of the electrode interfacial polarisation on the impedance measurement, the 4-point electrode or tetrapolar method can be adopted.¹⁶² As its name suggest, this electrode configuration is based on the use of four electrodes, with one electrode pair (W and C) serving as the CC electrodes, while the other two (Ws and R) measure the potential difference (**Figure 19.a** and **b**). This setup reduces the effects of interfacial polarisation by connecting both PU electrodes to a very high resistance ($\sim M\Omega$ range). This stops the current from flowing into the voltage measurement path. Consequently, both PU electrodes do not present interfacial impedance.¹⁶³ This in turn eliminates completely the contribution of electrode polarisation to the measured impedance, allowing the focus of the measurement entirely on the properties of the bulk of the investigated sample, unlike 2-point possible electrode configuration (**Figure 19.c**). Hence, we decided to utilise the 4-point electrode configuration for impedance measurements in this project.

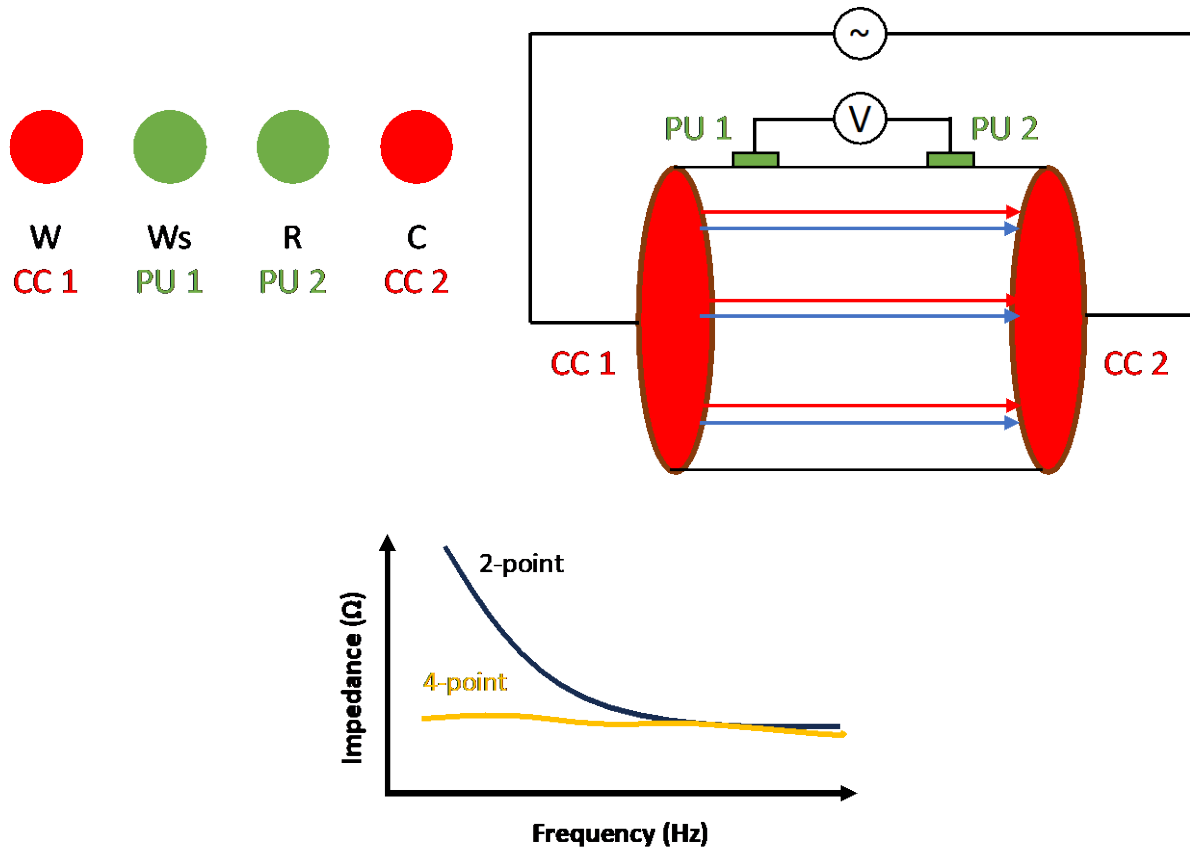


Figure 19. Schematic of a 4-point electrode configuration and resulting impedance spectra. (a) 4-point electrode configuration. W is the working electrode, C is the counter electrode, Ws the working sensing and R the reference electrode. The electric double layer is represented by the brown circle. **(b)** Use in measuring a generic cylindrical conductor. Red arrows represent the current density lines, blue arrows represent the electric field. Inspired from [163]. **(c)** Impedance spectra for 2- and 4-electrode configurations.

3.2.3. Electrode cell constant

The cell constant $\kappa [\text{m}^{-1}]$ of an impedance sensor is defined as the proportionality factor between the impedivity (specific impedance, i.e. impedance per unit volume) of the material and the measured impedance:¹⁶⁴

$$\kappa = Z\sigma_C^* \quad (15)$$

As we have previously seen, a common way to model the volume impedance of a non-ideal conductor is via an analogue circuit composed of a conductor in parallel with a capacitor (Figure 14). In this case, we have:

$$R = \frac{\kappa}{\sigma_C'} \quad (16)$$

and

$$C = \frac{\varepsilon'_C}{\kappa} \quad (17)$$

Interestingly, these equations closely resemble those of ideal conductors and capacitors. Indeed, a conductive material of length l , cross-section A and electrical conductivity σ has a resistance $R = \frac{l}{A\sigma}$. The cell constant κ is therefore equal to $\frac{l}{A}$.

An ideal capacitor with plates with a cross-section area S , separated by a dielectric material with relative permittivity ε_r over a distance d , we have a capacitance $C = \frac{S}{d} \varepsilon_0 \varepsilon_r$. The cell constant κ is therefore equal to $\frac{d}{S}$.

These relationships are of particular interest because they highlight the importance of cell constant as it enables the finding of material properties through the measurement of the volume's resistance and capacitance.¹⁶⁵ In addition, by using conformal mapping, any field distribution of a planar electrodes system can be converted to that of a corresponding parallel electrode system.¹⁶⁶ Therefore, the cell constant expression above can be used for all planar electrode systems. An important aspect of the cell constant is that a higher constant results in a higher sensitivity to small variations in σ^*_C and therefore the investigated sample's impedance.

3.3. Electrical bioimpedance

Bioimpedance is defined as the ability of a biological material to oppose the flow of electrical current, a property that arises from the intrinsic electrical characteristics of cells, tissues, and their surrounding environment.¹⁶² It can be quantified by applying an external electrical AC current across the biological sample and measuring the resulting voltage response. This impedance response is governed by a combination of resistive and capacitive components. Bioimpedance spectroscopy has been widely employed for diagnostic applications,¹⁶⁷ hydration and cancer detection.¹⁶⁸

Within the β -dispersion region (500 Hz to 100 kHz) range,¹⁶⁹ current primarily flows around cell rather than through them. This makes this frequency range particularly sensitive to the capacitive properties of the cell membrane and thus provides valuable information on membrane integrity and overall cell viability.¹⁷⁰ Although lower frequencies (α -dispersion) are

often dominated by electrode polarisation, as we chose to implement a four-point electrode setup, this parasitic capacitance does not significantly affect our measurements. Meanwhile, very high frequencies (γ -dispersion) are primarily governed by the reorientation of water molecules.

As we previously described, solid tumours have a heterogeneous and complex structure. They are comprised of cells embedded within an ECM, both of which are surrounded by extracellular fluids. These components possess distinct electrical properties, meaning that their individual and collective responses to an applied electrical current can be studied separately or in an integrated manner within a tissue model.

3.3.1. The extracellular fluid as an electrical component

The extracellular fluid can be considered as a bulk electrolyte. Thus, its electrical properties (conductivity and permittivity) depend on the chemical and physical properties of its different (bio)chemical components (i.e. electrical charge, ionic concentration, etc). At our working frequencies, it can be modelled as a simple resistor with resistance R_e . If we model a 4-point electrode system measuring the impedance of a bulk electrolyte, we have the following electrical circuit (**Figure 20.a**). As its impedance solely relies on its ion concentrations, a change of composition will directly affect the measured impedance, with an increase in ion concentration resulting in a decrease in impedance (**Figure 20.b**).¹⁷¹

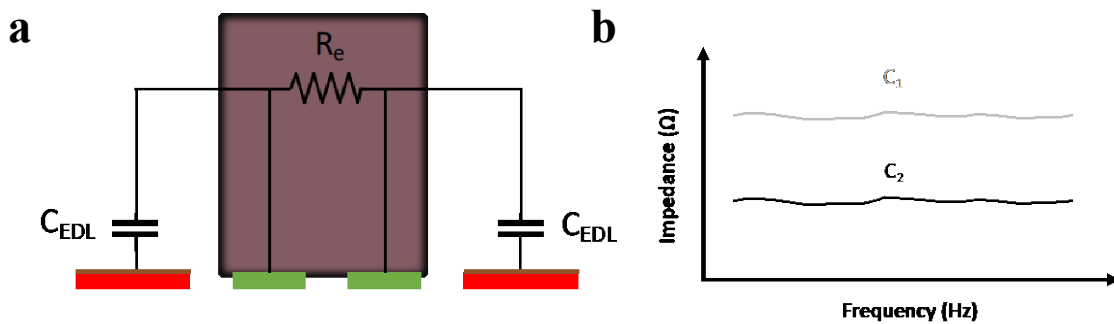


Figure 20. Equivalent circuit of a bulk electrolyte and bode diagram of impedance magnitude. (a) Electrical circuit representing the configuration of the impedance measurement of an electrolyte. Red and green rectangles represent CC and PU electrodes, respectively. The bulk electrolyte is represented as a resistance R_e . The measurement volume is represented by the pink rectangular area (b) Impedance spectrum of two electrolytes of varying ionic concentrations with $C_2 > C_1$.

3.3.2. The extracellular matrix as an electrical component

As previously mentioned, cancer cells are embedded within an ECM composed of proteins such as hyaluronic acid and collagen 1. This ECM is often mimicked by cultivating cells within a hydrogel such as MatrigelTM.⁴¹ We therefore have to consider this hydrogel as an electrical component to characterise a scaffold-based 3D culture.

Typically, cells are mixed within the hydrogel in its aqueous form. The mixture is then deposited in the culture system and placed in the incubator so that the hydrogel solidifies. As the hydrogel solidifies, its viscosity increases and reduces the ion mobility, which in turn reduces the electrical conductivity of the gel, making it less conductive.¹⁷² As the gel does not blend with the extracellular fluid, we can model it as an additional resistor with resistance R_{ECM} at our working frequency range (**Figure 21**).¹⁷³ As hydrogels restrict ion mobility compared to free electrolytes, $R_{ECM} > R_e$. Therefore, using a hydrogel results in an increase of the measured total impedance.¹⁷⁴ However, when adding a hydrogel to the ECM, we do not simply add a parallel conductive pathway but rather replace conductive fluid by less conductive hydrogel. This behaviour can be quantitatively described using the Bruggeman Effective Medium Approximation, which accounts for the conductivity of a heterogeneous medium composed of both extracellular fluid and hydrogel.¹⁷⁵

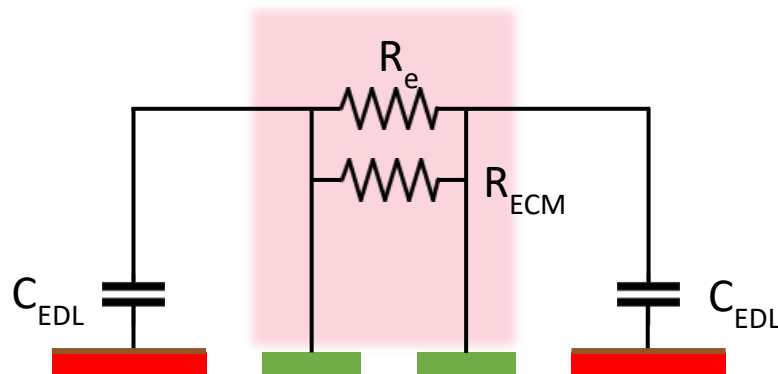


Figure 21. Equivalent circuit of a measured volume containing both an extracellular matrix and extracellular fluid. The extracellular matrix is represented by a resistance in parallel with the extracellular fluid resistance.

3.3.3. The cell as an electrical component

There are many different types of cells within a tissue, each with different sizes and functions. However, for descriptive purposes, we can introduce the concept of a 'generalised cell' that represents the main physical characteristics of all cell types (**Figure 22.a**). Our

‘generalised cell’ model consists of three main parts: a nucleus, a cytoplasm and a cell membrane.¹⁷⁶

The cytoplasm is mainly composed of a liquid phase containing ions, proteins and organic acids. Additionally, functional blocks of the cells – organelles and nucleus, are also contained within the cytoplasm. These blocks are delimited by membranes of dielectric materials (i.e. phospholipids). In the frequency range we are working, these blocks are not conductive materials and can be excluded from the cytoplasm volume. For the sake of simplicity, the cytoplasm is modelled as a simple resistor with resistance R_i .

The cell membrane is a barrier of approximately 10 nanometres that separates the cytoplasm from the extracellular fluid (passive role), and controls the exchange of different chemical species between the two (active role).¹⁷⁷ Both roles are played by different structures. Indeed, the passive part of the membrane is mainly composed of the phospholipid bilayer (**Figure 22.b**). Phospholipids are molecules whose structure is made up of two hydrophobic fatty acid chains linked to a hydrophilic phosphate group. This bilayer allows lipids and water through but not ions and can therefore be considered as a dielectric material surrounded by conductive aqueous materials and can consequently be modelled as a capacitor with a capacitance C_m ($\sim 1\mu\text{F.cm}^{-2}$ of membrane area¹⁷⁸). The membrane capacitance can vary between cell populations and depending on the chemotherapeutic treatment. This was shown by Lannin et al. who through the use of electrorotation showed that gemcitabine-resistant PDAC cells (BxGR360c) had a 17 % increase in membrane capacitance compared to non-gemcitabine resistant cells (BxPC3).¹⁷⁹

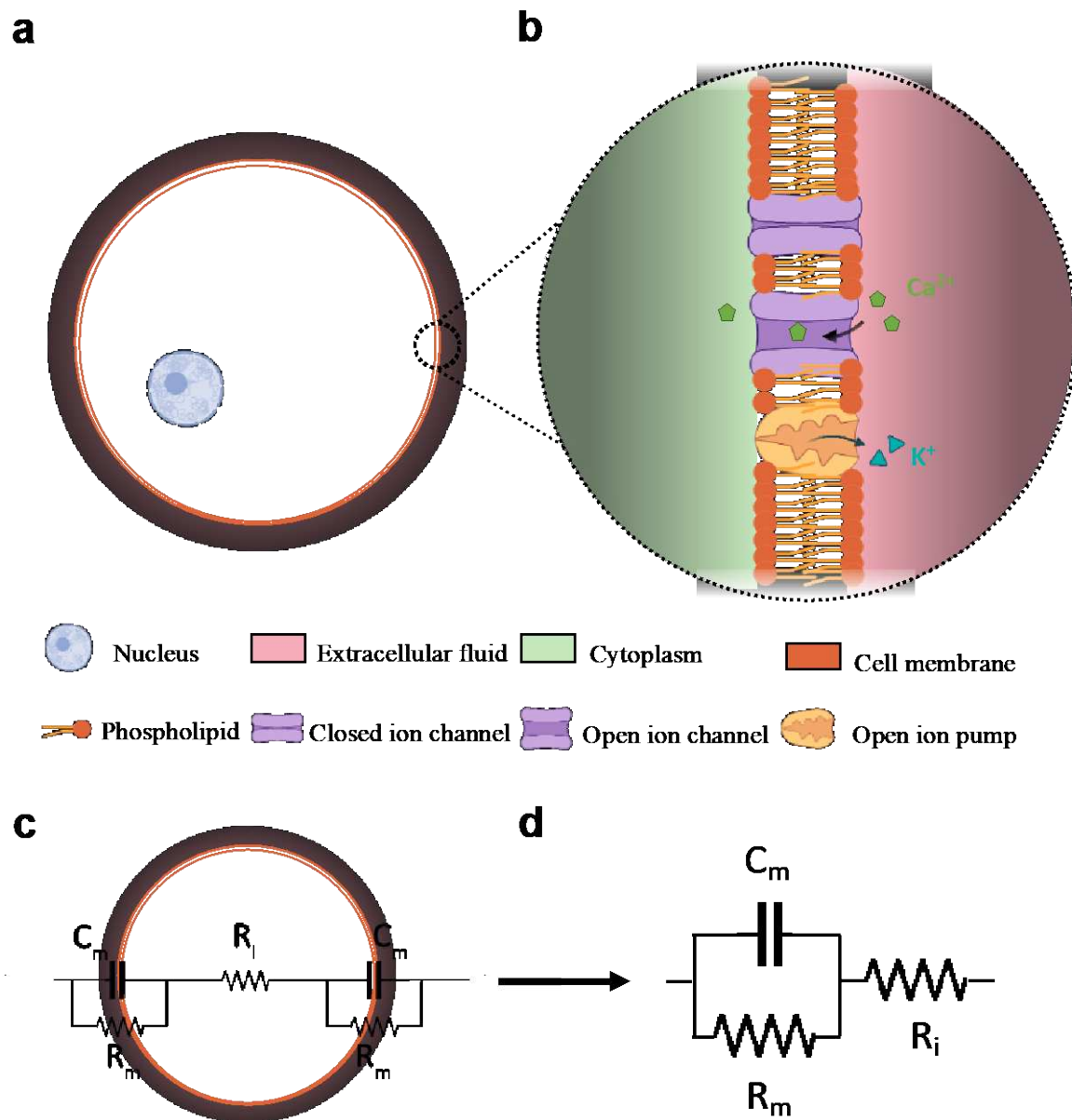


Figure 22. Equivalent electrical circuit of a cell. (a) Schematic of the "generalised cell" structure. (b) Zoom in on the cell membrane and its structure. (c) Equivalent electrical circuit of a cell with the electrical components corresponding to the cytoplasm (R_i) and cell membrane (R_m and C_m).

As for the active part of the cell membrane, it is mainly composed of embedded proteins that form ion channels and pumps. The ion channels are porous structures whose function is to passively allow the flow of ions either between the intra- and extracellular liquids, or between two cells (gap junctions). They can be in an open or closed state and are selective of a certain type of ions. On the other hand, ion pumps actively push ions in and out of the cell through the active release of ATP.¹⁸⁰ Both structure work to maintain cell integrity through maintenance of osmotic gradient between the extra- and intra-cellular liquids. They enable the active part of the membrane to be considered as a resistor with resistance R_m in parallel with the membrane capacitance C_m . Accordingly, it is possible to represent a single cell as an electrical circuit (Figure 22.c).

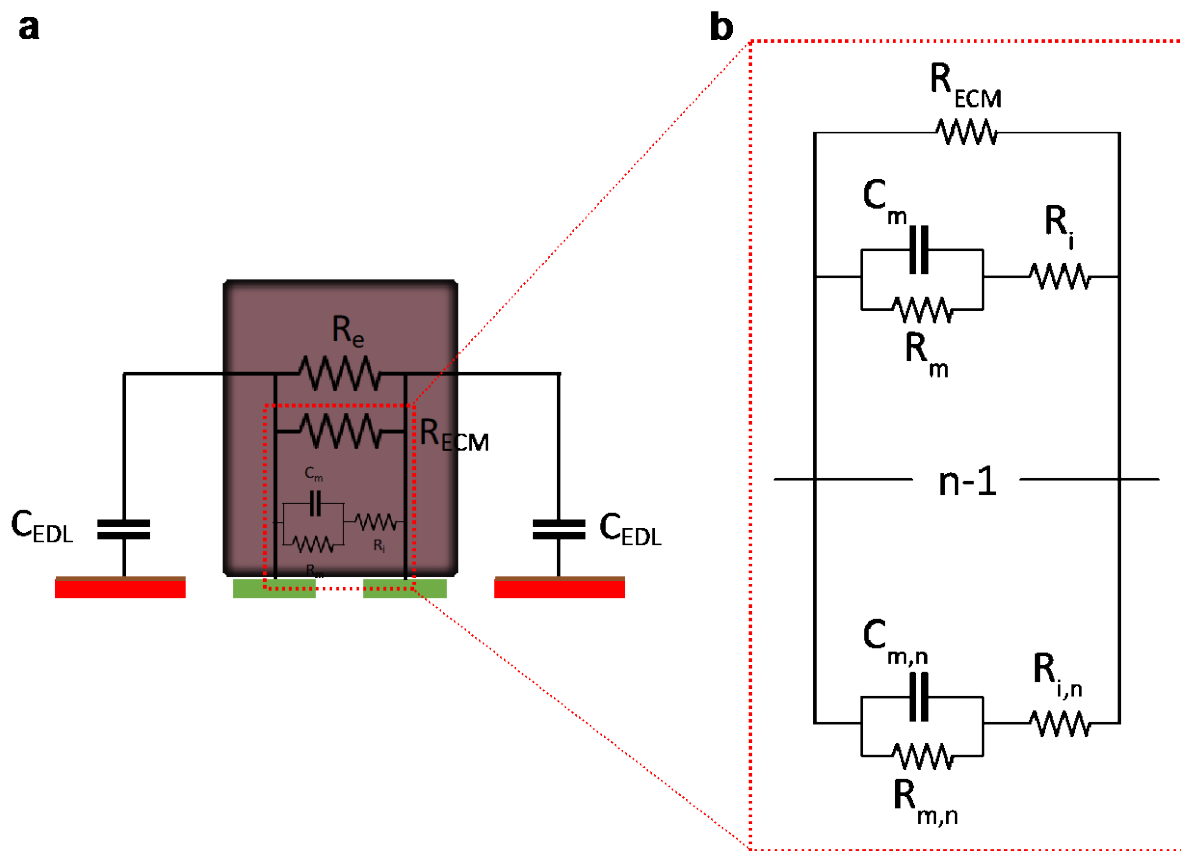


Figure 23. Bioimpedance measurement on scaffold-based 3D cell culture. (a) Equivalent electrical circuit of a 4-point impedance measurement setup to characterise cell culture within a hydrogel. The measurement volume is represented by the pink rectangular area. (b) Equivalent circuit of a tissue with cells in an ECM.

To represent a 3D cell culture as an equivalent electrical circuit, we can include this representation of a cell into the electrical circuit representing the ECM and extracellular fluid (Figure 23.a). However, as a tissue is made of many cells that proliferate, we can model them by putting their equivalent circuits in parallel (Figure 23.b).

Having described the equivalent circuit model of a 3D tissue, it becomes evident that cellular viability strongly influences impedance parameters. Indeed, when cells are exposed to drugs that induce cell death (via apoptosis, necrosis or other mechanisms), the immediate consequence is a disruption of membrane integrity. This leads to changes in membrane resistance and capacitance, as the insulating properties of the membranes are compromised. Consequently, bioimpedance measurements reflect these physiological changes, directly correlating with tissue viability. Thus, monitoring changes in impedance parameters can effectively serve as a non-invasive, real-time viability assay of 3D cell culture and tissues.

3.4. Bioimpedance measurements in solid tumour models

3.4.1. Bioimpedance viability measurements in static 3D culture

Multiple electrode setups have been utilised to study drug response of static 3D cell cultures.¹⁸¹ Kloß et al. developed a measuring method based on 3D cell culture being placed in a micro cavity array, with inclined walls on which the electrodes are patterned. This forces the electrical signal to pass through the spheroid directly, consequently measuring its impedance¹⁸². This setup was utilised by Pan et al., who studied lung cancer cells embedded in Matrigel™ in a micro cavity array (**Figure 24.a**).¹⁸³ They first measured the impedance of the cell-gel mixture and compared it to the impedance of cell-free gel. They observed a decrease in impedance when cells were embedded into the gel. Additionally, they observed that this decrease in impedance was proportional to the cell number, i.e. the more cells are present in the gel, the lower the cell-gel mixture's impedance. They attributed this decrease in measured impedance to the gap junctions between cells. Indeed, although cells are considered non-conductive at low frequencies due to their insulating membranes,¹⁸⁴ they form intercellular channels, known as gap junctions, that enable the bidirectional cytosolic exchange of ions, metabolites and secondary messengers.¹⁸⁵ When the cells are exposed to an electrical field, these gap junctions facilitate the passage of electric current by creating conductive pathways between connected cells and increase the overall electrical conductivity of the cell-gel mixture, hence the decrease in impedance.

They subsequently utilised a cell index, defined as the ratio of the 3D cell growth-induced impedance ($|Z_{cells}|$) change $|\Delta Z| = |Z_{cells} - Z_0|$ to the impedance of Matrigel™ ($|Z_0|$), that is, . Using this normalised readout, they temporally measured the effects of therapies on lung cancer cells with two distinct phases (**Figure 24.b**). Prior to the treatment administration,

they observed an increase in the CI. They attributed this to an impedance decrease of the cell-gel mixture ($|Z_{cells}|$) compared to cell-free gel ($|Z_0|$) due to the cell proliferation.¹⁸⁶ The absolute value of ΔZ therefore increases as does the CI (**Figure 24.c**). As previously mentioned, their explanation is that cell proliferation results in an increase in the number of gap junctions. However, another explanation that they did not investigate was the accumulation of ions within the cell-gel mixture due to cell metabolism. Indeed, the metabolic activity of cells leads to the accumulation of ions, such as hydrogen ions (H^+) and other metabolic byproducts, which would increase the ionic strength of the cell-gel mixture and therefore its electrical conductivity. This phenomenon may significantly affect impedance measurements by reducing Z_{cells} compared to Z_0 and could explain their observations.

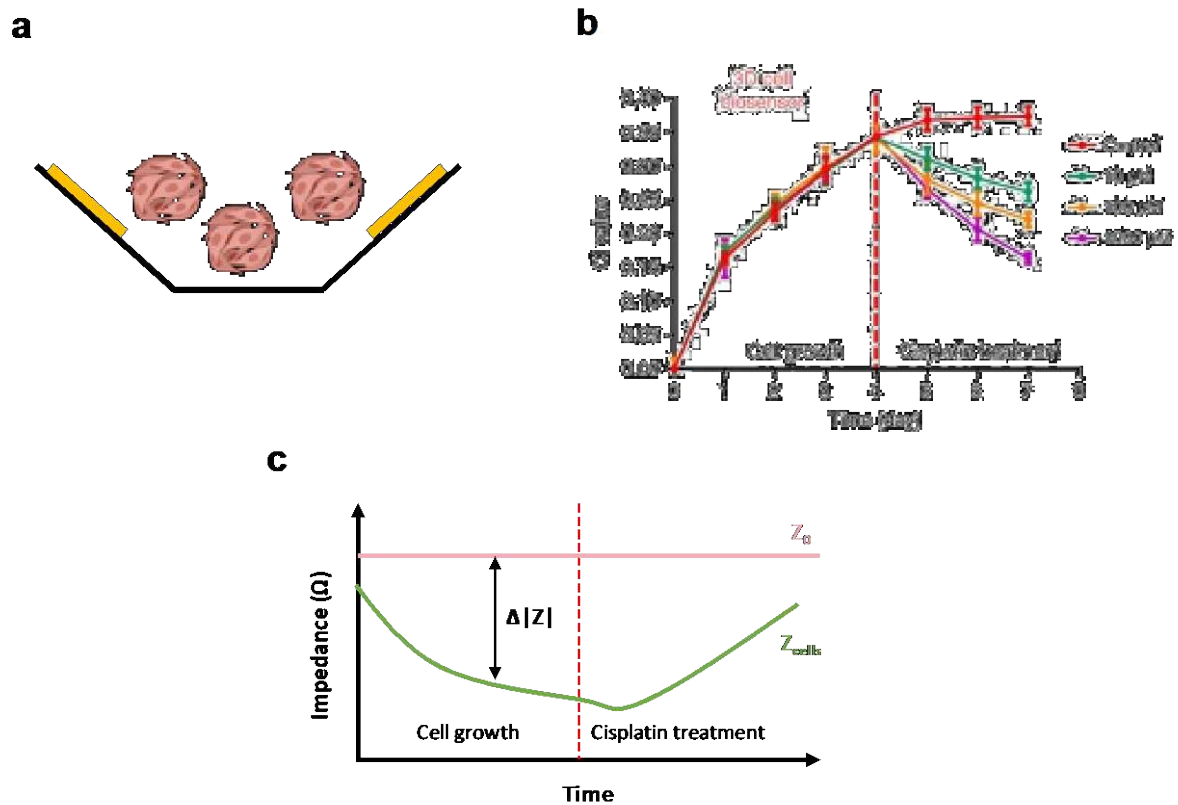


Figure 24. Micro cavity array bioimpedance monitoring of lung cancer cell drug response. (a) The micro cavity features inclined walls with electrodes, enabling for the electrical signal to be forced through the gel/cell mixture. (b) Subsequent cell growth and response to treatment performed in a micro cavity array. From [183]. (c) Changes in cell free Matrigel (Z_0) and cell/Matrigel mixture (Z_{cells}) over time and subsequent changes in ΔZ . As the CI is the absolute value of ΔZ normalised by Z_0 , CI increases as cells proliferate.

The second phase, after cisplatin treatment, was marked by a decrease in the CI. They attributed this to alterations to gap junctions due to the treatment-induced cell death, resulting in an increase of the cell/gel mixture impedance Z_{cells} such that the cell-gel mixture's

impedance returns to a value closer to the impedance of cell-free Matrigel™. However, they did not investigate the electrical conductivity of cisplatin. Although the conductivity of cisplatin itself is not well established, it is commonly reconstituted in dimethyl sulfoxide (DMSO), a weak conductor.¹⁸⁷ A simple control experiment measuring the impedance of the cisplatin solution would help assess its direct contribution to the observed impedance changes as the addition of a weak conductor would have an effect on the measured impedance and therefore on the CI.

Lei et al. decided on a different approach and utilised parallel plate electrodes to quantify the drug response of hepatoma cells spheroids embedded in agarose gel after seven days of culture (**Figure 25.a**).¹⁸⁸ They first cultured the cells in methyl cellulose gel, but as its impedance was too low to characterise cellular behaviour, they seeded the cells in agarose gel instead. They subsequently cultured them for seven days to obtain spheroid colonies prior to doxorubicin treatment. As they found that they obtained the best linear correlation between cell number and measured impedance at a frequency of 100 Hz, they defined two normalised quantities to describe both colony formation as well as treatment response: a colony index (impedance magnitude change at 100 Hz) and a colony size (impedance phase angle at 100 Hz). They then correlated a decrease in colony index and colony size with an increasing concentration of drug application, providing a quantitative and objective method to analyse both early tumour development and drug-response (**Figure 25.b**).

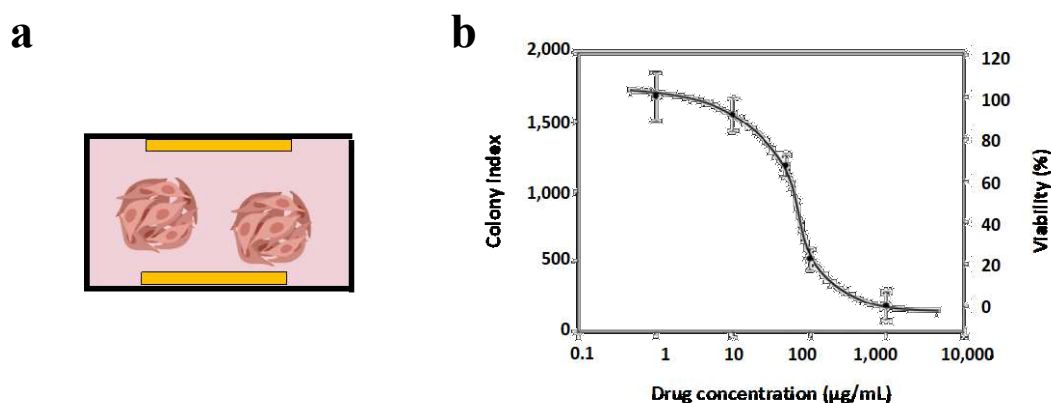


Figure 25. Parallel electrodes bioimpedance monitoring of hepatic cancer cell drug response. (a) Electrodes are placed at the bottom and top of the cell culture, enabling for the electrical signal to be forced through the gel/cell mixture. (b) Subsequent measurement of colony index with respect to drug concentration (doxorubicin) and consequent cell viability. From [188].

Other studies investigated 3D cell culture drug response by using coplanar electrodes to measure the impedance of spheroids cultured in hydrogels. One of these studies was performed by Jeong et al., who investigated human lung adenocarcinoma epithelial cells in a 1

% (w/v) alginate solution over coplanar gold electrodes (**Figure 26.a**). They treated the culture with erlotinib (EGFR tyrosine kinase inhibitor) and measured the resulting cell viability to establish an IC_{50} curve using impedance.¹⁸⁹ They compared it with optical measurements and a Cell Count Kit to assess the sensitivity of impedance to assess the cell viability of a hydrogel-based 3D model, and found similar IC_{50} values for all three cell viability assays (**Figure 26.b, c and d**).

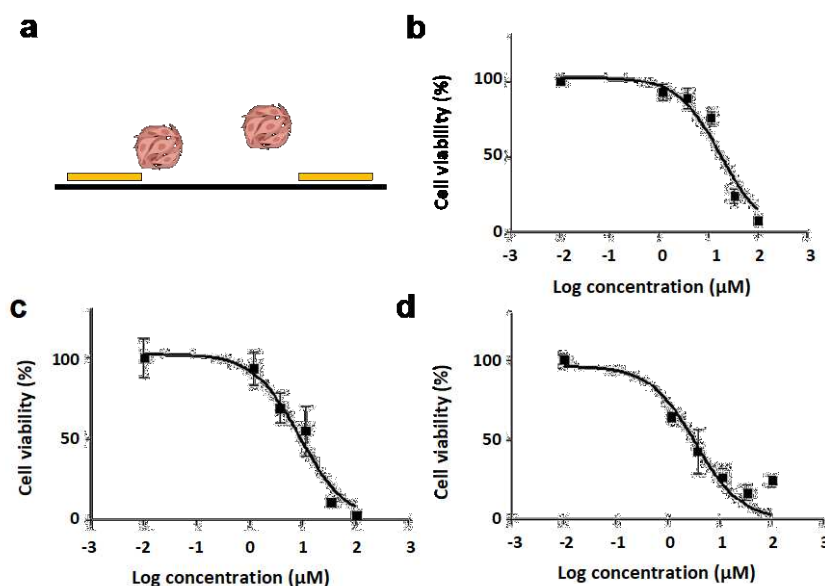


Figure 26. Coplanar electrodes bioimpedance monitoring of lung cancer cells. (a) Electrodes are placed at the bottom of the cell culture with the gel/cell mixture on top of it. (b) bioimpedance, (c) optical and (d) cell counting kit determination of IC_{50} of lung cancer cells when exposed to erlotinib. From [189].

Collectively, these studies showcased the ability of bioimpedance measurements to quantify the viability and consequent drug response of static hydrogel-based 3D *in vitro* tumour models. However, we have seen that 3D static models struggle to represent such certain tumour characteristics, such as IF, which are crucial for predicting treatment transport.

In response to this, microfluidic systems, through the precise control of their dimensions, enable a better reproduction of solid tumour characteristics as make it possible to add an essential component, i.e. IF through a dynamic perfusion of the culture medium. Furthermore, microfluidic systems, thanks to their fabrication materials (glass as a substrate), can be easily combined with bioimpedance microelectrodes as they can be fabricated by depositing and patterning a thin layer of metal onto a glass substrate and can therefore be a constitutive component of microfluidics systems to electrically characterise cancer cells.¹⁷⁰

3.4.2. Bioimpedance viability measurements in tumour-on-chips

Lei et al. developed a microfluidic chip with vertical electrodes in six culture chambers to assess the efficacy of cisplatin on a 3D cell/gel construct constituted of human oral cancer cells (OEC-M1) in agarose gel¹⁹⁰. They defined a cell index as $|Z_t|/|Z_r|$ with $|Z_t|$ being the impedance at time t and $|Z_r|$ the impedance at an arbitrary reference time point and used it to quantify the changes in impedance during the dynamic culture. Interestingly, they found that an increase in cisplatin concentration, hence a higher cell death, resulted in a higher measured impedance at a frequency of 500 Hz, which is coherent with measurements performed in static conditions by Pan et al. This correlates with studies which concluded that cells act as conductors when embedded in a hydrogel due to the electrical connections formed by the gap junctions between neighbouring cells.^{184,191}

Pandya et al. studied the drug response of WT human prostate cancer cells (DU-145) and drug-tolerant cells by culturing both in a collagen scaffold as individual cells in a microfluidic system¹⁹². They seeded individual cells in the hydrogel and exposed the cells to carboplatin, a cisplatin analogue while measuring the resulting viability via bioimpedance measurements over the following 12 h. To validate the use of bioimpedance measurement to extract the IC_{50} value of the cells to Paclitaxel, they also extracted it with a standard MTS viability assay. They obtained similar cell viability for both methods for the same concentration of drugs (80 % for a concentration of 10^4 nM), thus validating the usability of bioimpedance measurements to quantify the drug response of a dynamic 3D solid tumour model. However, in this study, the drug was applied to the cells immediately following seeding. Therefore, the impedance changes observed likely reflect the response of individual, isolated cells rather than the collective response of a fully formed tissue.

3.5. Conclusion

Bioimpedance is defined as the ability of a biological material to oppose the flow of electrical current, a property arising from the intrinsic electrical characteristics of cells, tissues, and their surrounding environment. It can be quantified by applying an external electrical AC current across the biological sample and measuring the resulting voltage response. This impedance response of a tissue is governed by a combination of resistive and capacitive behaviours that can be represented by an equivalent electrical circuit. By attributing different

values to these resistances and capacitors, it is possible to represent the tumour's cellular heterogeneity.

The impedance of a tissue at low working frequencies (500 Hz to 60 kHz) therefore depends on the characteristics of the tissue: ECM composition and physicochemical properties, cell types, and cellular density. The variations of impedance in this frequency range can be related to biological events such as cell proliferation or death, matrix densification, and changes in cellular metabolism. Importantly, these events can be quantified using impedance measurements over time in a non-destructive manner, as bioimpedance does not require the use of biomarkers.

This approach has been leveraged to characterise the drug response of both 2D and 3D static solid tumour models, illustrating that monitoring changes in impedance parameters can effectively serve as a non-invasive, real-time viability assay for 3D cell culture and tissues. Moreover, the fabrication of microelectrodes is inherently compatible with microfluidic systems, opening the door for non-destructive and more physiological drug response assays.

Work Objectives

Solid tumours are complex and heterogeneous, with mechanical cues such as IF playing a crucial role in their progression and resistance to treatment. While traditional models such as *in vivo* animal models, 2D *in vitro* models, and static 3D *in vitro* models have significantly advanced our understanding of cancer biology, each has inherent limitations in fully recapitulating the complexity of solid tumours. In response to these limitations, microfluidic systems have been more and more utilised to study key aspects of both tumour growth and development, and response to treatment. Indeed, through the precise control of their dimensions, they enable a better reproduction of certain solid tumour characteristics such as IF flow than traditional culture methods. Furthermore, microfluidic systems can be instrumented with actuators and biosensors, such as bioimpedance sensors, enabling real-time long-term monitoring of the culture in a non-destructive manner.

As we have seen, traditional microfluidic system fabrication methods such as SU-8 PDMS soft lithography have often faced criticism for their complexity, time-consuming and costly nature, and the necessity of accessing specialised equipment such as a clean room, raising the need for innovative fabrication pipelines. At the same time, current viability assays lack the capability to provide viability measurements in a real-time, non-destructive manner when measuring responses to therapy, limiting their utility in studying tumour progression and drug efficacy. Addressing these limitations through the development of more representative models and the incorporation of non-invasive viability assessment methods will enable more precise investigation of solid tumour therapeutic responses, highlighting the ongoing need for improved analytical methods to enhance reproducibility and sensitivity in drug efficacy assessment, particularly in physiologically relevant 3D models.

In this context, we identified three objectives for the thesis project:

The first objective focused on the development of a parallelised microfluidic system aiming to host solid tissue models. Additional requirements were to develop a structure allowing easy access to the biological model while also increasing the overall number of experiments on one system. To achieve this, we developed a novel fabrication and characterisation pipeline based on LCD 3D printing. We then validated the microfluidic system for cell culture by cultivating both a 2D and 3D PDAC model over a seven-day period.

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The second objective was the incorporation and characterisation of a bioimpedance microelectrode biosensor within the culture chambers of the microfluidic system. We first determined both electrode geometry and placement to maximise its sensitivity. We then developed a dedicated automated data acquisition and analysis pipeline to utilise this bioimpedance sensor to monitor long-term cell culture. Furthermore, the influence of key parameters, such as culture chamber height and alignment with the electrodes, were quantified.

The third objective was to incorporate a 3D solid tumour model within the microfluidic device, and measure cell proliferation and cellular death via bioimpedance monitoring. The first step was to monitor cell proliferation in a 2D cell monolayer PDAC model. We then characterised the electrical signature of a collagen 1 based ECM to select the most appropriate volume and collagen 1 concentration. Finally, we investigated the effects of incorporating PDAC cells in the hydrogel and monitored the 3D model when cultivated in dynamic conditions in the microfluidic systems.

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Results

Chapter 1: Development of a parallelised microfluidic chip using 3D printing as a dedicated fabrication method

The chapter was the fruit of a collaborative effort resulting in a manuscript currently submitted for publication: “*Design of a microfabrication pipeline combining PDMS soft lithography and vat photopolymerization for the engineering of various microfluidic systems to study tissues and multicellular*”. 3D printer comparison data acquisitions were realised by **Robin Houssier** (D3) and **Orégane Bajeux** (D1). Cell viability assays in contact with 3D printed components were realised by **Dr Thomas Meynard**. Fluorescein numerical simulation was implemented by **Anthony Leroy** (M2).

In this chapter, we have developed a new versatile 3D microfluidic device, using LCD-based 3D printing with TR300 resin for PDMS moulding and Bio-Med Clear (BMC) resin for constitutive parts. We treated BMC parts to decrease its cytotoxicity and autofluorescence, and then validated the use of the device for cell culture. This device is also adapted to modern robotics and microscopes with new and tailored-made culture chamber geometries. Overall, this versatile microfluidic platform can be used to study various complex biological models and establish dynamic culture conditions, allowing to address a variety of use and applications in many biological fields such as cancer.

1.1. Design of microfluidic device geometry

The first step of a microfluidic system fabrication is to define the geometry of the culture chambers. We first established a list of requirements that we used to guide the geometry's conceptualisation:

1. Parallelised and independent culture chambers,
2. Structure that allows easy access to the biological model,
3. Increased culture chamber dimensions to increase the amount of biological material.

To that effect, the chip can be divided into two distinct parts: a PDMS bottom part (**Figure 27**) and a cap (**Figure 28**). This structure allows easy access to the biological sample at any given time-point of the culture, thus validating requirement 2.

The bottom part's initial dimensions were 70 x 50 mm and it featured 12 individual channels (**Figure 27.a**), with each culture chamber 15 mm long and 2 mm wide (**Figure 27.b**). As we intended to develop a user-friendly microfluidic system, and although this geometry complied with all three previously mentioned requirements, we noticed that the culture chambers were not properly aligned with standard Society for Biomolecular Screening (SBS) 96 well plates (**Figure 27.c**). Consequently, we modified both culture chamber dimensions and placements to align them with a 96 well plate's wells. This resulted in an intermediary geometry (**Figure 27.d**) with six independent channels. Each individual channel is 20.6 mm long, 2 mm wide and is aligned with the walls of a 96 well plate well (**Figure 27.e**). Although all channels were aligned with a 96 well plate (**Figure 27.f**), we deemed the channel spacing too inefficient. To improve channel spacing, we increased the number of channels to eight chambers (**Figure 27.g**) while decreasing their lengths to 19.98 mm (**Figure 27.h**). This allowed us to align both inlets and outlet's centre point with an SBS 96 well plate well's centre point (**Figure 27.i**). This maximised both efficiency and usability, as the device is easily inserted in modern microscopes enabling automatic image acquisition.

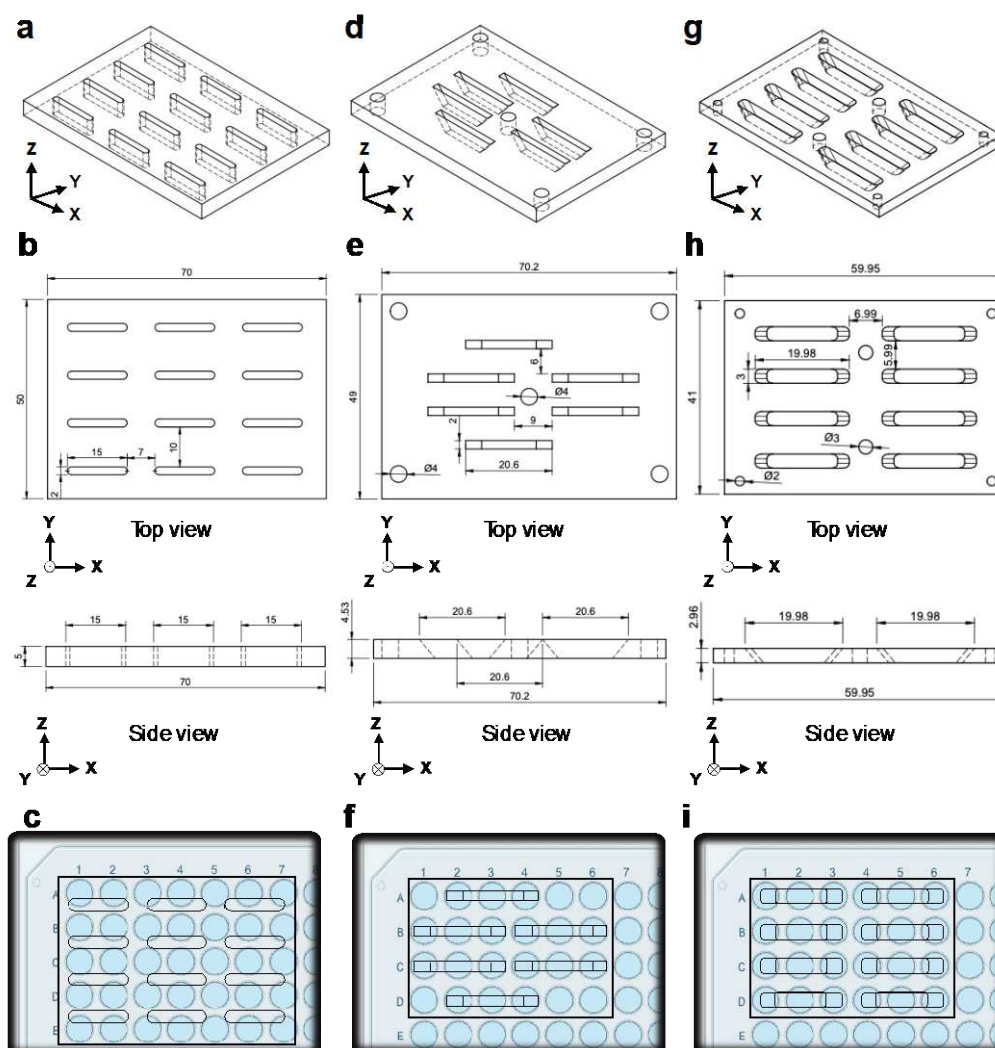


Figure 27. Three parallelised microfluidic geometries. Technical diagrams of the isometric (a), top and side (b) projections of the 12-channel geometry. This design was not properly aligned with the wells of a 96-well plate (c). Technical diagrams of the isometric (d), top and side (e) projections of the 6-channel geometry. This design ensured that all six channels are aligned with the wells of a 96-well plate (f). Technical diagrams of the isometric (g), top and side (h) projections of the 8-channel geometry. This design ensured that all eight channels are aligned with the wells of a 96-well plate (i). All dimensions are in mm.

The second component of the microfluidic chip is the cap (**Figure 28.a**). The first material used for its fabrication was PDMS, but it was later changed to a 3D printing resin as we will see further in this chapter. Its dimensions are similar to the bottom part's, with one inlet and one outlet per channel (2 mm internal diameter) separated by 17.98 mm centre to centre while all channels are 8.99 mm apart and are surrounded by a leak-proof cord (**Figure 28.b**). The culture chamber is produced by inserting the cap into the PDMS bottom part (**Figure 28.c**). The height of the culture chamber is the crucial dimension in the closed microfluidic chip, as it influences the physical constraints imposed on the biological model. It depends on how deep

the cap is inserted into the PDMS bottom. It therefore depends on both the cap's protrusion height and the PDMS bottom part height and can be modified based on user need to generate solid stress by increasing the depth at which the cap is inserted and pressing down onto the biological model. Once closed, the culture chamber (yellow), where the biological components are loaded, is created. Its dimensions are 15.01 mm long, 3.00 mm wide, and 0.60 mm high (Figure 28.d).

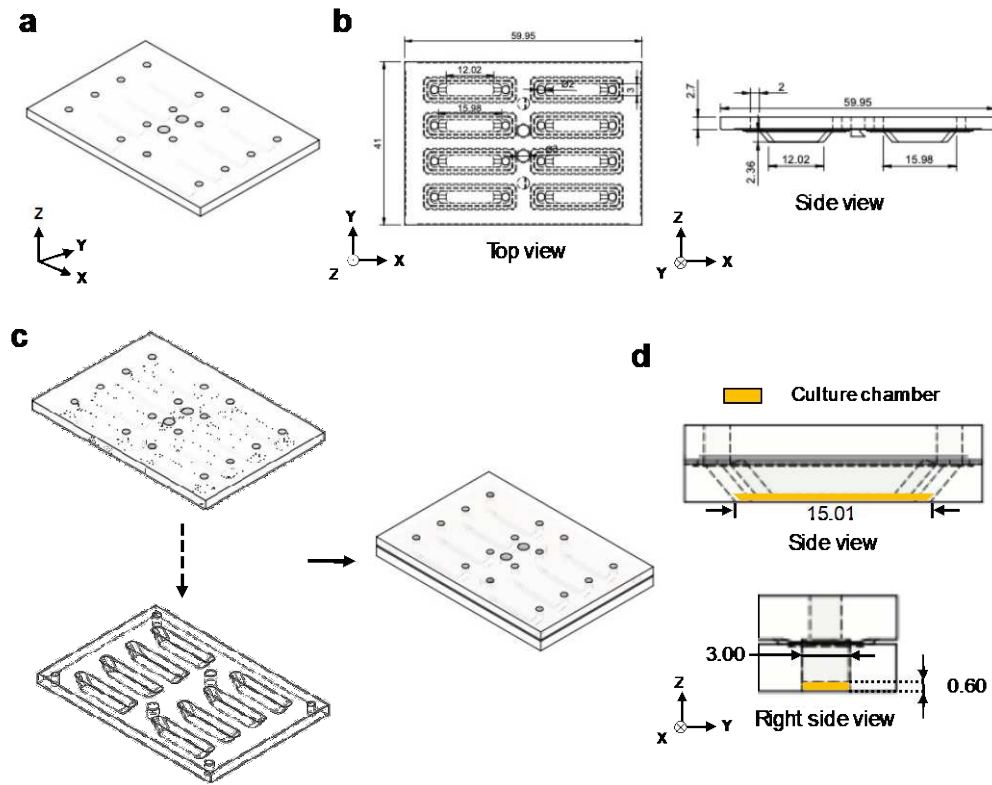


Figure 28. Microfluidic system cap for the culture chamber formation. Technical diagrams of the isometric (a), top and side (b) projections of the microfluidic chip cap. The cap is inserted into the bottom part until both components are in contact (c). This enables the formation of the microfluidic culture chamber (d) with its height being modulated by the cap's protrusion height. The culture chamber has a reference height of 0.60 mm. All dimensions are in mm.

To validate the culture chamber geometry, we numerically simulated the fluid flow inside the channel to evaluate both flow velocity and shear stress. To determine which physics to implement (Laminar or Turbulent flow), we calculated the channel's Reynolds number when a fluid flows at $5 \mu\text{m.s}^{-1}$, and obtained a value of 6×10^{-3} indicating a laminar flow profile. After implementing the channel geometry in the numerical simulation (Figure 29.a), the simulation of fluid flow in the culture chamber was carried out according to the Stokes equations (the flow of an incompressible Newtonian fluid with a low Reynolds number):

$$\mu \nabla^2 \vec{u} = \nabla p - \vec{f} \quad (18)$$

with μ the dynamic viscosity of the fluid (Pa.s), $\vec{u}$ its velocity (m.s^{-1}), p its pressure (Pa), ρ its mass density (kg.m^{-3}) and $\vec{f} = \rho g$ the bulk forces being applied to the fluid (N). The parameters used in the simulation are recapitulated in **Table 3**:

Table 3. Parameters used for numerical simulation of laminar flow.

Parameter	Symbol	Value	Units
Dynamic viscosity	μ	10^{-3}	Pa.s
Outlet pressure	p	0	Pa
Mass density	ρ	10^3	kg.m^{-3}
Inlet flow rate	q	3.74	$\mu\text{L.min}^{-1}$

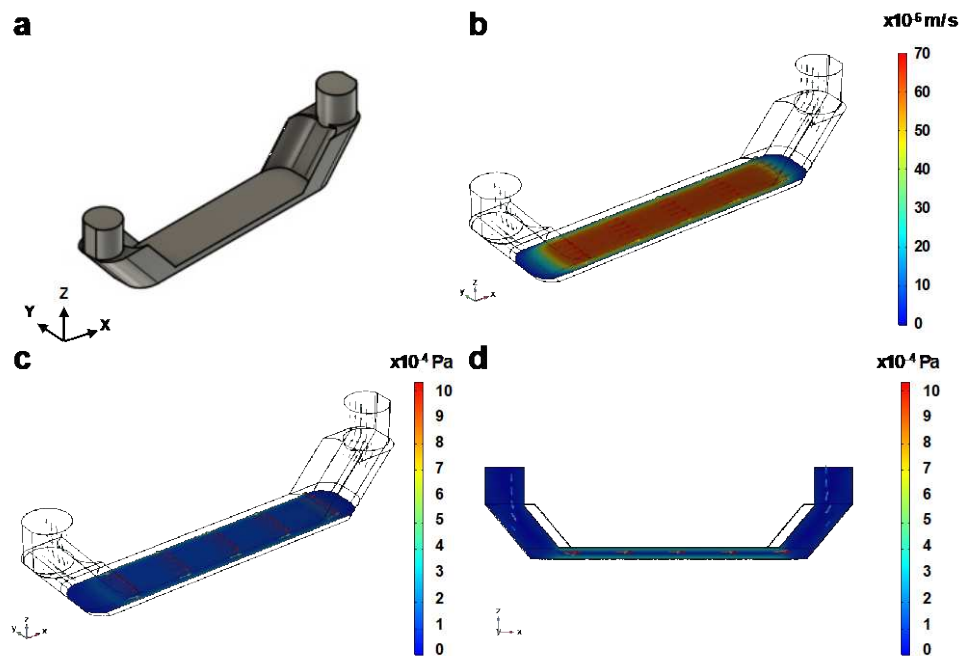


Figure 29. Numerical simulation of the flow profile across the microfluidic channel using COMSOL Multiphysics®. (a) Extraction of the fluidic channel geometry from the ‘.sat’ file to import it in COMSOL. The geometry is then meshed to implement the laminar flow physics to calculate the flow velocity profile (b) in the channel. The shear stress profile can also be extracted in both isometric (c) and xz (d) orientations.

This enabled us to plot the velocity profile (**Figure 29.b**) within the culture chamber. We observed that the average fluid velocity in the culture chamber volume was $34.42 \mu\text{m.s}^{-1}$. Additionally, this model enabled us to extract the shear stress distribution in the culture chamber (**Figure 29.c**). Although the average shear stress across the chamber’s volume was $1.95 \times 10^{-4} \text{ Pa}$, it was mainly concentrated at the channel’s wall, with an average value of $3.60 \times 10^{-4} \text{ Pa}$ at the channel’s bottom (**Figure 29.d**). This value is lower than *in vivo* values for

IF-induced shear stress in solid tumours (1.00×10^{-2} Pa),²⁵ but this difference can be explained by the fact that our model only considers fluid flow and no other physical parameters such as surface roughness that have been shown to increase shear stress.¹⁹³ Additionally, we did not include cells that increase the *in vivo* shear stress by reducing the *in vivo* interstitial space. The sequence of COMSOL commands for this modelling is given as an ‘.mph’ file in **Appendix 3**.

We first designed the microfluidic chip to align with an SBS 96-well plate while meeting our specific requirements. The chip was split into two distinct sections to ensure easy access to the biological material. Next, we numerically simulated fluid flow through the channel to assess both flow velocity and shear stress, to validate the channel’s geometry. The following step was to optimise the fabrication of the microfluidic chip.

1.2. Development of a dedicated fabrication method based on LCD 3D printing

1.2.1. PDMS mould fabrication method

Due to its two-components nature, as well as its dimensions (PDMS bottom is 2.96 mm thick), the microfluidic chip could not be fabricated using traditional SU-8 moulding. We therefore decided to use 3D printing to produce PDMS moulds. We first evaluated two commercially available 3D printers to determine which produces the most precise pattern dimensions: the DWS XFAB 200, based on SLA technology, and the Phrozen Sonic Mini 8K, which employs LCD technology. Their specifications are defined in **Table 4**.

Table 4. Specifications of XFAB 2000 and Sonic Mini 8k 3D printers.

Printer \ Specifications	Technology	Resolution (μm)	Printer size (cm)	Weight (kg)	Printing volume (mm)	UV source	Layer thickness (μm)	Retail price (\$)
DWS XFAB 2000	Laser SLA	10	40x60.6x64.2	31	18x18x18	Solid State BlueEdge®	10-100	5000.00
Phrozen Sonic Mini 8K	LCD SLA	22	29x29x43	13	16.5x7.2x18	405 nm	10-300	484.99

To compare both printers, we decided to use the Precisa 780 resin, as it is specifically made for the SLA 3D printer. We first optimised its printing parameters for the LCD-based printer because as Precisa 780 resin has a high viscosity compared to resins made for LCD 3D printing, it required a higher energy dose to properly cure (**Table 5** and

Table 6).

Table 5. Physical properties of the photosensitive resins studied provided by suppliers.

Cells	Properties	Density (g/cm ³)	Viscosity (mPa.s)	Surface Hardness (Shore D)	Tensile Stress at Break (MPa)	Elongation at Break (%)	Tensile Modulus (MPa)	Heat Deflection Temperature (°C)	Flexural Strength (MPa)	Flexural Modulus (MPa)	Price (\$/kg)
Precisa 780		1.12	1500 - 2200	82 - 85	35 - 45	6 - 7	1700 - 2000	48 - 53	60 - 80	1500 - 1850	160
TR300		1.17	123.6	80	59.9	3.9	3443.7	160	x	x	94.22
Bio-Med Clear		1.18	475	85	55	5 - 10	2000	62	78	2200	122

Table 6. Printing parameters used on the Phrozen Sonic Mini 8k LCD printer for the 3 resins studied.

Parameters	Resins		
	Precisa 780	TR300	BIOMED - Liqcreate
Layer Height (mm)	0.050	0.050	0.050
Bottom Layer Count	6	6	6
Exposure Time (s)	60	1.75	12
Bottom Exposure Time (s)	150	25	60
Transition Layer Count	6	6	6
Transition Type	linear	linear	linear
Transition Time Decrement	x	3.320	6.860
Waiting Mode during Printing	resting time	resting time	resting time
Rest Time Before Lift (s)	0	0	0
Rest Time After Lift (s)	0	0	0
Rest Time After Retract (s)	6	2	2
Bottom Lift Distance (mm)	6	6	6
Lifting Distance (mm)	6	6	6
Bottom Retract Distance (mm)	6	6	6
Retract Distance (mm)	6	6	6
Bottom Lift Speed (mm/min)	15	30	25
Lifting Speed (mm/min)	15	30	25
Bottom Retract Speed (mm/min)	35	75	65
Retract Speed (mm/min)	35	75	65
Anti-Aliasing	no	no	no
Shrinkage Compensation	no	no	no
Tolerance Compensation	no	no	no
Bottom Tolerance Compensation	no	no	no
Print Time Compensation	no	no	no

The required energy dose is provided in SLA printing by a powerful laser, but as LCD 3D printing utilises LEDs to generate UV light, it delivers a lower dose of UV energy. Additionally, both temperature and hygrometry were recorded regularly in the printing room over a 3-month period using a digital thermometer and hygrometer (Atome 3D, Toulouse, France, AFAI1040). These are important parameters as they will directly impact the resin's viscosity and therefore its reticulation. The obtained temperature was stable over time with an average of 20.82 °C as the laboratory is heat-regulated, with only local variations mainly due

to day/night cycle and current weather (**Figure 30.a and b**). The humidity increased over time due to the seasonal weather changes, with an average of 44.97 %.

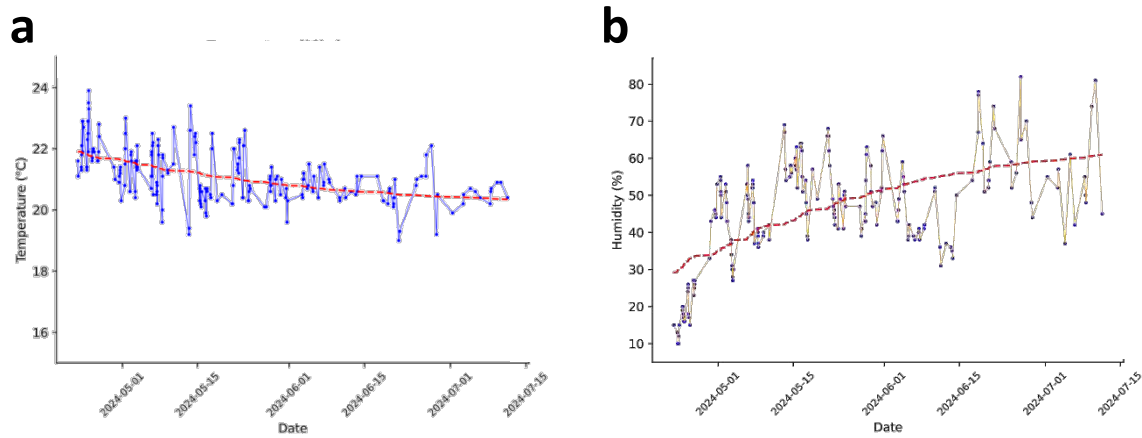


Figure 30. Measured temperature and humidity in the 3D printing room over 3 months. (a) Temperature in Celsius degrees (°C) and (b) humidity in %.

With this in mind, we increased the exposure times for both bottom (layers serving as anchor to the building platform) and normal (3D printed object's composite layer) layers to obtain a satisfactory print, with a respective exposure time of 150 and 60 s (**Figure 31.a, b, c and d**). As the aim was to identify the 3D printer most capable of creating components with the highest geometrical accuracy, we measured the fabrication error of a characterisation platform (CP) made of positive and negative patterns, and we based our analysis on indicators such as printing orientation and pattern type (**Figure 31.e and f**). The averaged measured error for negative patterns in pattern height demonstrated that the accuracy was significantly better for the LCD-based printer compared to the SLA with measured errors of $-11.5 \pm 2.2 \mu\text{m}$ (LCD) and $-16.7 \pm 0.7 \mu\text{m}$ (SLA) for XY- (**Figure 31.g**). However, for Z-patterns, we measured errors of $-9.4 \pm 3.8 \mu\text{m}$ (LCD) and $-52 \pm 10.8 \mu\text{m}$ (SLA), respectively, but found no statistical difference between both 3D printers (**Figure 31.h**). As for positive patterns, the averaged measured error in pattern height demonstrated that the two printers could not be significantly discriminated in accuracy for horizontal printing but a significant difference was found for vertical printing: $-33.9 \pm 0.4 \mu\text{m}$ (LCD) and $-29.6 \pm 2.3 \mu\text{m}$ (SLA) for XY+ ; -120.1 ± 5.0 (LCD) and -152.3 ± 20.3 (SLA) μm for Z+ (**Figure 31.i and j**).

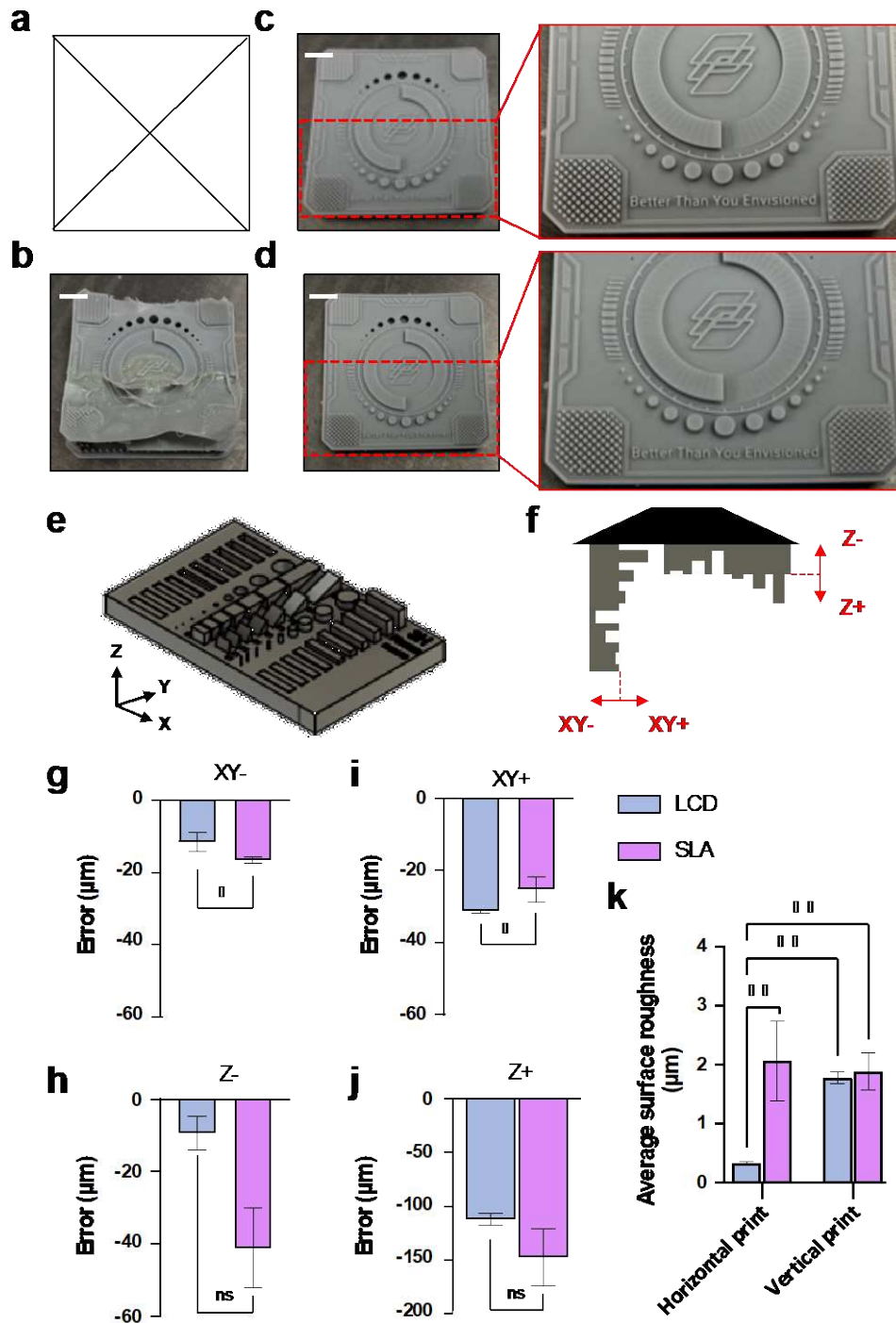


Figure 31. Assessment of fabrication precision of obtained parts with SLA and LCD printer technologies using Precisa 780 resin. (a, b, c and d) Optimisation process of the LCD printing parameters for the use of Precisa 780 resin. (e) Design of the characterisation platform. (f) Schematic of the indicators used to characterise the precision. Comparison of the precision of the printers (Phrozen Sonic Mini 8K (LCD), XFAB 2000 (SLA)) with (g) 'XY-' cavities in length, (h) 'Z-' cavities in height, (i) 'XY+' protrusions in length and (j) 'Z+' protrusions in height as a function of the pattern design dimension and averaged on the whole dimension range (n=3). (k) Comparison of the surface roughness depending on the printing orientation (n=3). Error bars denote SD. * for $p < 0.05$ and ** for $p < 0.01$.

PDMS mould surface roughness is also an important factor to consider, as it will have an impact on PDMS bonding. We therefore decided to measure the average surface roughness of components fabricated using both printers. We found that the measured average surface roughness was significantly lower for platforms printed horizontally using the LCD printer: $0.33 \pm 0.0 \mu\text{m}$ (horizontal LCD) compared to $1.78 \pm 0.1 \mu\text{m}$ (vertical LCD), $2.06 \pm 0.7 \mu\text{m}$ (horizontal SLA) and $1.88 \pm 0.3 \mu\text{m}$ (horizontal SLA) (**Figure 31.k**). No significant differences were observed for the two printers for vertical print, or for the SLA printer for both orientations. Additional characterisation performed in the lab indicated that the LCD-based 3D printer resulted in a lower curvature than the SLA printer (**Appendix 5**). We also considered this an important factor as incurvation of PDMS mould would result in worst bonding to the substrate, subsequently generating leaks.

In conclusion, the LCD-based printer yielded: (i) a better geometrical accuracy, (ii) a lower curvature and (iii) a lower surface roughness. We thus selected the LCD 3D printer for the fabrication of the PDMS moulds using TR300 resin.

To further validate the use of TR300 for PDMS mould fabrication, we decided to fabricate moulds using aluminium milling (**Figure 32.a** and **b**), and to compare the fabrication errors of both methods. Although statistically different, the average height errors measured for both methods were similar to each other, with $-5.32 \mu\text{m}$ for TR300 and $6.52 \mu\text{m}$ for aluminium (**Figure 32.c**). However, aluminium resulted in a significantly lower fabrication error in terms of width, with a mean error of $1.95 \mu\text{m}$, while TR300 3D printing resulted in a $70.26 \mu\text{m}$ error (**Figure 32.d**). Similarly, average surface roughness was significantly better for aluminium moulds than TR300: $0.51 \mu\text{m}$ and $1.69 \mu\text{m}$, respectively (**Figure 32.e**). Although aluminium milling resulted in more precise fabrication, we found TR300 3D printing performance satisfactory. Furthermore, 3D printing enabled us to more freely change the design, allowing for more rapid prototyping, while being less time consuming and less expensive than aluminium milling. Thus, we continued fabricating PDMS moulds using TR300 3D printing.

As the culture chambers are created by inserting the cap into the bottom part, the contact interface between both components must be flat. This flatness ensures that the contact surface area between both components is maximised, and that no fluid leakage occurs at the channels' borders. Thus, a flat surface is required for our system to avoid leakage when parts are assembled. The PDMS moulding protocol was modified accordingly, with the introduction of

the use of 3D printed rings. Briefly, the moulds are closed by a 200 μm thick polyvinyl chloride (PVC) sheet. This PVC sheet is held in place by the newly fabricated rings by sliding them around the PDMS mould. Subsequently, the uncured PDMS is poured in the mould and placed in an oven for reticulation (**Figure 32.f**). We specifically chose PVC as a confining material as it has a low surface roughness ($\sim 1.5 \mu\text{m}$ according to the manufacturer). This ensures that both surfaces of our PDMS parts have a smooth surface. However, because the glass transition temperature of PVC is typically set between 70°C and 90°C , the curing protocol was modified to a lower temperature than the manufacturer's recommendation (from 150°C to 70°C) and longer curing time (10 min to 180 min).

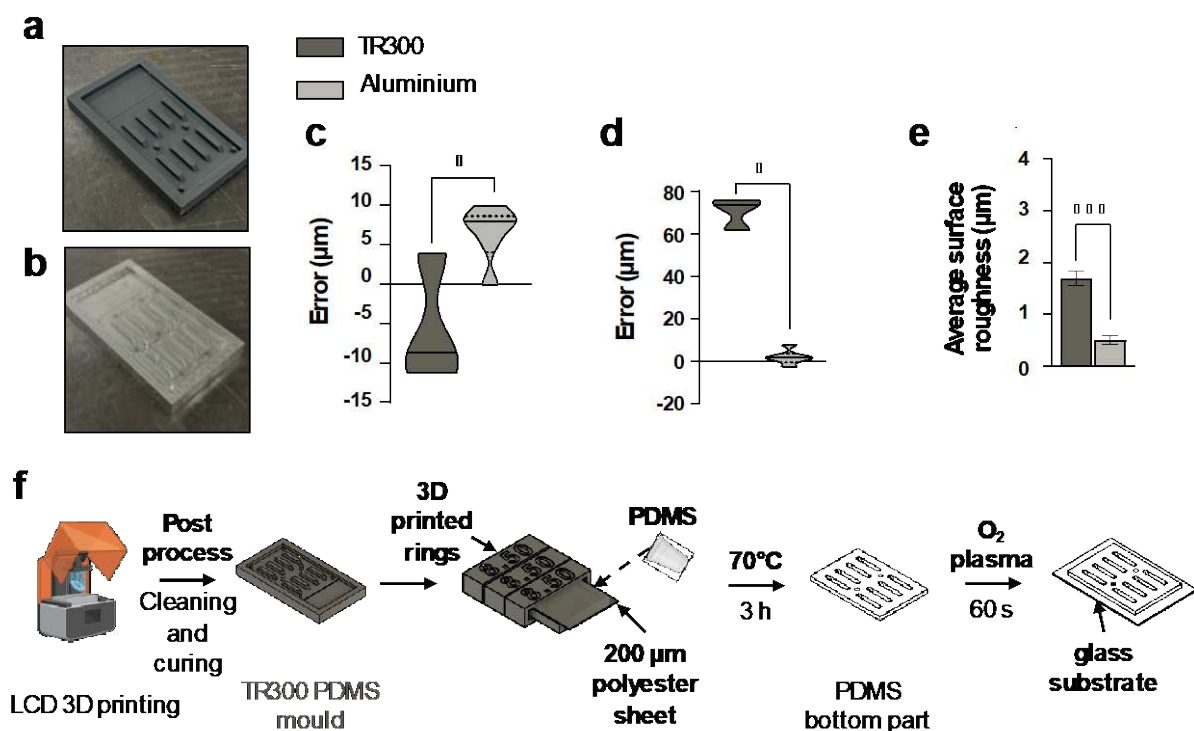


Figure 32. 3D printed and aluminium milled PDMS mould for bottom part fabrication. 3D printed (a) and aluminium milled (b) PDMS moulds. Comparison of height (c) and width (d) error for both types of moulds. (e) Comparison of the surface roughness ($n=3$). (f) Once the moulds have been fabricated and cleaned, they are filled with uncured PDMS and sealed with a 200 μm thick polyester sheet and 3D printed rings. It is then cured for 3h at 70°C , after which the PDMS is demoulded to obtain the bottom part of the microfluidic chip. Once demoulded, the PDMS is bonded to a glass substrate after a O_2 plasma. Error bars denote SD. * for $p<0.05$ and *** for $p<0.001$.

To hold both bottom part and cap together and prevent leaks during dynamic perfusion, a dedicated closing system, composed of two aluminium plates (**Figure 33.a** and **b**) and an aluminium wedge (**Figure 33.c**) were developed. Both aluminium plates feature eight openings to allow microscopy observation, as well as a 2 mm notch to reduce the focal distance between the microscope's objective and the biological model within the culture chamber. As the bottom

plate is threaded, the system is screwed shut using a dynamometric screwdriver at 0.5 N.m to ensure even distribution of the closing force without breaking the glass substrate. Once closed, the theoretical spacing between both closing plates' sides is 2.96 mm. Indeed, the cap is 3.00 mm thick, while the bottom part of the system and the glass substrate are 2.96 and 1.00 mm thick, respectively. As the sum of the aluminium closing plates is equal to 4.00 mm, we can subtract both quantities and obtain a theoretical spacing between both closing plates of 2.96 mm. To ensure proper sealing of the microfluidic system, we decided to be slightly over-tightened together to ensure uniform contact across the entire interface between the lower part and the cap, and thus obtain a theoretically leak-proof closure of the device. Hence, we settled on a thickness 2.80mm for the aluminium wedge.

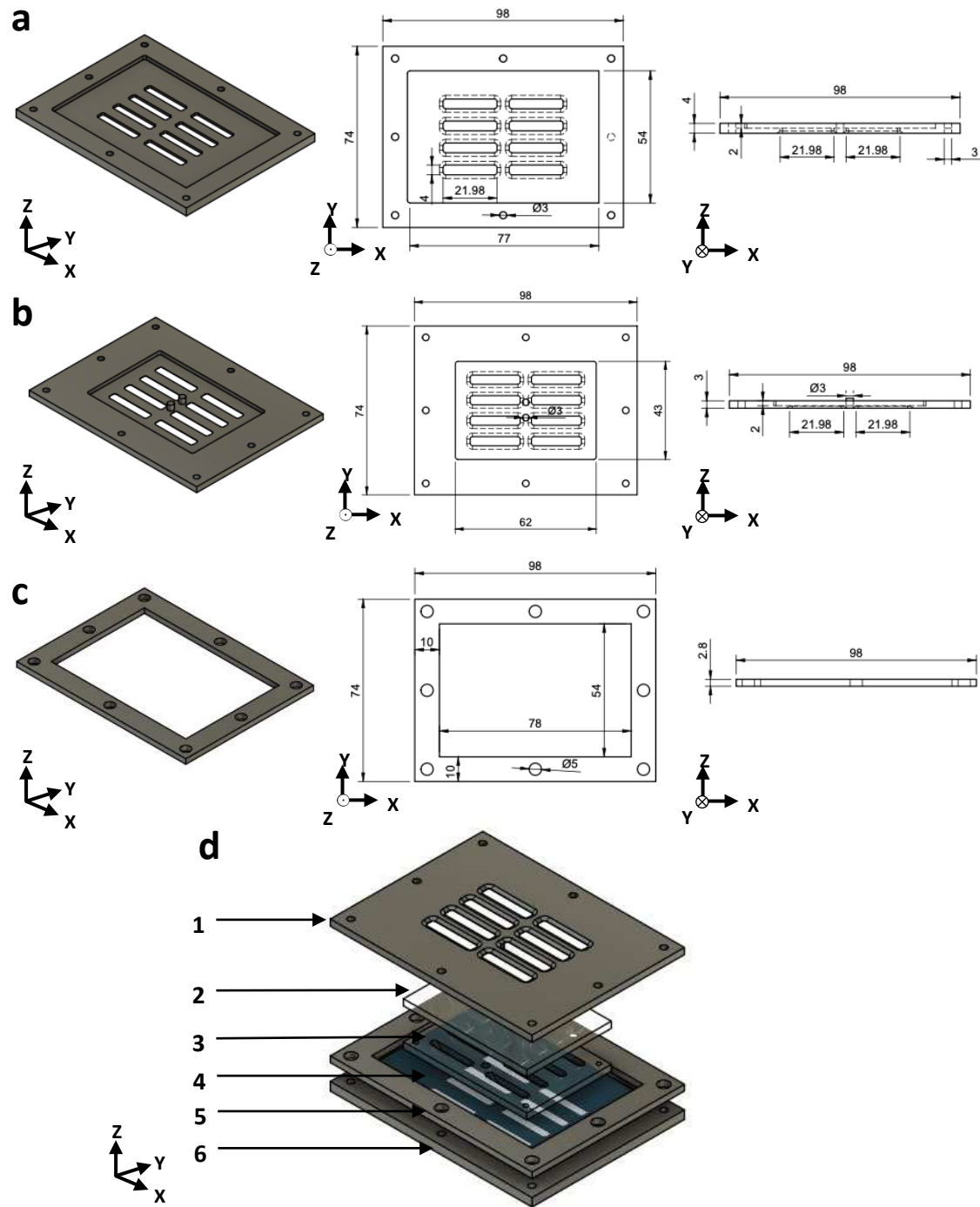


Figure 33. Dedicated microfluidic chip closing system and exploded view of assembled microfluidic chip. (a) The lower and (b) upper aluminium closing plates have a 2 mm deep notch to align with the glass substrate and cap, respectively. Additionally, the notch reduces the distance between the culture chambers and the microscope objectives to improve image quality. Both plates also feature eight openings aligned with the culture chambers to allow imaging to be performed. Both plates are separated by a 2.8 mm thick wedge (c) to ensure proper closing precision. (d) Exploded view of the system showing the six parts composing the microfluidic chip: 1: aluminium upper closure plate 2: PDMS cap 3: PDMS bottom made from 3D printed mould 4: glass substrate 5: aluminium wedge for closure precision 6: aluminium lower closure plate.

1.2.2. Incorporation of 3D printed components as constitutive parts of the microfluidic system

First dynamic perfusion tests revealed that leaks occurred more often than initially anticipated. Our hypothesis was that the leaks were caused by a deformation of the PDMS cap as the device was closed. Indeed, as PDMS is considered as a flexible material, the slight overclosure of the system would result in a bending of the PDMS cap, breaking the seal formed by the contact between PDMS bottom and cap. Additionally, a deformed cap would result in a non-uniform distribution of chamber culture height which is undesirable. To reduce cap deformation, we fabricated it using a biocompatible 3D printing resin (**Figure 34.a and b**), BMC, as it has a higher Young's modulus than PDMS ($\sim 2,000$ MPa and ~ 2 MPa, respectively). This means that a 3D printed cap will not deform as much under bending, thus resulting in a more uniform distribution of culture chamber heights. Consequently, we assembled chips with both 3D printed and PDMS caps and measured the resulting chamber culture heights using a digital microscope. We then generated heatmaps to visualise the height distribution for both caps, which showcased a more uniform distribution when using a 3D printed cap (**Figure 34.c and d**). Furthermore, the 3D printed cap resulted in an average culture chamber closer to the intended $600\ \mu\text{m}$ than a PDMS cap, with averages of $606.15 \pm 14.60\ \mu\text{m}$ and $646.15 \pm 66.40\ \mu\text{m}$, respectively (**Figure 34.e**). This result validated the replacement of PDMS to BMC resin as the material for the chip's cap.

Additionally, we investigated where did the deviation in culture chamber height originate from. The culture chamber's height depends on the 3D printed cap's protrusion height, the PDMS bottom part height, both influenced by the characterised precision of the fabrication protocols, and the precision of the closing system (**Figure 34.f**). Similar to the characterisation of the dimensional precision of the printers and resins, we measured fabrication errors on both the PDMS bottom and 3D-printed cap's dimension of interest. We found that the cap and PDMS bottom part fabrication error was on average -5.98 ± 2.35 and $-42.63 \pm 15.83\ \mu\text{m}$, respectively (**Figure 34.g and h**). From these measurements, we calculated the expected culture chamber height h_{expected} of four pairs of caps and PDMS parts with the formula:

$$h_{\text{expected}} = 600 + \text{error}_{\text{PDMS}} - \text{error}_{\text{cap}} \quad (19)$$

with $error_{PDMS}$ and $error_{cap}$ the previously measured fabrication errors of the respective parts used for. We found that, on average, the expected chamber culture height was $638.61 \pm 15.25 \mu\text{m}$ (**Figure 34.i**). Although this differed from the measured chamber culture height, which was closer to the theoretical $600 \mu\text{m}$, it can be explained by the thickness of the aluminium wedge placed between both closing plates. As it is 2.80 mm thick instead of the theoretical 2.96 mm separating the plates, and considering the flexible nature of PDMS, we anticipated measuring a lower culture chamber height than what was derived from the cumulative manufacturing errors of the cap and the PDMS bottom part. This result validated the utilisation of the closing system, as we obtained a more uniform chamber height when using a 3D printed cap.

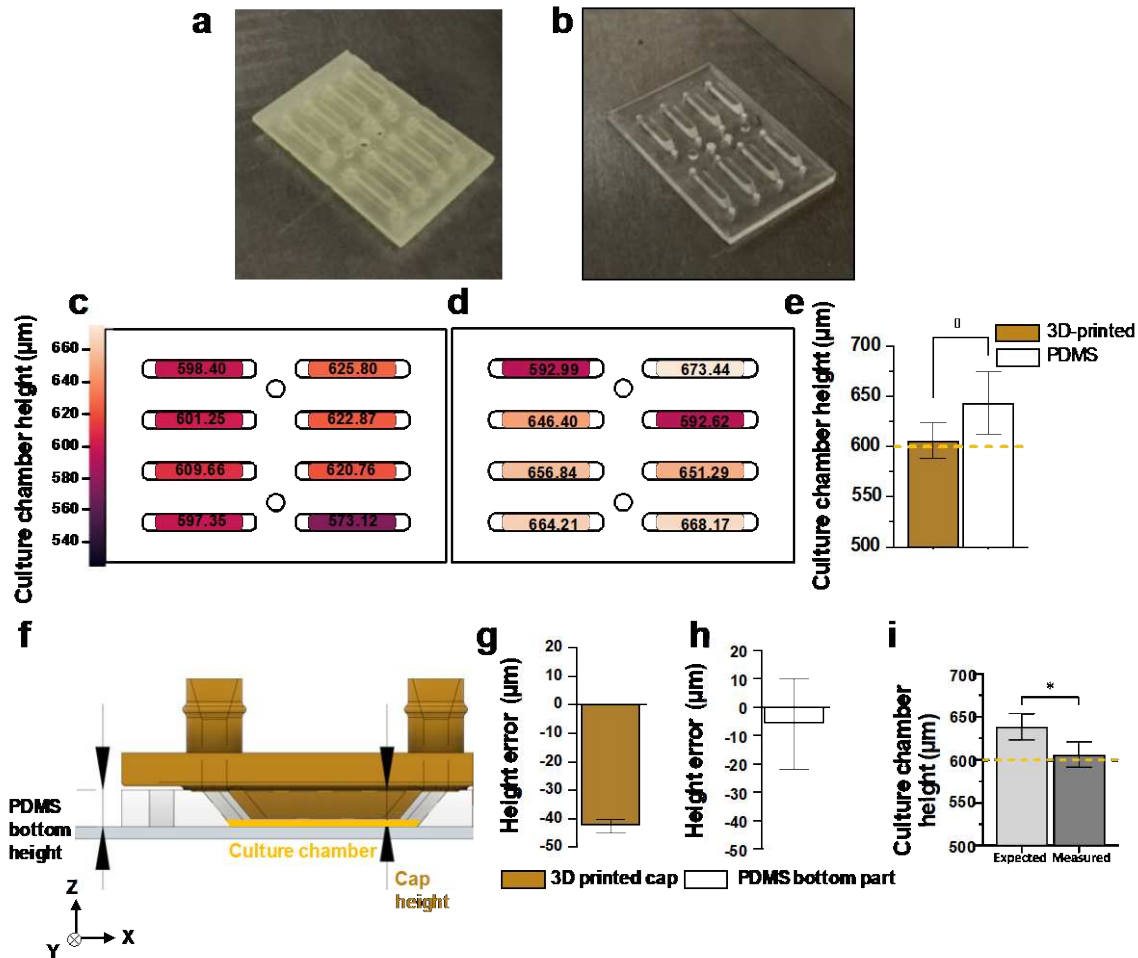


Figure 34. Comparison of culture chamber heights with a 3D-printed or PDMS cap and characterisation of microfluidic chip's components' key dimensions. (a) Cap using 3D printed Bio-Med Clear resin and (b) PDMS. (c) Heatmap of the measured heights of the culture chambers and their annotations (A1 to D2) of a closed device with a 3D-printed and (d) PDMS cap (n=4). (e) Comparison of the culture chamber heights for both 3D-printed and PDMS cap. (f) Schematic of the cap (dark yellow), PDMS bottom section (transparent) and culture chamber (gold) with their dimensions of interest. (g) Analysis of the fabrication error of the PDMS bottom part (n=4) and (h) of the 3D printed cap (n=4). The error is calculated with respect to the dimensions specified in the CAD file. (i) Comparison of the expected culture chamber heights (n=4) with the measured culture chamber heights (n=4) once the device is closed using aluminium plates. The expected height of the culture chambers is calculated using the measured fabrication errors shown in graph (g and h). The gold line represents the theoretical height of 600 µm. Error bars denote SD. * for $p < 0.05$.

Although BMC resin is labelled as a biocompatible resin according to the ISO 10993-5:2009, we assessed its cytotoxicity to validate its long-term use in the microfluidic device. Additionally, we found that 3D-printed components were auto fluorescent when subjected to illumination by a 469 nm wavelength laser, mainly due to remaining photo-initiators even after post-printing reticulation. We performed two surface treatments on 3D-printed components to resolve these issues, and compared both the resulting viabilities, and the mean pixel intensity measured at three wavelengths (excitation at 388, 468 and 567 nm) commonly used in fluorescent microscopy. This consisted in immersion in a Sudan Black B (SBB) solution preceded by an oxygen plasma, after which we coated them with a 2 μ m Parylene C film (**Figure 35.a**). SBB has been previously described to reduce autofluorescence of polymers either by absorbing emitted photons after illumination or by changing the polymer's refractive index.¹⁹⁴ As for Parylene C, it has been previously shown to improve viability in both short and long-term cell cultures by forming an insulating protective polymer layer that prevents toxic phenomena induced by the 3D-printed component.¹⁹⁵

Inserts, designed by a Ph.D. student in the team (Thomas Meynard, Ph.D. thesis 2025), were 3D printed and placed in a 48-well plate to reproduce the dimensions of a 96-well plate's well. Fluorescent HPAC-eGFP cells were then seeded into the inserts and cell viability was assessed after 72 h of culture to compare the cytotoxicity of both treated and untreated components (**Figure 35.b**). SBB treatment resulted in reduced viability after 48 h when compared to untreated inserts, with values of 70.1 % for HPAC-eGFP cells. However, this was compensated by the Parylene C coating, which ensured high cell viability with 90.5 % for HPAC-eGFP cells (**Figure 35.c**). Furthermore, the treatment with both SBB and Parylene C resulted in a lower reduced autofluorescence, as well as a cell behaviour similar to that of the control group at both 24 and 48 hours, with maintenance of cell morphology when compared to the control (**Figure 35.b**).

The reduction of autofluorescence was further evaluated by imaging untreated and treated components using an automated microscope and extracting the mean average pixel intensity. We first observed a notable decrease in autofluorescence when using DAPI (used for nuclei staining) and GFP dedicated lasers when coating the components with SBB, with no visible effect by the additional Parylene C coating. As for the DsRed laser, since there was no fluorescence on uncoated parts, we could not visually determine a decrease (**Figure 35.d**).

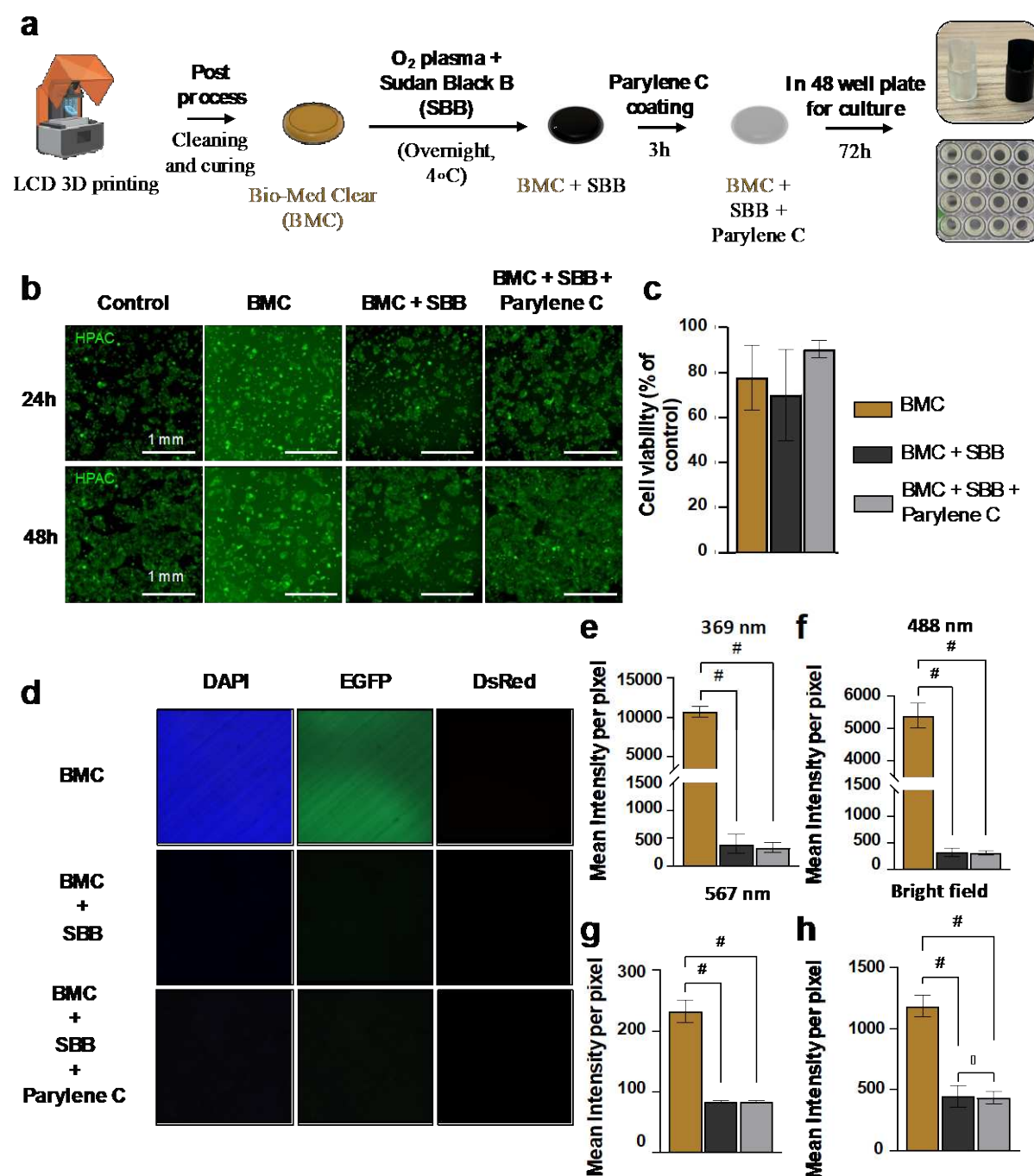


Figure 35. Reduced autofluorescence and increased cytocompatibility of LCD 3D-printed parts through post-processing (Sudan Black B and parylene coating). (a) Upon completion of the printing process, the insert is positioned within a 48-well plate, resulting in a 96-well 3D-printed resin well, maintaining uniform inner dimensions. (b) Images of fluorescent HPAC-eGFP cells at 24 and 48 h in wells made with untreated and treated components. (c) Histogram of cell viability (compared to control) at 72 hours for HPAC-eGFP cells. Scale bars = 1 mm (n=3). (d) Fluorescence microscopy of untreated and treated (SBB and SBB + parylene) pellets using three laser (369 nm, 488 nm and 567 nm). (e) Mean intensity per pixel of untreated and treated pellets exposed with 369 nm laser. (f) Mean intensity per pixel of untreated and treated pellets exposed with 488 nm laser. (g) Mean intensity per pixel of untreated and treated pellets exposed with 567 nm laser. (h) Mean intensity per pixel of untreated and treated pellets exposed with brightfield light. Error bars denote SD. * for $p < 0.05$ and '#' for $p < 0.0001$.

This was confirmed calculating the mean intensity for each condition and illumination (DAPI, GFP, DsRed and brightfield). SBB treatment resulted in an average 79.2 % decrease in autofluorescence across all four channels, with a more drastic reduction in DAPI and GFP channels, with a reduction of 96.3 % and 93.9 %, respectively, enabling better visualisation of fluorescent cells. This showed that a significant decrease occurred when coating components with SBB, whereas Parylene C coating did not significantly alter the SBB effect (**Figure 35.e, f, g and h**). Measurements are recapitulated in **Table 7**. Hence, we incorporated O₂ plasma, followed by SBB treatment, and finally a Parylene C coating in the post-process treatment of 3D-printed components destined to be incorporated in either the microfluidic chip or microfluidic circuit.

Table 7. Mean pixel intensity of untreated and treated 3D-printed components.

Condition	Mean pixel intensity			
	DAPI	GFP	DsRed	Bright field
BMC	10,716.07 ± 705	5,402.45 ± 386	232.09 ± 18	1,184.74 ± 89
BMC + SBB	391.80 ± 172	329.80 ± 75	83.33 ± 2	446.00 ± 89
BMC + SBB + Parylene C	330.16 ± 94	309.38 ± 38	83.30 ± 2	431.58 ± 51

In addition to using 3D printing to manufacture the microfluidic chip's cap, the upper closing plate's material was also changed to BMC. This enabled us to create more complex structures, such as a reinforcement cross to reduce the bending induced by the screwing of the plates (**Figure 36 – component 1**).

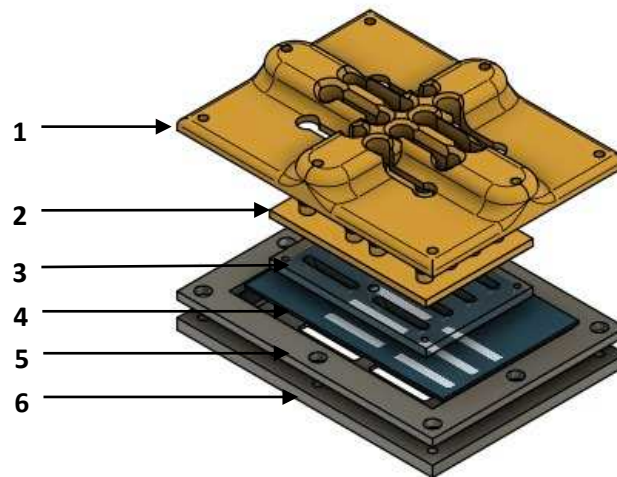


Figure 36. Exploded multi-component parallelized microfluidic chip. The microfluidic chip is composed of six independent components: **1:** 3D printed upper closure plate **2:** 3D printed resin cap **3:** PDMS bottom made from 3D printed mould **4:** glass substrate **5:** aluminium wedge for closure precision **6:** aluminium lower closure plate.

Following the characterisation and validation of the manufacturing protocol for the microfluidic chip, i.e. the manufacture of the PDMS mould in TR300 and the cap in BMC, we worked on building the microfluidic circuit. This enabled us to evaluate the capacity of 3D printing to generate intricate (hollow) geometries to incorporate components within the circuit.

1.3. Parallelised microfluidic device for cell culture

1.3.1. Parallelised microfluidic circuit

Prior to utilising both the microfluidic chip and circuit for the culture of a biological model, we wanted to characterise the flow within the culture chamber. We first measured the flow rate of both peristaltic pumps (Ismatec, 8- and 24-channels) using a flowmeter (Fluigent, Flow sensor M). This consisted in a closed microfluidic circuit connected to the microfluidic chip, both filled with water, with the flowmeter placed after the pump to have the flow pushed through it. We measured the average resulting flow rate for four indicated flow rates (3.74, 10, 25 and 50 $\mu\text{L}\cdot\text{min}^{-1}$) over three individual channels and compared them to the indicated flow rates. We found that on average, the 8-channel pump had a -9.58 % difference in flowrate compared to flow rate indicated by the peristaltic pump, while the 24-channel pump had a -6.71 % difference, indicating that both pumps generated similar flow rates (**Figure 37**). The 8-channels pump had a lower difference for 3.74 and 25 $\mu\text{L}\cdot\text{min}^{-1}$ flow rates (-6 and -7 %, respectively) than for the 10 and 50 $\mu\text{L}\cdot\text{min}^{-1}$ (-11 and -15 %, respectively). On the other hand, the lowest difference for the 24-channels pump was for the highest flow rate (+1 % for the 50 $\mu\text{L}\cdot\text{min}^{-1}$) while the highest difference was for the 10 $\mu\text{L}\cdot\text{min}^{-1}$, with a measured difference of -18 %.

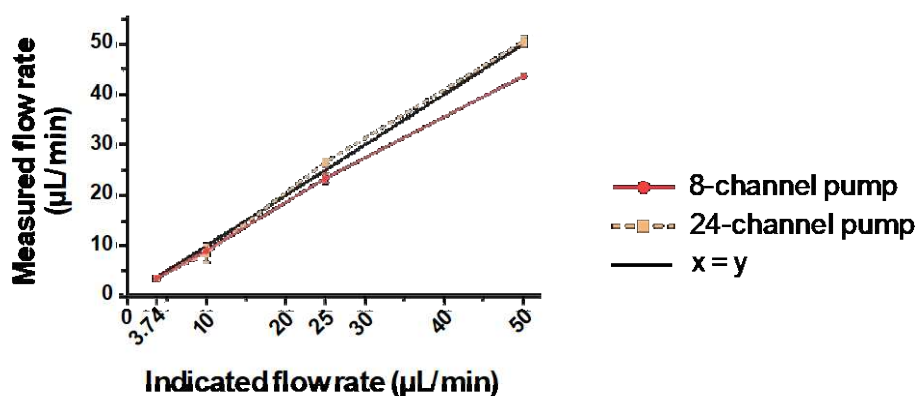


Figure 37. Flow rate characterisation of both 8-channel and 24-channel pump using dedicated flowmeters. (n=3). Error bars denote SD.

We subsequently developed a closed-loop microfluidic circuit to allow for the recirculation of the medium, saving a considerable volume (3.5 mL in a closed loop vs. 37.5 mL for an open circuit with $3.53 \mu\text{L}\cdot\text{min}^{-1}$ flow rate over a seven-day culture) and allowing continuous actuation of cell secretions (metabolites, growth hormones, etc) (**Figure 38**). The circuit is composed of a 16-in-1 microfluidic connector (**Figure 39.a**), tubing connectors and an 8-tank holder that were designed by a Ph.D. student in the team (Thomas Meynard, Ph.D. thesis 2025¹⁹⁶) and subsequently manufactured. This enabled the connection of each individual channel to one single growth medium tank to form the circuit (**Figure 39.b**). The flow is established by the peristaltic pump and the flow rate can be set up according to the specific biological application.

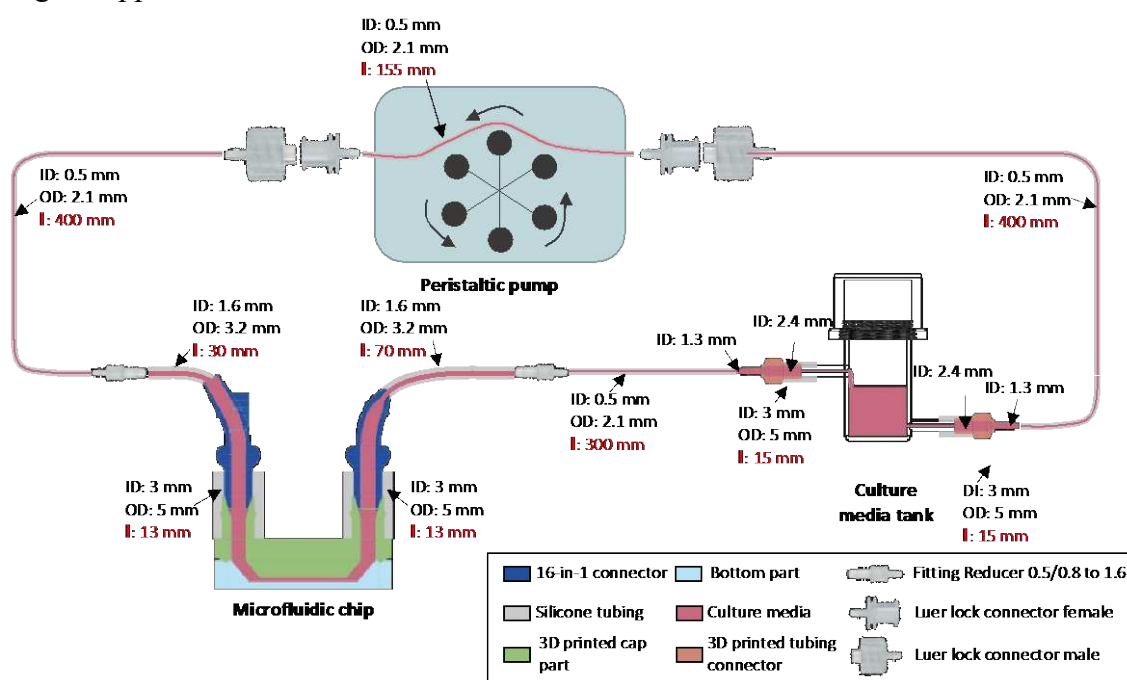


Figure 38. Functional representation of 1 of the 8 independent microfluidic circuit of a culture chamber. The culture chamber, formed by closing the different parts, is surrounded by inlets and outlets. These inlets and outlets are connected to the 16-in-1 connector by silicone tubes. The connector is then connected to the rest of the circuit by 1.6 mm ID tubes and connected to the 0.5 mm ID tubes by fitting reducers. The connection to the culture medium reservoir is made via 3D printed connectors and silicone tubes. The circuit continues with 0.5 mm tubing, connected to male and female luer lock connectors so that the tubing remains in the peristaltic pump. The circuit ends by returning to the connector. Thomas Meynard, Ph.D thesis 2025.¹⁹⁶

We then characterised the flow velocity within the culture chamber. To do so, we injected a 100 μL bolus with a concentration of $4.26 \times 10^{-10} \text{ mol}\cdot\text{m}^{-3}$ fluorescein using an in-line injector (**Figure 39.c**) and established a flow across the chamber with different flow rates imposed by the pump ($3.53 \mu\text{L}\cdot\text{min}^{-1}$, $9.02 \mu\text{L}\cdot\text{min}^{-1}$, $23.39 \mu\text{L}\cdot\text{min}^{-1}$ and $43.64 \mu\text{L}\cdot\text{min}^{-1}$). We then imaged the culture chamber at intervals of 4 s over a 60 min time period (**Figure 39.d**).

We then extracted the average fluorescein fluorescence intensity within two predefined regions of interest separated by a distance d of 4 mm centre to centre. We then filtered out the fluorescence values that were below 500 as well as the values for which the time is greater than the time of maximum fluorescence in zone 1. We were then able to extract the meantime delta between the average intensity curves of both regions of interest and subsequently calculate the average flow velocity within the culture chamber over the experiment duration (**Figure 39.e**).

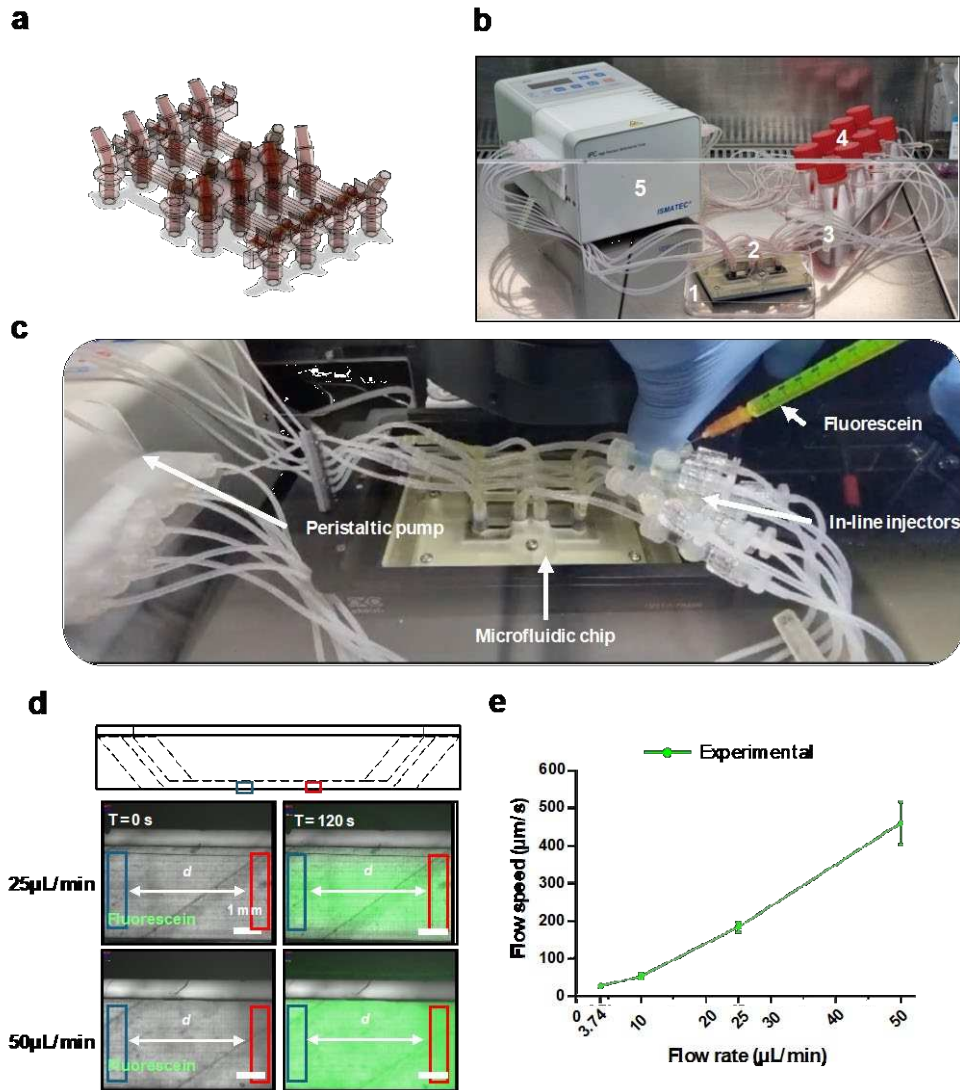


Figure 39. Microfluidic circuit composition and circuit flow analysis. (a) Design of the 16-in-1 connector enabling the connection of the microfluidic chip to the circuit. Thomas Meynard, Ph.D thesis 2025.¹⁹⁶. (b) Assembled microfluidic circuit and its components: 1: closed microfluidic chip 2: 16-in-1 connector that connects the chip to the circuit 3: microfluidic tubing 4: 8 glass tanks in tank holder, one for each culture chamber to have 8 independent parallelised conditions 5: peristaltic pump to generate the flow across the entirety of the circuit. (c) Experimental setup utilised to characterise the flow velocity within the culture chamber. An in-line injector was added to the microfluidic circuit to inject a 100 µL fluorescein bolus. (d) Fluorescein fluorescence imaging inside a culture chamber with two flow rates (25 and 50 µL/min) at two time points (T=0 and T=120 seconds) using two regions separated by a distance d to measure fluorescein intensity (blue and red rectangle). (e) Flow speed calculation using fluorescein measurements (n=3). Scale bars = 1 mm. Error bars denote SD.

To validate those measurements, we implemented two numerical simulations. In order to recreate the experimental measurements in the most accurate way, we included the microfluidic system hydraulic resistance by incorporating the connector, the tubing as well as the in-line injector (**Figure 40.a**). We then defined two measurements volumes and placed them at the same location as the experimental measurement regions. However, rather than measuring the fluorescein intensity, we measured its average concentration over time in both measurement volumes. The injection of the fluorescein solution was represented by a 100 μL bolus with a concentration of $4.26 \times 10^{-10} \text{ mol.m}^{-3}$ that initially starts in the in-line injector and then moves through the system based on the set flow rate (**Figure 40.b**). To incorporate the mass transport of fluorescein and measure its concentration, we implemented a model described in the literature by Baydoun et al.¹⁹⁷

$$0 = -u\nabla c_i + \beta D_i^a \nabla^2 c_i \quad (20)$$

with u the flow velocity, D_i^a the diffusion coefficient of the species i in the liquid phase a , β an empirical factor taking into account the retardation effect observed in the diffusion of molecules (equal to 1 in the culture medium) and c_i the fluorescein concentration of the injected bolus. The parameters values utilised in both simulations are recapitulated in **Table 8**:

Table 8. Parameters used for numerical simulations of fluorescein.

Parameter	Simulation 1	Simulation 2
$c_i \text{ (mol.m}^{-3}\text{)}$	4.26×10^{-10}	4.26×10^{-10}
β	1	1
$D_i^a \text{ (cm}^2\text{.s}^{-1}\text{)}$	0	4.25×10^{-6}

The first simulation only took the advection of fluorescein into account. Therefore, we set the variable D_i^a to 0. However, fluorescein has a diffusion coefficient of $4.25 \times 10^{-6} \text{ cm}^2\text{.s}^{-1}$ that needs to be taken into account to model its profile.¹⁹⁸ Therefore, the second numerical simulation, developed by an intern, implemented the convection (advection and diffusion) of the fluorescein by setting D_i^a equal to the fluorescein diffusion coefficient. We then extracted and plotted the concentration of both regions over time (**Figure 40.c**). As expected, we observed that a slower flow rate resulted in a longer time for both measurement volumes to reach the maximum concentration value. Indeed, the maximum concentration when using a flow rate of $3.53 \mu\text{L.min}^{-1}$ was reached after around 6,000 s, while it only took 450 s to reach it with a flow rate of $43.64 \mu\text{L.min}^{-1}$. We then filtered out the concentration values that were below

$5 \times 10^{-3} \text{ mol.m}^{-3}$ as well as the values for which the time is greater than the time of maximum concentration in zone 1. To calculate the subsequent flow velocity across the culture chamber, we then measured the time Δt between both measurement regions to reach the same concentration (**Figure 40.c**). We then divided the distance d with this average Δt and averaged out all values to obtain the average flow velocity across the culture chamber and subsequently plotted the calculated flow velocity and compared it to the experimental values (**Figure 40.d**). All values are recapitulated in **Table 9**. We found little to no difference between both numerical models when calculating the flow velocity. This suggests that diffusion has little to no influence on the fluorescein concentration profile. However, we found a 25 % difference in the flow velocities calculated by both numerical models and experimental measurements.

Additionally, we analytically calculated the flow velocity. Indeed, if we assume the velocity v ($\mu\text{m.s}^{-1}$) to be uniform on the channel's cross-section, we can calculate it with:

$$v = Q \times S \quad (21)$$

with Q the flow rate ($\mu\text{L.min}^{-1}$) and S the channel cross section area (mm^2). All results are shown in **Table 9**. The sequence of COMSOL commands for this modelling is given as an ‘.mph’ file in **Appendix 3**.

Table 9. Culture chamber flow velocity according to calculation method.

Flow rate ($\mu\text{L.min}^{-1}$)	Cross section area (mm^2)	Flow Velocity ($\mu\text{m.s}^{-1}$)			
		Analytical	Advection	Advection + Diffusion	Experimental
3.53	1.80	34.63	34.42	34.43	25.22 ± 1.32
9.02		92.59	92.59	94.64	36.27 ± 15.67
23.39		231.48	231.47	240.67	178.72 ± 39.61
43.63		462.96	462.93	463.44	431.32 ± 22.04

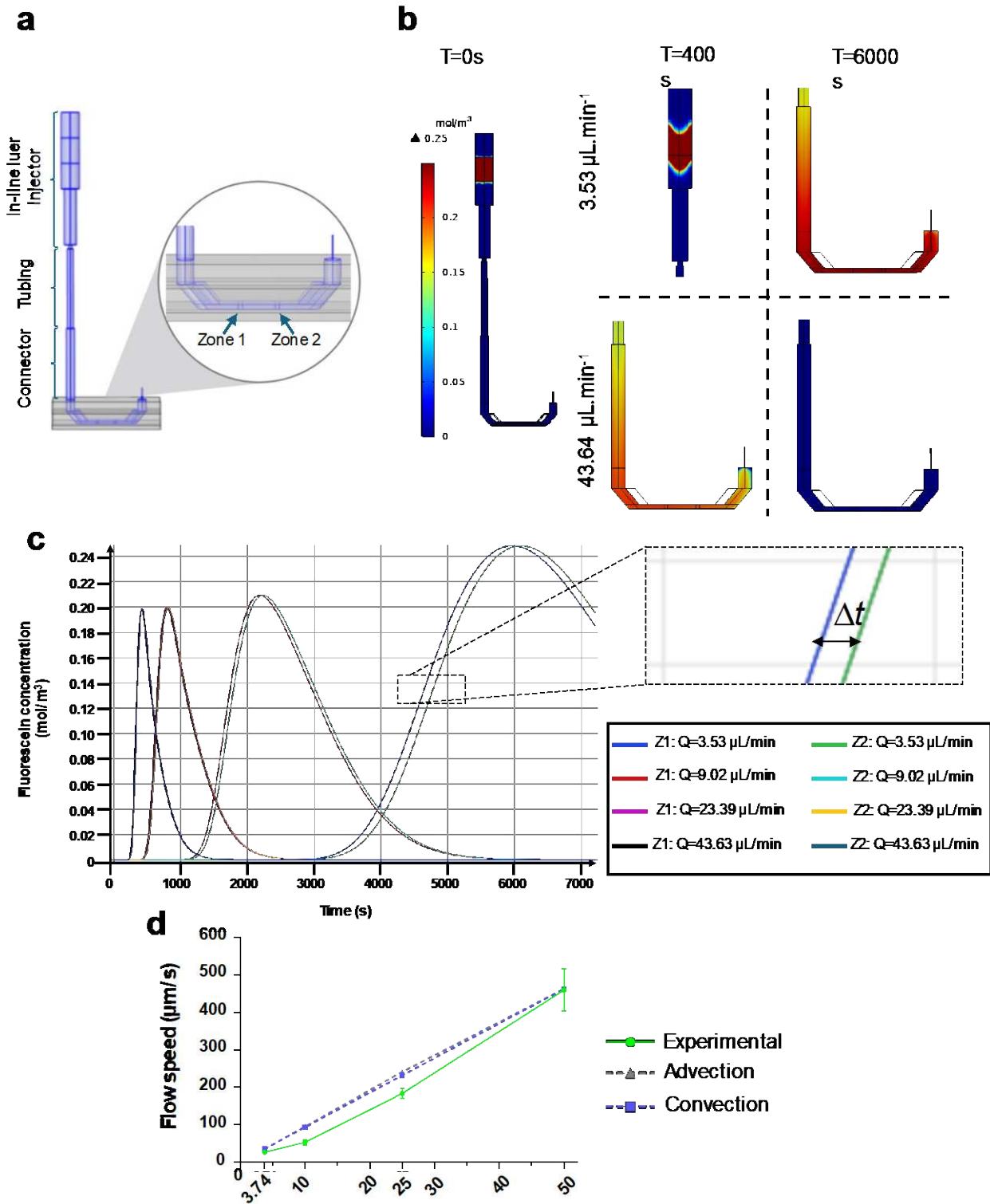


Figure 40. Flow velocity profile calculation in the culture chamber based on the fluorescein concentration. (a) Incorporation of the 16-in-1 connector, tubing and in-line injector to represent the circuit hydraulic resistance. (b) Simulation of the mass transport of fluorescein in the culture chamber for different flow rates over time. (c) Plotting of the fluorescein concentration in both zone 1 and 2 in the culture chamber for different flow rates (3.53, 9.02, 23.39 and 43.63 $\mu L/min$). We extracted the Δt between two curves to calculate the flow velocity for each flow rate. (d) Comparison of the calculated flow velocities between both numerical models and experimental measurements. Error bars denote SD. Anthony Leroy, M1 internship 2025.

After connecting the microfluidic chip to the microfluidic circuit and studying the flow velocities in the culture, the subsequent step was to validate the use of the microfluidic device and circuit for cell culture.

1.3.2. 2D cell culture within the microfluidic system

To this effect, we developed a cap to create two chamber heights on the same microfluidic chip (600 μm and 2400 μm) to generate two different flow velocities to study the effect of flow velocity on the adhesion and proliferation of PDAC cells in a 2D monolayer. We first numerically simulated both geometries with a $3.53 \mu\text{L}.\text{min}^{-1}$, and found that the 2400 μm geometry resulted in a lower average flow velocity than the 600 μm , with $9.86 \mu\text{m}.\text{s}^{-1}$ and $34.42 \mu\text{m}.\text{s}^{-1}$, respectively (**Figure 41.a** and **b**). Additionally, this increase in channel height also resulted in a 92.5 % decrease in average shear stress on the glass substrate surface, with shear stresses of $2.70 \times 10^{-5} \text{ Pa}$ and $3.60 \times 10^{-4} \text{ Pa}$, respectively. Furthermore, we calculated the average flow velocity along the culture chamber's length (see 1D line section in **Figure 41.d**). For both geometries, we observed an increase in flow velocity as the flow reaches the cap's protrusion compared to the flow velocity near the culture chamber's inlet and outlet (**Figure 41.c**). This occurs because of the reduction of the cross-section area ((21)). The sequence of COMSOL commands for this modelling is given as an '.mph' file in **Appendix 3**.

After numerically evaluating the flow velocity profile for both culture chamber heights, we cultivated HPAC-eGFP cells in a 2D monolayer in both geometries. 100 μL of culture medium with a concentration of HPAC-eGFP cells of $5 \times 10^5 \text{ cells.mL}^{-1}$ were seeded in the culture chambers. We let them adhere to the glass substrate for 24 h before activating the dynamic perfusion ($3.53 \mu\text{L}.\text{min}^{-1}$). After 7 days of culture, we observed that HPAC-eGFP cells cultured in the 2400 μm high culture chamber were more confluent than in the 600 μm high chamber (**Figure 41.d** and **e**). Fluorescence microscopy revealed that in the 600 μm chamber, HPAC-eGFP cells proliferated more near the inlets and outlets of the chamber for the whole experiment duration (**Figure 42**). This correlates with the chamber's regions with the slower flow velocity, further indicating that a high flow velocity has a detrimental effect on HPAC-eGFP cells. This difference in cell proliferation between both conditions was confirmed by measuring both the surface area (% of the channel) covered by cells and nuclei counting (**Figure 41.f** and **g**). After 24 h of dynamic culture, we performed nuclei staining and subsequently imaged and counted the nuclei number in both conditions. We observed that the

number of nuclei decreased in the 600 μm culture while it increased in the 2400 μm , with 24,932 and 83,788 cells, respectively, compared to the initially 50,000 seeded cells.

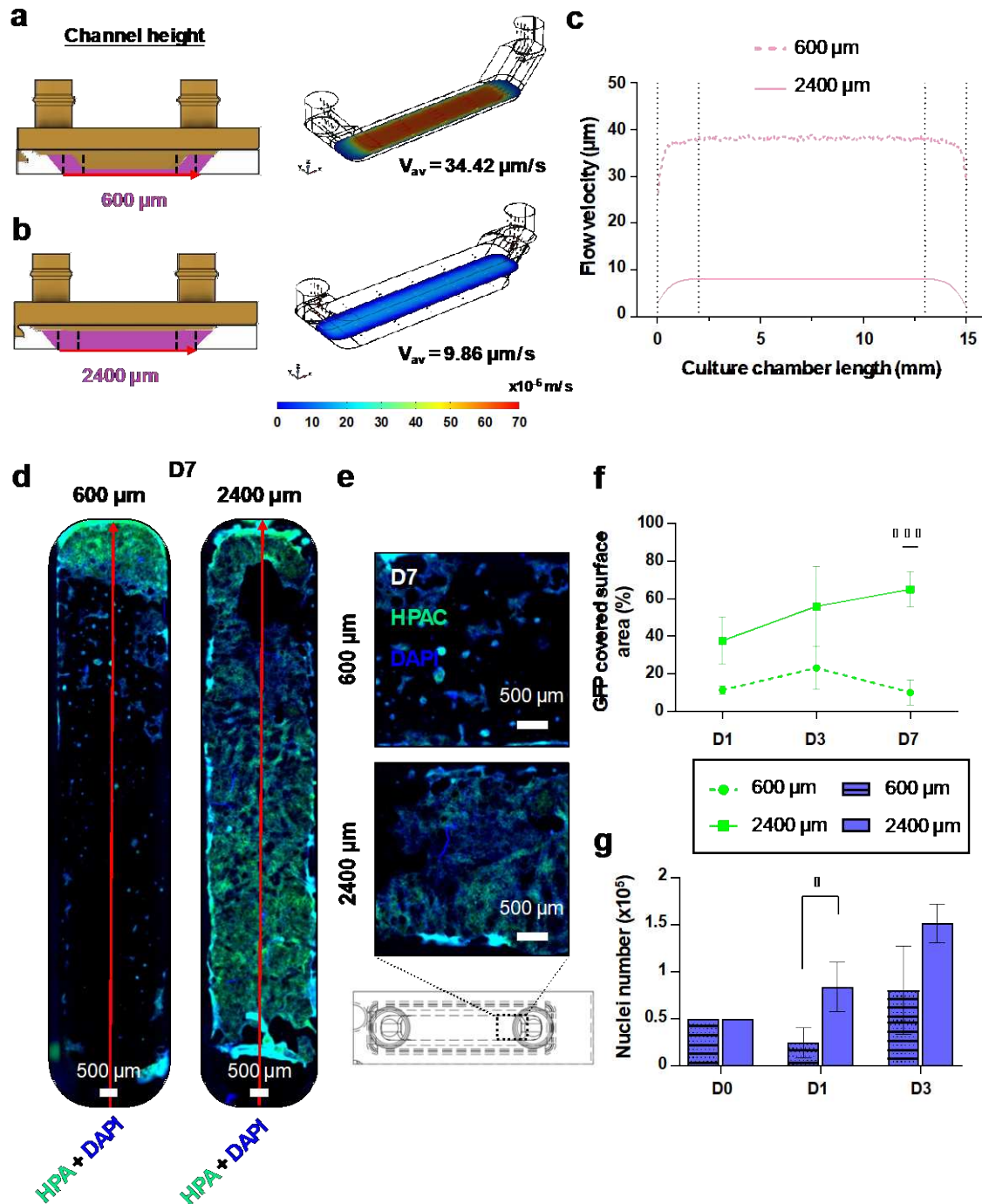


Figure 41. 2D HPAC-eGFP cell culture in the microfluidic device using two channel heights. The first configuration is the geometry with a chamber height of 600 μm (a) resulting in an average flow velocity of $34.42 \mu\text{m.s}^{-1}$ across the chamber volume. To reduce the flow velocity, the chamber height was increased to 2400 μm (b), resulting in an average flow velocity of $9.86 \mu\text{m.s}^{-1}$. (c) The flow velocity was calculated along the culture chamber length (red arrow in (d)) placed 0.1 mm above the glass substrate surface. We observed two distinct regions: one at the culture chamber inlet and outlet (dotted lines) and the other underneath the cap protrusion. The flow is considerably faster as the flow reaches the cap due to a decrease in the cross-section area. HPAC-GFP cells were subsequently grown in a 2D monoculture for 7 days. (d) full imaging and (e) channel zoom. (f) The channel surface area % covered by the cells was calculated using eGFP fluorescence and nuclei counting (g) was performed using DAPI staining (n=3). Error bars denote SD. * for $p < 0.05$ and *** for $p < 0.001$.

This was further confirmed by the difference in surface area covered by HPAC-eGFP cells, with 37.6 % of the 2400 μm covered and only 11.3 % of the 600 μm covered. This indicated that the flow velocity profile in the 2400 μm high culture chamber was better suited to HPAC-eGFP cells than the 600 μm high culture chamber. After three days, an increase was observed in both covered surface area, 55.9 % and 23.1 %, respectively, and nuclei staining with 151,510 and 80,347, respectively. This indicated that cells were viable and proliferative for both conditions. However, after seven days of culture, we observed a decrease in surface area covered in the 600 μm culture chamber. On the other hand, in the 2400 μm culture chamber, HPAC-eGFP cells coverage increased to 65 %.

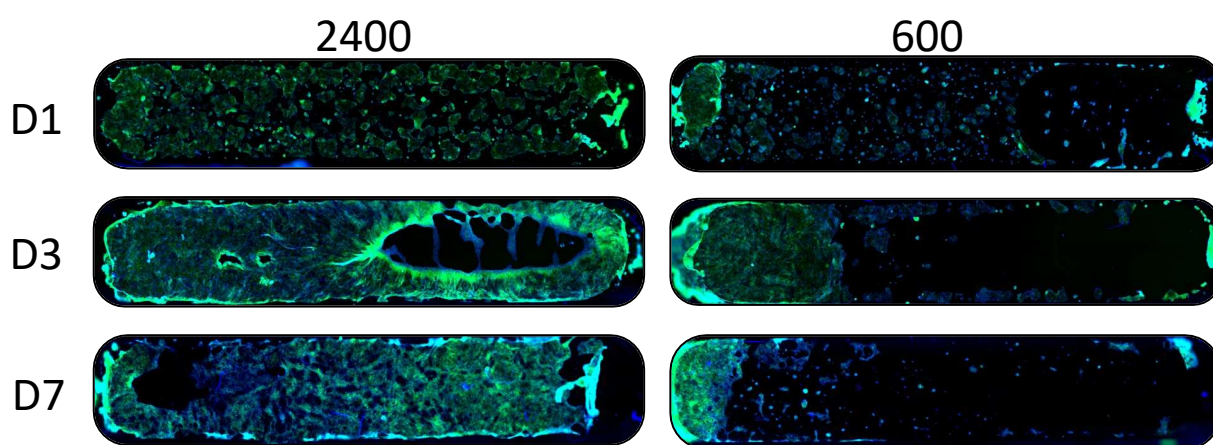


Figure 42. 2D monoculture of human HPAC-eGFP (green) and nuclei DAPI staining (blue) in the microfluidic device with two channel heights (600 μm and 2400 μm).

1.3.3. 3D cell culture

After validating the microfluidic system for 2D cell culture, we wanted to validate for 3D cell culture. We initially wanted to use a porous hydroscaffold (BIOMIMESYS Oncology), that can withstand direct perfusion with no degradation, hence the simple rectangular shape of the culture chamber. However, the manufacturing company, *HCS Pharma*, went bankrupt. We therefore had to switch from their hydroscaffold, to a 50:50 (v/v) mixture of HPAC-eGFP cell culture medium (1X-DMEM) with rat tail collagen 1, resulting in a final collagen 1 concentration of 1.86 mg.mL^{-1} .

Since collagen 1 has a low permeability ($6.10 \times 10^{-11} \text{ m}^2$),¹⁹⁹ we cannot perfuse it directly without rapid mechanical degradation under flow conditions, making it unusable for our experiments. To overcome this rapid degradation, we utilised a technique developed within the team based on the use of a cell strainer (pores of $70 \text{ }\mu\text{m}$ diameter) as a protective barrier. More specifically, we modified the cap, and particularly the protrusion that is inserted into the lower part of the PDMS. We hollowed out this protrusion along its length (**Figure 43.a**). By gluing a cell strainer onto the remaining platforms using BMC resin (**Figure 43.b**), we created a protective barrier for the hydrogel and reduced its degradation over time, while still allowing molecules such as O_2 and glucose to diffuse into the hydrogel. To validate this culture method, $50 \text{ }\mu\text{L}$ of 50 % (v/v) collagen 1/culture medium solution with a cell concentration of 1×10^6 HPAC-eGFP cells were seeded in the culture chamber and cultured for 7 days (**Figure 43.c**). We performed fluorescence microscopy and observed that cells were uniformly seeded within the culture chamber. Furthermore, by zooming on the cell culture, we could see that cell aggregates that were initially clear became blurry (**Figure 43.d**). This indicated that they were no longer on the same focal plane, thus validating the 3D nature of the culture. Blue spots in the pictures correspond to added 3D printing resin to glue the cell strainer to the cap.

After three days of culture, we observed both a stronger GFP and DAPI fluorescence compared to day 0, indicating that cells were viable. We also observed that cells started forming larger aggregates, mostly at the culture chamber inlet and outlet. Similar to day 0, we could observe cells on different focal planes on the same image, indicating that the cells are still in 3D. The aggregation of cells continued up to seven days of culture, with even larger aggregates being imaged. Furthermore, the localisation of the aggregates towards the inlet and outlet could suggest a migration of the cells. As for day 0 and 3, we could observe the 3D nature of the

culture by zooming in after seven days of culture. Overall, our approach using a modified cell strainer effectively protected the collagen hydrogel against degradation from dynamic perfusion, successfully validating our microfluidic device for 3D culture of HPAC-eGFP cells.

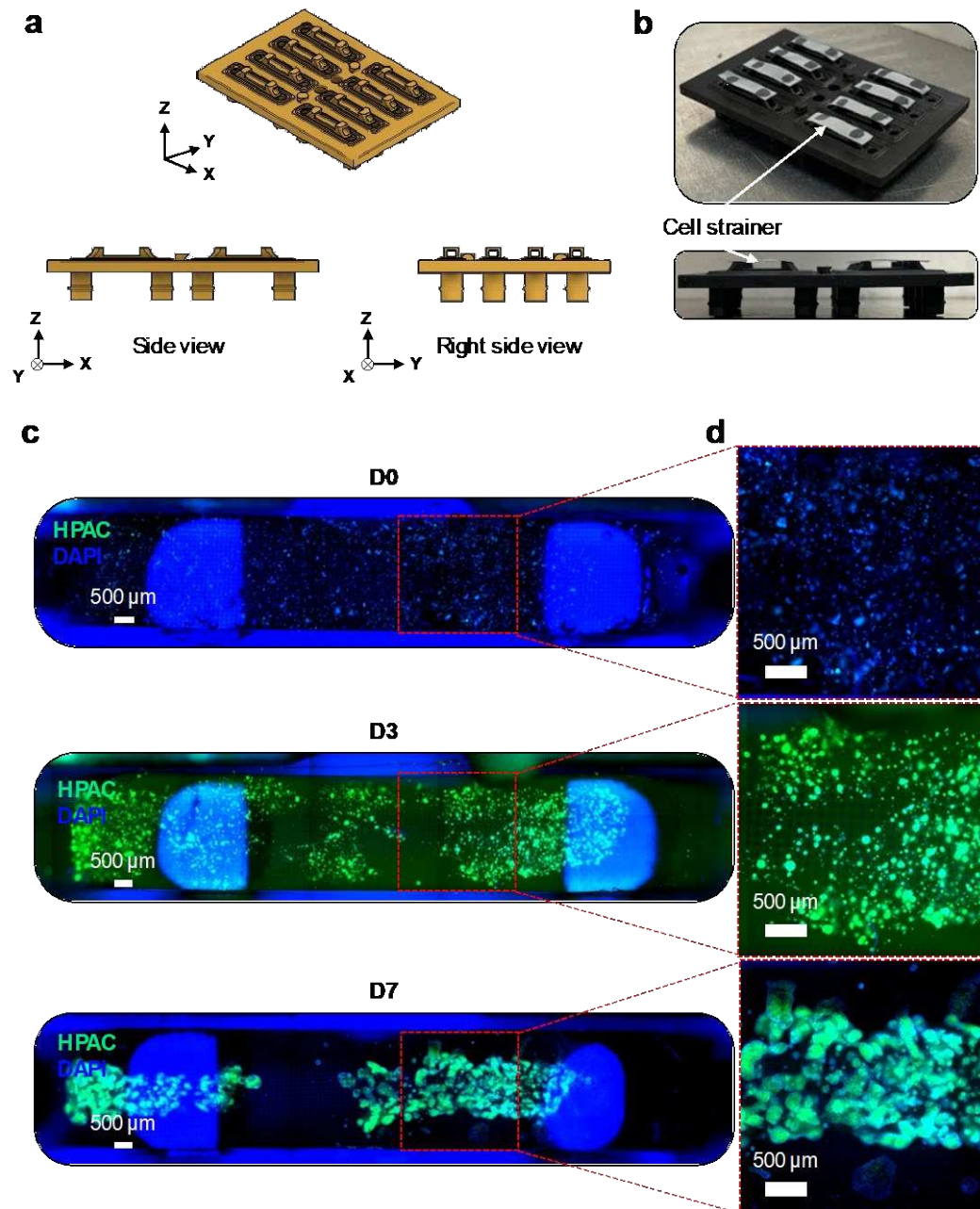


Figure 43. 3D HPAC-eGFP cell culture in the microfluidic system using collagen 1 as an extracellular matrix. (a) Modifications made to the cap to reduce the mechanical degradation of collagen 1 hydrogel under flow conditions. The cap's protrusion is hollowed out in along the x-direction to create a pathway for the cell culture medium. (b) To reduce the degradation of collagen 1, a cell strainer (70 μm pore diameter) is glued using 3D printing resin and creates a protective barrier at the culture medium – collagen 1 interface. HPAC-GFP cells were subsequently grown in a 3D monoculture for 7 days. (c) full imaging and (d) channel zoom revealed a growth in HPAC-eGFP spheroids as well as the 3D nature of the culture for the whole duration of the culture ($n=2$).

1.4. Discussion

In this first chapter of the results, we utilised 3D printing for PDMS mould fabrication in TR300 resin, but also for the fabrication of constitutive components of the microfluidic system and circuit with BMC resin. We subsequently treated BMC parts to decrease its cytotoxicity and autofluorescence to incorporate 3D printed components within the device. Furthermore, we designed the device such that it is also adapted to modern robotics and microscopes, with the culture chambers being aligned with the 96-well plate SBS format in order to facilitate its use. We then validated the use of the microfluidic device for cell culture by cultivating cells under two flow velocities for seven days. Finally, we adapted the microfluidic system to reduce mechanical degradation of a collagen 1 based ECM to dynamically culture an *in vitro* solid tumour model.

The LCD-based printer has proved easier to use thanks to the overall control over printing parameters provided by the software, contrary to the SLA printer. We successfully adapted the Precisa 780 resin, originally intended for the SLA printer, and used it in the LCD printer to subsequently characterise the printing errors of both printers. However, as the resin is manufactured for a printer with a much more powerful laser than the LCD's LEDs, printing times have increased drastically, from 7 h on SLA to 42 h on LCD, making the use of this resin too restrictive. Our characterisation demonstrated that the cheaper LCD-based printer was more accurate in regard with pattern dimension error regardless of printing orientation, which is in agreement with other studies.²⁰⁰ In addition, we evaluated the impact of part orientation during printing. Vertical printing generally resulted in more accurate patterns, which again is in accordance with the literature,²⁰⁰ while horizontal printing allowed to minimise the surface roughness. This enabled us to choose the printing orientation depending on the targeted application as well as choosing TR300 as a resin for PDMS mould fabrication. Nonetheless, we did not investigate all commercially available 3D printers. A combined investigation of both 3D printer and 3D printing resins could highlight a better suited combination for ToCs fabrication.

As leaks were a recurring limitation due to the impossibility of sealing two PDMS parts together in a leak-proof manner, we investigated using 3D printed components as a cap and consequently measured cell viability when in contact with BMC resin. Although the threshold for cytocompatibility is usually set at 80 % for biomedical applications,¹⁴⁷ we considered this

viability still insufficient for long-term incorporation into the chip and its circuit for culture purposes. We therefore investigated modifying the BMC resin post 3D printing to increase cell viability when in contact with BMC components. Several options had been explored by other research teams to increase the resin's cytocompatibility such as modifying the post-printing washing and curing.²⁰¹ Rather than drifting from the manufacturer's recommendations, which could result in unwanted fabrication errors, we coated our BMC components with Parylene C. Indeed, Parylene C has previously been shown to improve viability in both short and long-term cell cultures by forming an insulating protective polymer layer that prevents toxicity phenomena induced by the material.²⁰² This enabled us to enhance the viability of BMC components to over 90 % for cell culture, approaching that of traditional 2D *in vitro* culture systems and thus validating our protocol.¹⁹⁵

We also had to improve the quality of fluorescent imaging as we noticed strong autofluorescence when performing fluorescence-based microscopy with BMC components, questioning its use in the microfluidic chip. We therefore decided to treat the resin with SBB which had been described to reduce the autofluorescence of polymers either by absorbing emitted photons, or by changing its refractive index.²⁰³ This allowed us to strongly reduce the emission intensity at the wavelengths of interest for cell culture applications, which is consistent with a previously established SBB coating protocol.²⁰⁴ The images obtained after SBB immersion were more contrasted and allowed precise nuclei counting.

Our SBB and Parylene C protocol enabled us to functionalise the BMC for incorporation in the microfluidic system. Additionally, this protocol could easily be adapted to other transparent 3D printing resins that could result in a more precise fabrication. Indeed, most 3D printing vendors offer transparent resins (Phrozen – Aqua Clear plus, Formlabs – Clear Resin V5), with some such as the BioMed Clear Resin by Formlabs being labelled biocompatible by the USP Class VI certification.¹³⁹ Another improvement could be to manufacture our home-made 3D printing resin. This would enable us to modulate the resin's composition in order to control both its cytotoxicity and autofluorescence. Indeed, 3D printing resins consists in a mixture of monomers and oligomers, which crosslink using radicals provided by a photoinitiator when exposed to a UV light.²⁰⁵ However, these photoinitiators are often auto fluorescent after the crosslinking, which is detrimental for microscopy-based analysis of cell cultures.²⁰⁶ Solutions to suppress this autofluorescence can be categorised in two groups : pre-processing and post-processing ones. We decided to investigate the latter by

applying an SBB coating to our components, but other teams chose to explore pre-processing solutions. Flamourakis et al. replaced the photoinitiator of the SZ2080 resin with SBB.²⁰⁷ This enabled them to obtain structures exhibiting much lower fluorescence than with the standard resin composition. What is even more interesting is the optical transmittance of these structures. Indeed, as we have shown in **Figure 35**, a side effect of SBB coating is a reduction in brightfield mean pixel intensity. This indicates that less light is transmitted through our SBB coated components, which is an unwanted side effect of reducing the autofluorescence. Meanwhile, components fabricated using SBB as a photoinitiator by Flamourakis et al. were found to be almost completely transparent, with a transmittance of almost 90 % in the visible spectrum, showcasing the potential of complex custom-made 3D printing resin. Another study performed by Warr et al. this time investigated the potential of a custom-made 3D printing resin as a noncytotoxic material.²⁰⁸ They observed an excellent cytocompatibility of the resin right after printing, with no further processing post fabrication. Although they did not specifically investigate autofluorescence, we can see from some of their images that there is a background fluorescence when imaging 3D printed components. We could therefore imagine a combination of both these studies to eliminate both autofluorescence as well as resin cytotoxicity without needing for post printing processes involving specific machinery such as Parylene C coating.

One of our requirements for the design of the microfluidic system was that it needed to be parallelised. Indeed, parallelised microfluidic systems on varying scales (channel number) enable a larger number of experiments or conditions to be carried out simultaneously on more complex models, limiting bias of sequential experiments.²⁰⁹ Although the culture chamber geometry is of a simple rectangular shape, we chose its dimensions in order to ease the parallelisation of the system and to fit with the 96 well plate SBS format. Furthermore, contrarily to commercially available parallelised microfluidic systems, we have shown that we could easily adapt the connection between the circuit and our device by developing dedicated tubing connectors such as the 16-in-1 connector. This also served to demonstrate the capability of 3D printing to control all aspects of a microfluidic platform compared to other fabrication methods (SU-8 lithography, aluminium milling). As for the microfluidic circuit, we opted for a closed loop, allowing continuous actuation of metabolites and cell secretions. To provide a unidirectional flow to the circuit, we opted to utilise a peristaltic pump, which is a common flow actuator used in recirculation circuits.²¹⁰ This allowed for the recirculation of the medium,

saving a considerable volume over the experiment duration (3.5 mL in a closed loop vs. 37.5 mL for an open circuit with $3.74 \mu\text{L}\cdot\text{min}^{-1}$ flow rate over a 7-day experiment).

We subsequently characterised the flow velocity profile within the culture chamber and compared the experimental measurements to two numerical simulations. The values calculated in the culture chamber using both the numerical models were consistent, with relatively small differences between the two numerical methods. However, we found the simulated values were on average 25 % higher than the experimental values for each flow rate, apart from the $9.02 \text{ mL}\cdot\text{min}^{-1}$ flow rate. This can be explained by simplifications in the modelling or the omission of certain phenomena such as wall roughness or fluctuations due to the pumping system. Indeed, as we implemented a uniform flow rate in both the numerical models, we did not represent the pulsatile behaviour of the peristaltic pump. We therefore did not represent the experimental sinusoidal shape of the flow rate, but rather a linear and continuous perfusion. Furthermore, we determined the flow velocity in the channel using fluorescence time-lapse microscopy, which was limited by the minimum interval between captured images (4 s). Given the pulsatile nature of the flow, this interval introduced potential measurement inaccuracies, as imaging may not have been synchronised with the periodic peaks and valleys of the flow rate. For instance, if imaging consistently coincided with flow minima, the resulting velocity calculations would underestimate the true average velocity, while imaging during maxima would yield an overestimation. This lack of synchronisation could explain why velocities obtained from our numerical model, which does not account for pulsatile flow, were on average 25 % higher than experimental measurements for the four flow rates we investigated. It would therefore be interesting to perform this study but calculate the flow velocity by recording a video rather than taking images. Finally, although beneficial for our application, the measured values were still higher than *in vivo* values, which is why we performed our study on the effects of two flow velocities.²⁵

Additionally, we designed the microsystem to be made up of two separable parts. This concept is in line with recent microfluidic systems that have investigated different clamping strategies to avoid irreversible sealing of the system.²¹¹ This is particularly beneficial when aiming to perform specific biological analyses other than microscopy. Although we did not perform such analyses, we intend in future works to open it for biological analyses and perform protein or RNA extraction, which are difficult to do when the system is closed. For example, it has been shown that atomic force microscopy analyses can be carried out using an openable

device, which is impossible if the system is irreversibly closed.^{212,213} However, this compression technique is utilised for end point analysis of the culture and not to recreate the characteristics of solid tumours. In our system, having two separable components enables us to easily apply a compressive stress to the entirety of the tissue, simply by changing the cap's protrusion height. This could be leveraged to study the combined effects of shear stress and compression on tumour development and drug response (Thesis project of Orégane Bajoux).

This reversible sealing of our system was rendered possible by the incorporation of a dedicated closing system composed of one aluminium plate and one 3D printed plate separated by a wedge and screwed together. This is a strategy that is known for its high-pressure capacity and robust leak-tightness (**Figure 44.a**).^{214,215} The combination of the dedicated closing system with the rigid 3D-printed cap and leak-proof cordons greatly reduced the number of leaks occurring during cell culture under flow conditions. Although we did not quantify the number of leaks, we noticed a drastic reduction in their occurrence. For example, and for information purposes only, before the combination described above was put in place, leaks occurred on the majority of the culture chambers (over 80 %). However, in the last two dynamic cell cultures (eight microfluidic chips – 64 individual chambers), leaks only occurred on 5 individual channels, which represents 7.8 % of chambers with leaks. Although this remains higher than what we would ideally want, this reduction in leak occurrence validates the closing method we have developed using screws.

Nonetheless, the need for multiple fasteners and careful torque control can become labour-intensive in parallel experiments. In comparison, vacuum clamping works on the principle of utilising a continuous negative pressure and pliable elastomeric parts (**Figure 44.b**).²¹⁶ However, vacuum clamping often requires the incorporation of complex channel networks as well as the use of a dedicated vacuum device, hindering the use for long term cell culture. By employing permanent magnets or magnetised elastomers, magnetic clamping simplifies closure but can also result in an uneven load distribution (**Figure 44.c**).²¹⁷ Meanwhile, Lock-and-Play devices integrate latches or hinges within the housing for single-step assembly (**Figure 44.d**). Although they excel in ease of use and reduce setup time, their maximum sealing strength can be lower than screw-based clamping. Although they demand high-precision fabrication,²¹⁸ Lock-and-Play mechanisms, especially when combined with injection-moulded thermoplastics and standardized gaskets, are typically the most

straightforward to scale and automate, providing a clear path to high-throughput ToCs manufacturing.

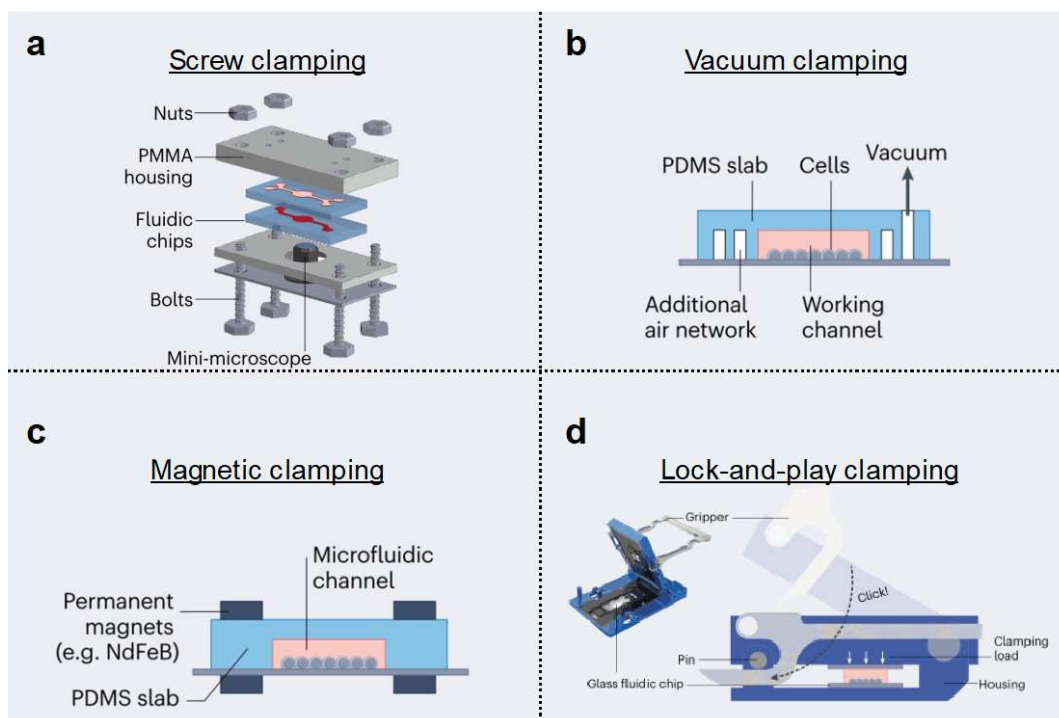


Figure 44. Clamping strategies to create reversibly sealed microfluidic systems. (a) Screw based clamping. The microfluidic chip is sandwiched between two PMMA plates that are screwed together. (b) Vacuum clamping of a microfluidic chip. Air channels on each side of the working culture channel are exposed to a vacuum to create an adhesive surface. (c) Magnetic clamping. Magnets are placed at the top and bottom of the chip to close the microfluidic chip. (d) Lock-in-play device currently commercialised by Micronit (Enschede, Netherlands). From [211].

To validate the use of our microfluidic system for 2D cell culture, we incorporated a 2D PDAC cell culture. As we noticed that the minimum flow velocity obtained in the $600\ \mu\text{m}$ was higher than the *in vivo* values of IF found in the literature ($34.63\ \mu\text{m}\cdot\text{s}^{-1}$ vs 1 to $5\ \mu\text{m}\cdot\text{s}^{-1}$)²⁵, several options were investigated to reduce it. We firstly investigated using dedicated tubing to reduce the flow rate, but quickly abandoned this solution. Indeed, as the tubing's inner diameter were too narrow, the resulting hydraulic resistance of the microfluidic circuit as a whole was too high, resulting in a pressure too high to fill it with culture medium. We could have reduced the pressure difference between the channel's inlet and outlet by incorporating an additional hydraulic resistance after it, but that would have implicated modifying the microfluidic circuit. We therefore decided to increase the channel's cross section area to reduce the flow velocity all the while maintaining the minimum flow rate allowed by the peristaltic pump ($3.53\ \mu\text{L}\cdot\text{min}^{-1}$). Rather than increasing the chamber's width, which could become an issue with the closing plates' openings for microscopy, we increased the culture chamber height

to 2400 μm . Indeed, increasing the width of the culture chambers would have meant enlarging the openings for microscopy in both closing plates. However, this would have reduced the distance between these openings, which could have reduced the structural integrity of the lower closure plate. This is why we decided to modify the height of the culture chamber rather than its width. We then produced a cap geometry capable of generating both chamber heights and investigated the impact of the resulting flow velocities on the culture of PDAC cells. By generating two different flow velocities, we observed a difference in both proliferative and adhesive properties of the cells. More specifically, they were enhanced when the flow velocity decreased. Nonetheless, we performed this study on only one cell type (HPAC-eGFP cells), which is not representative of a solid tumour. Indeed, it would be interesting to perform the same study on various PDAC cell lines, such as Panc-1 and MIA Paca-2, as they represent different stages of tumour development; and study their response to dynamical flow. Indeed, the effects of IF has previously been investigated on pre- and post-EMT breast cancer cells by Tchafa et al.²¹⁹ They subjected both cell types to IF and subsequently performed an invasion assay for both. Although they found that subjecting both cell phenotypes to IF increased their invasive properties, they observed that this increase was obtained through different machineries. Indeed, post-EMT cells were found to take advantage of both PI3K activation and chemokine gradients while pre-EMT cell only relied on PI3K activation. This suggests that to block flow-induced invasion in a tumour with more post-EMT cells, it would be necessary to inhibit both PI3K and chemokine gradients, highlighting the necessity to study different cell stages to elucidate the effects of IF on solid tumours.

To make our experiment more complex and to enable a more relevant reproduction of the solid tumour tissue, we cultivated HPAC-eGFP cells in a collagen 1 ECM. To reduce the mechanical degradation of the gel under flow conditions, we incorporated a cell strain that acts as a protective barrier for the hydrogel while still allowing diffusion of the necessary nutrients for cell proliferation and viability. Similar works utilising porous membranes to separate the hydrogel for the aqueous phase have been previously done. For example, Meyer et al. utilised a cell strainer with 3 μm pores, and subsequently cultivated fibroblasts in an alginate-based hydrogel.²²⁰ They only cultivated the cells in their microfluidic system for three days when we were able to cultivate for at least seven days. However, they performed a Live/Dead staining and observed no differences in viability between cells in the chip and cells cultivated in a 96 well plate as a control. Although we did not quantify the cell viability in our device, we

observed larger spheroids after seven days of culture compared to day 0 and 3, thus validating our methodology for cell culture. It will therefore be of interest to perform a viability assay to quantify the cell viability at different stages of the dynamic culture. Furthermore, ECM has previously been shown to play a crucial role in drug response, with studies observing a higher drug resistance in 3D cell culture compared to 2D culture.^{47,221} It would therefore be interesting to expose our cell culture to a chemotherapy, and to compare the response in the 2D cell monocultures with the 3D cell culture.

Chapter 2: Development of a bioimpedance measurement system within the microfluidic chip

In view of the need to develop new viability assays for measuring the drug response of 3D solid tumour models in microfluidic systems, we have previously seen that bioimpedance poses as a serious candidate in this respect. Indeed, as we have discussed, bioimpedance can provide quantitative information on the viability of cancer cells, whether it is a 2D or 3D model. It was in this context that I underwent a one-year mobility in the *Oslo Bioimpedance and Medical Technology Group* team directed by the Pr. Ørjan Grøttem Martinsen at the University of Oslo. Their research focuses on measuring the passive electrical properties of biological materials. Their expertise lies in impedance theory, impedance measurement instrumentation and electrode systems and modelling.

We have previously seen that an impedancemeter is compulsory to bioimpedance measurements. To develop a suitable impedancemeter, we collaborated with the company *Sciospec Scientific Instruments GmbH*. It is a company specialising in solutions for electrical impedance spectroscopy. Their products are mainly used for bioanalysis, medical research and component testing. They also offer solutions for integrating impedance measurement tools compatible with 96-well SBS plates and OoCs. We therefore approached them to develop and manufacture a measuring system that would enable us to carry out bioimpedance measurements in our microfluidic chip.

In this chapter, we have designed bioimpedance biosensor using the 4-point electrode configuration. We determined its geometry by calculating its sensitivity S using a numerical model, resulting in the incorporation of 8 electrodes in each culture chamber. We then fabricated the biosensor with titanium/platinum (Ti/Pt) using the bilayer liftoff lithography technique and connected it to the impedancemeter with a printed circuit board. We then characterised it to quantify the impact of the system's geometry and change in temperature on the electrical measurement. Finally, initial preliminary results validated that the instrumented microsystem could be used to monitor changes in cell density in dynamic 2D and 3D cultures of PDAC cells.

1.1. Integration and fabrication of the microelectrode system

1.1.1. Choice of the electrode geometry

Similar to the microfluidic chip, the first step of a microelectrode system fabrication is to define the geometry and placement of the electrodes within the culture chamber. As discussed in Chapter 3, we decided to utilise the 4-point electrode configuration to circumvent the electrode polarisation impedance. Hence, we utilised at least four electrodes per culture chamber. However, as the impedancemeter enabled the use of 64 electrodes and that the microfluidic chip has eight individual channels, we had the possibility to increase the number of electrodes per channel to eight.

To that aim, we considered three electrode configurations. All three patterns were designed to have the electrical measurement carried out underneath the cap's protrusion to ensure that it would not be affected by changes such a flow velocity induced by the change in the channel's cross-section. This volume was designed with dimensions of 9.00 x 2.50 x 0.49 mm. It is centred relative to the channel's centre and its bottom is set 10 μm above the glass substrate surface (**Figure 45**).

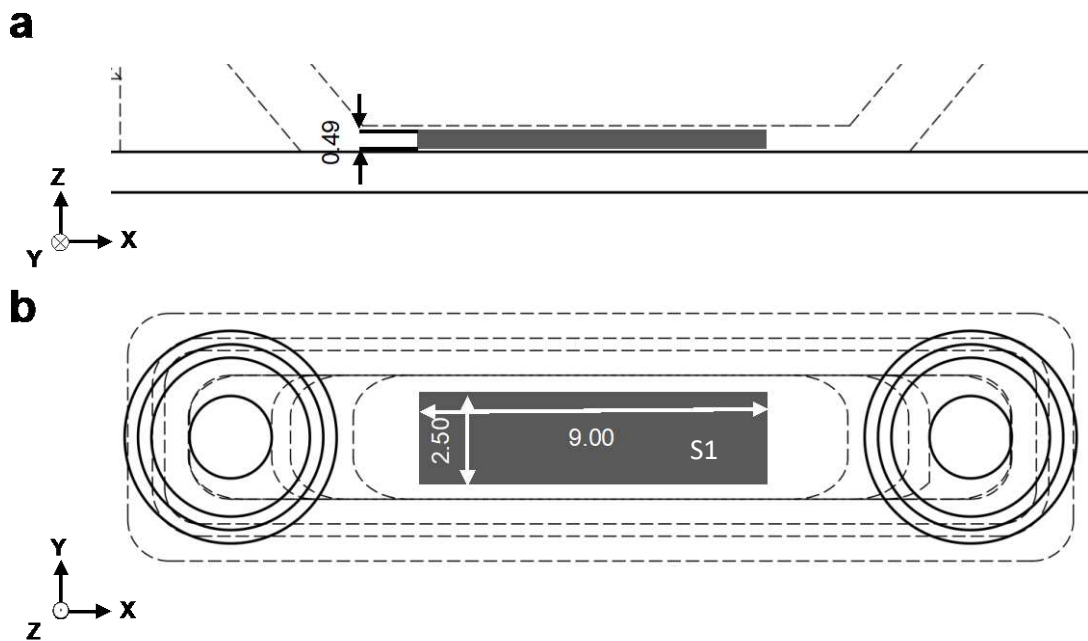


Figure 45. Definition of the volume of interest. The volume of interest is represented by the grey rectangles in both (a) xz and (b) xy views. The corresponding volume has dimensions 9.00 x 2.50 x 0.49 mm and is centred with respect to the culture chamber. S1 is the xy surface area of the volume of interest. All dimensions are in mm.

The first electrode configuration featured four squared electrodes of dimensions 0.50 x 0.50 mm, with one pair placed towards the culture chamber inlet and the other at the chamber outlet (**Figure 46.a**). This way, one CC electrode and one PU electrode were placed on either side of the chamber with a 9 mm gap between them in the x-direction. In the y-direction, a 0.50 mm gap was designed in-between CC and PU electrodes.

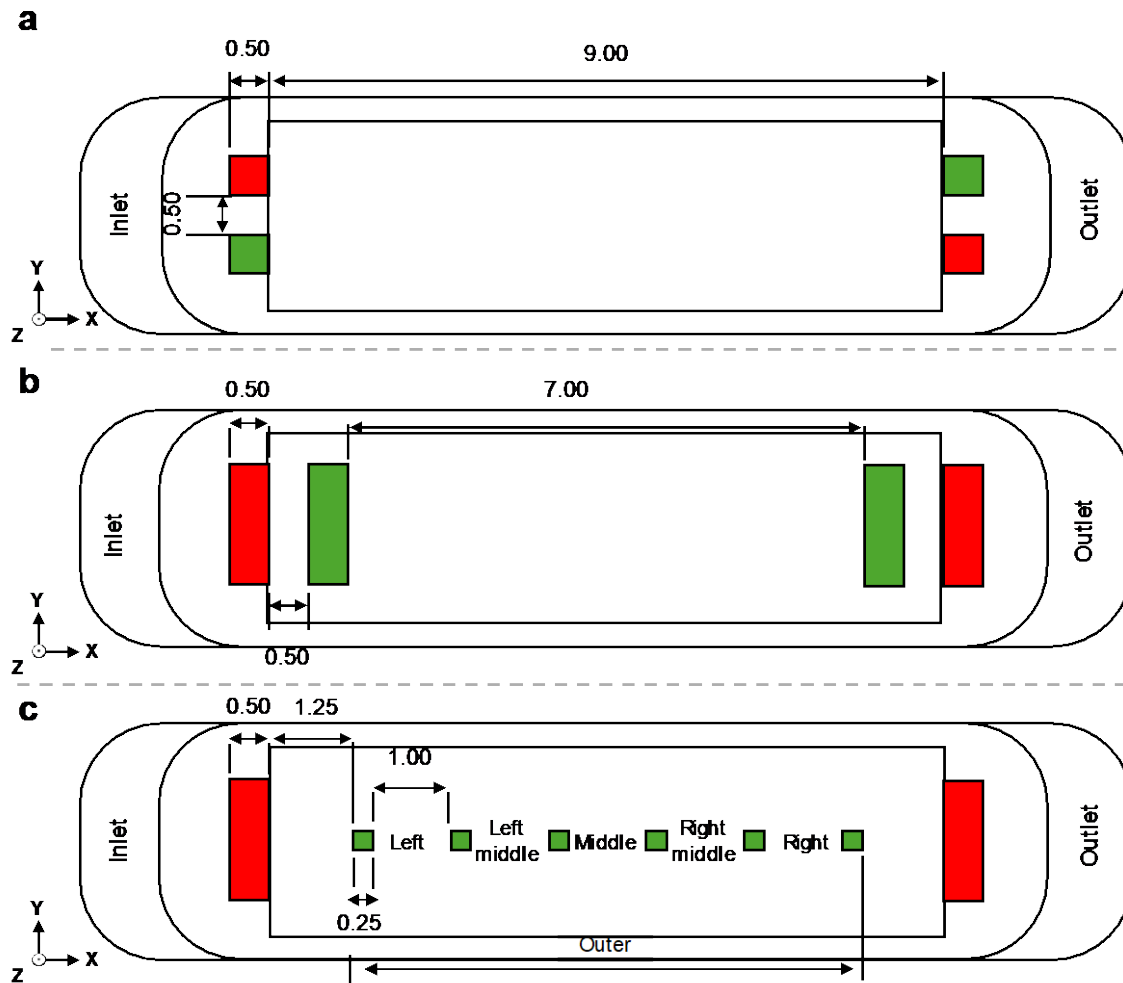


Figure 46. Three electrode geometries considered for incorporation within the microfluidic channel. Red electrodes are CC electrodes and green electrodes are PU electrodes Both are placed so that the electrical measurement is located within the volume of interest (grey rectangles). Three patterns were considered. The first has four square electrodes (**a**) of 0.50 x 0.50 mm separated by 9.00 mm. The second pattern features four rectangular electrodes (**b**) of 1.50 x 0.50 mm separated by 0.50 mm. PU electrodes are spaced by 7.00 mm. (**c**) The third configuration has a total of eight electrodes: two rectangular CC electrodes (1.50 x 0.50 mm) and six square PU electrodes (0.25 x 0.25 mm). All dimensions shown are in mm.

The second considered electrode pattern also featured four electrodes, but rather than having squared electrodes, we decided to consider using rectangular electrodes (**Figure 46.b**). This enables us to increase their dimensions to 1.50 x 0.50 mm. Similar to the first configuration, one CC electrode and one PU electrode were placed on either side of the

chamber. Both PU electrodes, spaced by 7.00 mm, were placed in-between both CC electrodes. In the x-direction, a 0.50 mm gap was designed in-between CC and PU electrodes.

The third considered electrode pattern featured eight electrodes instead of four (**Figure 46.c**). Two electrodes, placed at the culture chamber's inlet and outlet, were designed as the CC electrodes. They were designed to be rectangular in shape and to have dimensions of 1.50 x 0.50 mm. The other six electrodes were designed to be evenly spaced between both CC electrodes, with a 1.00 mm separating them. They are designed as squares with dimensions of 0.25 x 0.25 mm. This configuration theoretically enabled the segmentation of the culture chamber volume in smaller adjacent volumes as named in the figure, and to spatially differentiate them when performing impedance measurements.

However, we incorporated a dedicated ruler to the electrode geometry to measure the alignment between the PDMS bottom and the microelectrode system to study its effect on the electrical measurement as we will see later. This forced us to offset the electrodes from the central axis of the channel by 0.50 mm (**Figure 47**). We calculated the average sensitivity as described below for both centred and off-axis geometries using the surface area S1 at different z-heights (10, 250 and 500 μm) and did not notice any significant differences. All sensitivity averages are recapitulated in **Table 17**.

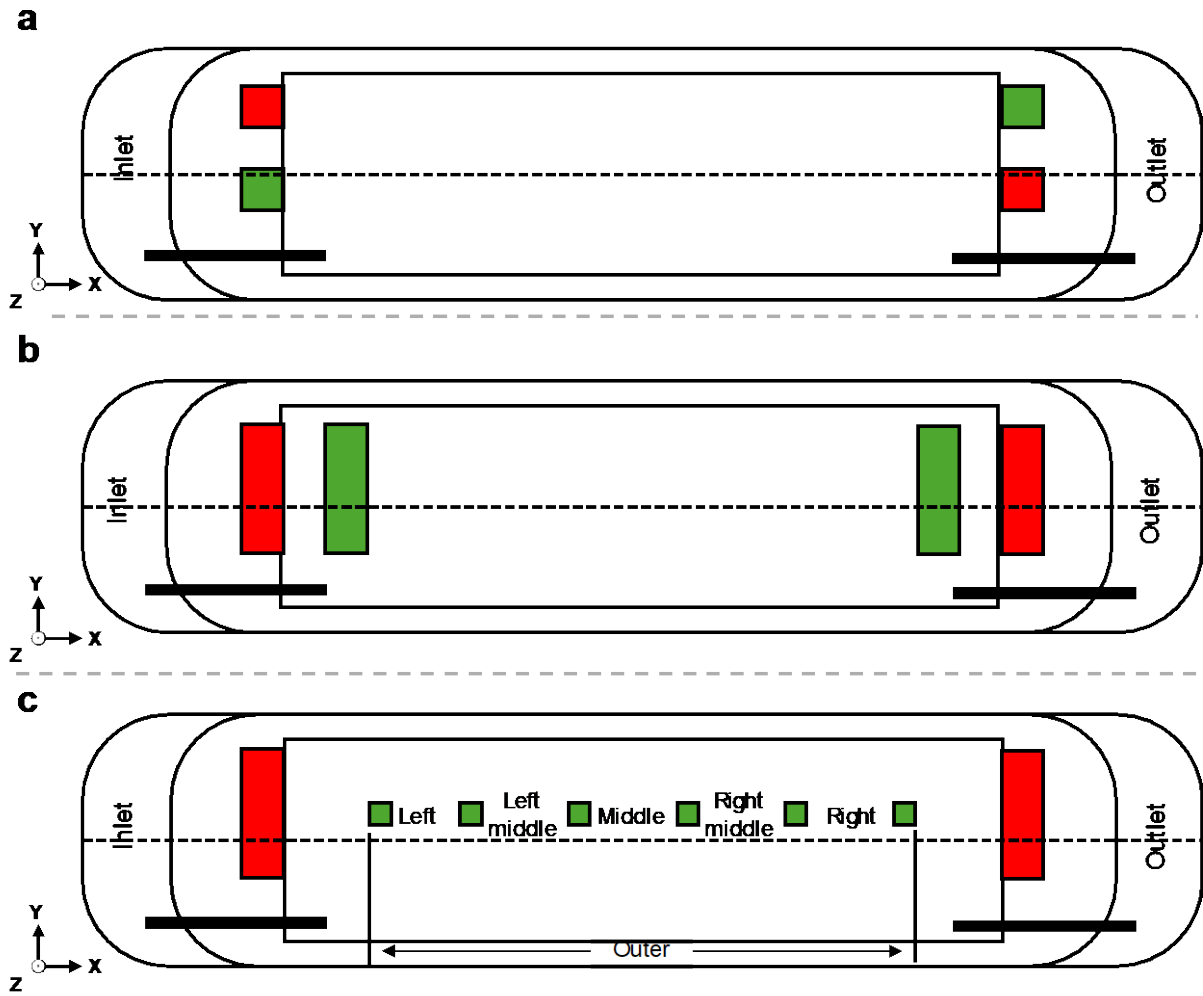


Figure 47. Off-axis electrode patterns. (a) 4-square, (b) 4-rectangle and (c) 8 electrode configurations were offset by 0.50 mm to incorporate the dedicated ruler (black rectangles). Grey rectangle represents the xy cross section of the volume of interest and dotted line represents the culture chamber centre line.

To determine which electrode geometry was best suited for bioimpedance measurements in the culture chamber, we analysed their corresponding sensitivity (S) fields. The sensitivity S [m^{-4}] of a measurement setup is a factor determining the influence of the local impedivity on the total impedance measurement.¹⁵⁶ In other words, if the sample volume is divided into smaller volumes (voxels), we can determine which voxels participate the most to the measured impedance by calculating their respective sensitivities. This makes the sensitivity field a valuable tool for comparing electrode configurations independently of the sample's actual conductivity distribution.

The sensitivity field is defined as:

$$S = \frac{\vec{J}_{CC} \cdot \vec{J}_{PU}}{I_{CC} \cdot I_{PU}} \quad (22)$$

with $\vec{J}_{CC}$ [A.m⁻²] the current density field when applying a unit current I_{CC} [A] between the CC electrodes and $\vec{J}_{PU}$ the current density field when applying a unit current I_{PU} between the PU electrodes. This definition is based on the lead field theory proposed by Geselowitz for ECG applications.²²² This sensitivity S can be used to calculate the changes in impedance Z when the complex conductivity (σ^*) of the sample volume V changes due to dynamic events:²²³

$$\Delta Z = \int_V \frac{S}{\Delta \sigma^*} dV \quad (23)$$

In a 4-point electrode, S can either be positive, negative or zero null depending on the angle between both density fields. If they are:¹⁶³

- In the same direction, S will be positive,
- In the opposite direction, S will be negative,
- In perpendicular direction, S will be 0.

Of note, in the regions of positive sensitivity, an increase in the local impedivity results in an increase in the measured impedance. On the other hand, an increase in the local impedivity in regions with negative sensitivity results in a decrease in impedance. It is therefore necessary to maximise the sensitivity field in the volume of interest to be able to detect small impedance changes. In our case, the volume of interest is located at the centre of the culture chamber, and more specifically, underneath the cap's protrusion (**Figure 46**). Furthermore, as we want to perform bioimpedance measurements on a 3D solid tumour model, it is important to have a high sensitivity along the channel height as well. We therefore investigated the sensitivity fields in the x-, y- and z-directions in all three previously presented geometries.

We implemented the channel geometry and the three considered electrode patterns in a numerical model (**model 1**) using the AC/DC module of COMSOL MULTIPHYSICS using the 'electric current (ec)' interface. Two electric currents interface were utilised, one for each electrode pairs. One electrode set to ground ($V = 0$), while the other was utilised to apply an electrical potential of 25 mV for each electric currents interface. Electrical insulation was applied to the culture chamber walls and the initial potential in the culture simulation volume was set to 0 V. The simulation of the sensitivity fields was carried out according to the current conservation equation for conductive media in the frequency domain:

$$\nabla \vec{J} = Q_{j,v} \quad (24)$$

$$\vec{J} = \sigma \vec{E} + j\omega \vec{D} + \vec{J}_e \quad (25)$$

$$\vec{E} = -\nabla V \quad (26)$$

$$\vec{D} = \epsilon_0 \epsilon_r \vec{E} \quad (27)$$

with $\vec{J}$ the total current density (A.m^{-2}), $Q_{j,v}$ the volumetric current source (A.m^{-3}), σ the electrical conductivity (S.m^{-1}), $\vec{E}$ the electric field (V.m^{-1}), $\vec{D}$ the electric displacement field (C.m^{-2}), $\vec{J}_e$ the externally applied current density (A.m^{-2}) and V the electric potential (V). The model then computed the sensitivity field S according to **equation 22** assuming the culture chamber was filled with an electrical conductivity standard ($\sigma = 20 \text{ mS.cm}^{-1}$ and $\epsilon_r = 80$). The sequence of COMSOL commands for this modelling is given as an ‘.mph’ file in **Appendix 3**.

We first simulated the four-squared electrode geometry and plotted the current density lines for both the CC and PU electrodes (**Figure 48.a**). We observed that the two current density vectors had opposite directions within the 0.5 mm space separating the CC and PU electrodes on each side of the culture chamber. We also observed that in the volume of interest, the current density vectors were both pointing in the same direction. We observed a negative sensitivity in the space separating the CC and PU, a positive sensitivity in the volume of interest, and a sensitivity equal to 0 outside elsewhere, when we represented the sensitivity field as a heatmap in both xy- and xz-directions. We also plotted the sensitivity field in the xy-direction at three different heights ($z = 10, 250$ and $500 \text{ }\mu\text{m}$). We extracted the average sensitivity of the cross section S1 at each z-height and obtained values of 3.14×10^{11} , 2.80×10^{11} and 2.80×10^{11} , respectively. This showed a decrease in sensitivity as we go up the culture chamber height (**Figure 48.b**). This was also observed when we plotted the sensitivity field heatmap in the xz-direction (**Figure 48.c**). This can be explained by the fact that as you move away from the surface of the electrodes the current density is reduced and, as a result, so is the sensitivity.

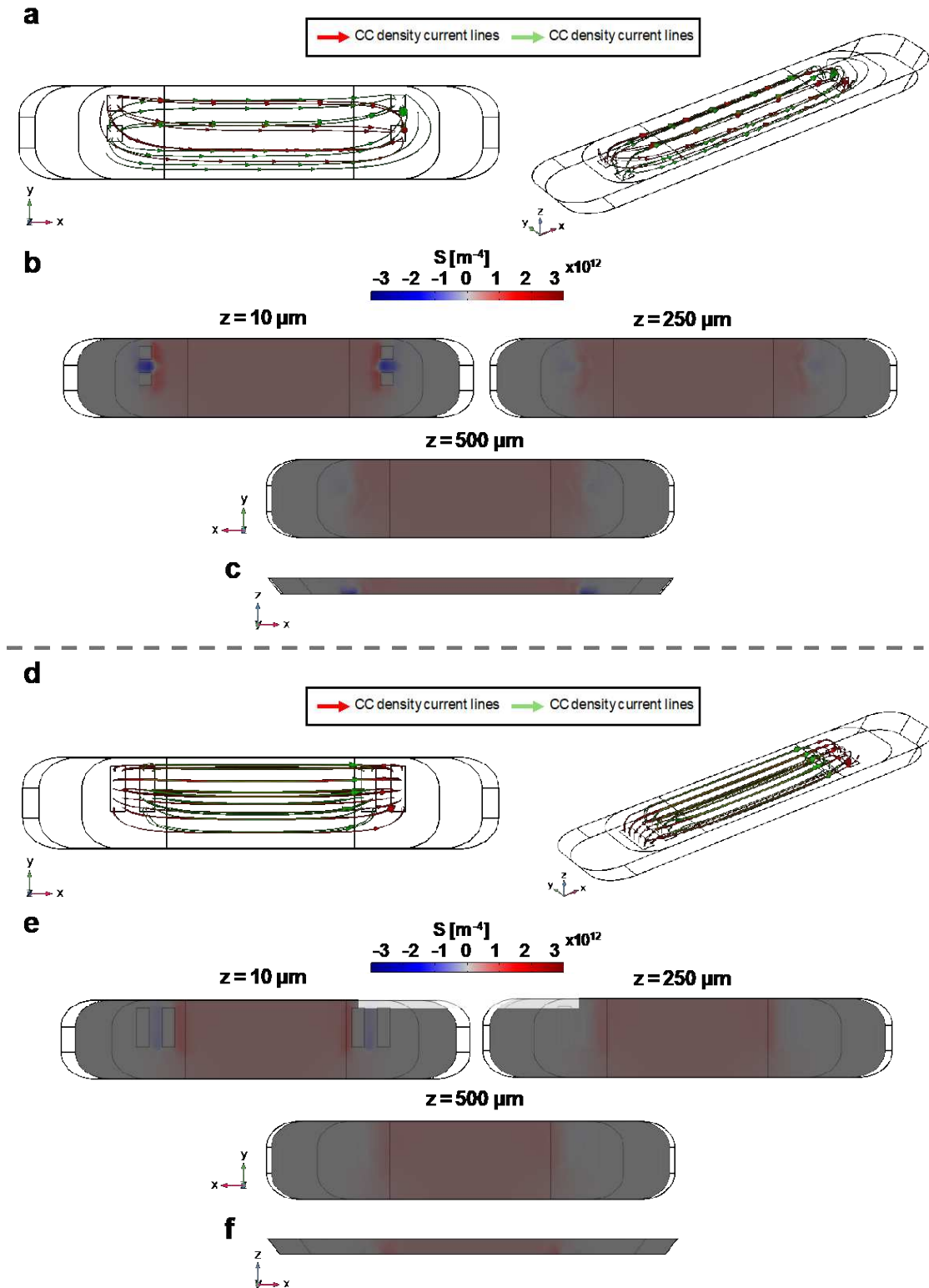


Figure 48. Sensitivity field analysis of 4-square and 4-rectangle geometries. Using the 4-square pattern, we visualised the (a) current density field lines both xy- and 3D-view of the channel (red lines represent current density of CC electrodes and green of PU electrodes) and the (b) sensitivity [m^{-4}] heatmap in the xy-direction for three z-positions (10, 250 and 500 μm) as well as in the (c) z-direction. A similar study was performed on the 4-rectangle pattern. We visualised the (d) current density field lines both xy- and 3D-view of the channel and the (e) sensitivity [m^{-4}] heatmap in the xy-direction for three z-positions (10, 250 and 500 μm) as well as in the (f) z-direction.

We noticed a similar behaviour in the 4-rectangle pattern. Both current density vectors had opposite directions in the 0.5 mm space separating the CC and PU, in the same direction between the two PU electrodes (**Figure 48.d**). Similar to the 4-squared pattern, we observed a negative sensitivity in the space separating the CC and PU, a positive sensitivity in the volume of interest, and a sensitivity equal to 0 outside elsewhere. This was confirmed when we plotted the sensitivity field heatmaps in the xy-direction at three different heights ($z = 10, 250$ and $500 \mu\text{m}$). We extracted the average sensitivity of the cross section S1 at each z-height and obtained values of 2.44×10^{11} , 2.27×10^{11} and 2.22×10^{11} , respectively. We observed a decrease as we went higher in the culture chamber (**Figure 48.e**). This was further observed when we plotted the sensitivity field heatmap in the xz-direction (**Figure 48.f**).

For the 8-electrode pattern, we performed a similar analysis for each PU electrode pair as defined in **Figure 46**. Sensitivity fields analysis for all PU electrode pairs can be found in **Annexe 1 (Figure 75 and Figure 76)** but only the outer and middle PU pairs are represented in **Figure 49** as we considered them representative. Similar to both 4-squared and 4-rectangle configurations, the current density lines for both outer and middle PU electrodes were in opposite directions between the CC and PU electrodes and in the same direction between the two PU electrodes (**Figure 49.a and d**). We observed a negative sensitivity in the space separating the CC and PU, a positive sensitivity in the volume of interest, and a sensitivity equal to 0 outside elsewhere. We extracted the average sensitivity of the cross section S1 at each z-height for the outer PU electrode pair and obtained values of 3.44×10^{11} , 3.18×10^{11} and 3.09×10^{11} , respectively. This showed a decrease in sensitivity as we go up in the culture chambers for both PU electrode pairs (**Figure 49.b, c, e and f**). A similar decrease was found for the middle PU electrode pair with values of 5.15×10^{11} , 3.45×10^{11} and 2.80×10^{11} . The sensitivity values for the middle PU electrode were found to be higher as this configuration reduced the distance between both PU electrodes compared to the outer electrodes. The sensitivity values for the other PU electrode pairs are summarised in **Table 10**.

Table 10. Sensitivity values [m^{-4}] for the 8 electrode geometry PU electrodes pairs at different heights.

z height (μm)	Left	Left middle	Middle	Right middle	Right
10	5.15E+11	5.02E+11	5.15E+11	4.94E+11	5.20E+11
250	3.47E+11	3.38E+11	3.45E+11	3.42E+11	3.43E+11
500	2.88E+11	2.87E+11	2.80E+11	2.87E+11	2.88E+11

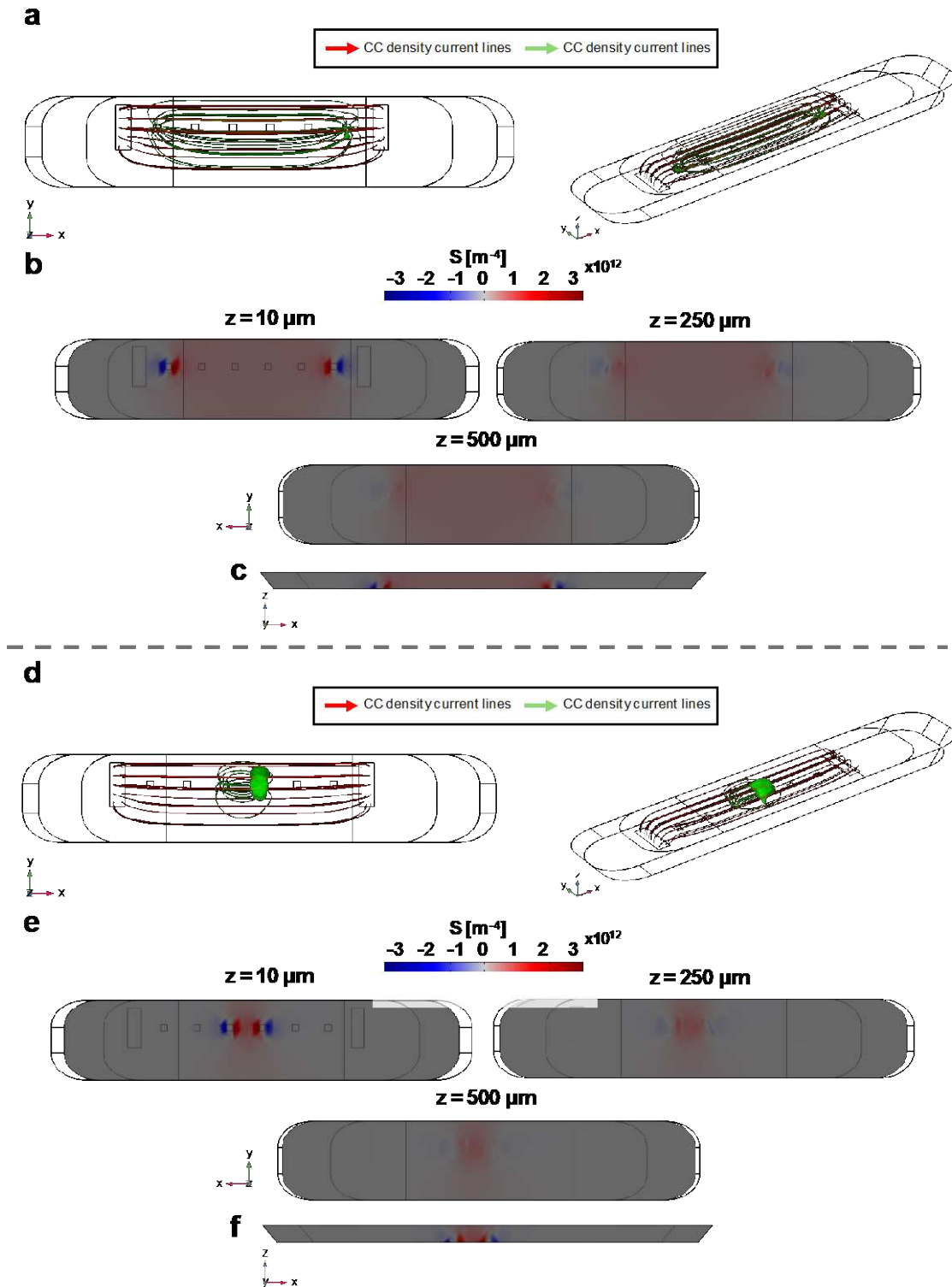


Figure 49. Sensitivity field analysis of 8-electrode pattern. Using the outer PU, we visualised the (a) current density field lines both xy- and 3D-view of the channel (red lines represent current density of CC electrodes and green of PU electrodes) and the (b) sensitivity $[\text{m}^{-4}]$ heatmap in the xy-direction for three z-positions (10, 250 and $500 \mu\text{m}$) as well as in the (c) z-direction. A similar study was performed on the other PU electrode pair. Here, we only show the result for the middle PU electrode pair. We visualised the (d) current density field lines both xy- and 3D-view of the channel and the (e) sensitivity $[\text{m}^{-4}]$ heatmap in the xy-direction for three z-positions (10, 250 and $500 \mu\text{m}$) as well as in the (f) z-direction.

One difference we noticed on the generated heatmaps was that the 4-rectangle configuration generated the lower sensitivity, while the 8-electrode pattern generated the highest in the xy-direction. This was especially true when choosing PU electrodes that were closer to each other such as the middle PU electrode pair. This was also true in the z-direction.

To validate this observation and quantitatively compare all three electrode geometries, we normalised the extracted average sensitivities for the three z-positions with the maximum sensitivity. This maximum sensitivity was 3.44×10^{11} and was found for the 8-electrode pattern (outer PU electrode pair) at $z = 10 \mu\text{m}$. Normalised sensitivity values are summarised in **Table 11**. The 4-rectangle configuration having the lowest average sensitivity for each z-positions, with values of 0.71, 0.66 and 0.64, respectively. The 8-electrode pattern however had the highest average sensitivity for a similar surface area (outer PU electrodes), with values of 1.00, 0.92 and 0.90, respectively.

Table 11. Normalised sensitivity values of each electrode geometry.

z height (μm)	4-square	4-rectangle	8-electrode
10.00	0.91	0.71	1.00
250.00	0.81	0.66	0.92
500.00	0.81	0.64	0.90

The 8-electrode pattern resulted in the higher average and peak sensitivities compared to the other two geometries, as well as allowing us to separate the culture chamber volume into smaller volumes and therefore a higher spatial resolution. This led us to choose this geometry. Furthermore, this geometry, as opposed to the other two, utilised the full 64 available electrodes, making it more efficient. We did not however investigate the placement of the six PU electrodes in this configuration.

1.1.2. Fabrication and integration of the bioimpedance measurement system

Having decided on the electrode geometry for each fluidic channel, we designed the microelectrode system such that all eight fluidic channels featured eight electrodes (**Figure 50.a**). As we previously shown, two of these electrodes served as CC electrodes while the other six served as PU electrodes (**Figure 50.b**). Additionally, we added two rulers placed at each channel inlet and outlet. These rulers featured digits that are $10\ \mu\text{m}$ wide that are evenly spaced with $100\ \mu\text{m}$ between them (**Figure 50.c**). As we will see later, these rulers served to measure the alignment between the microfluidic chip PDMS bottom and the electrodes.

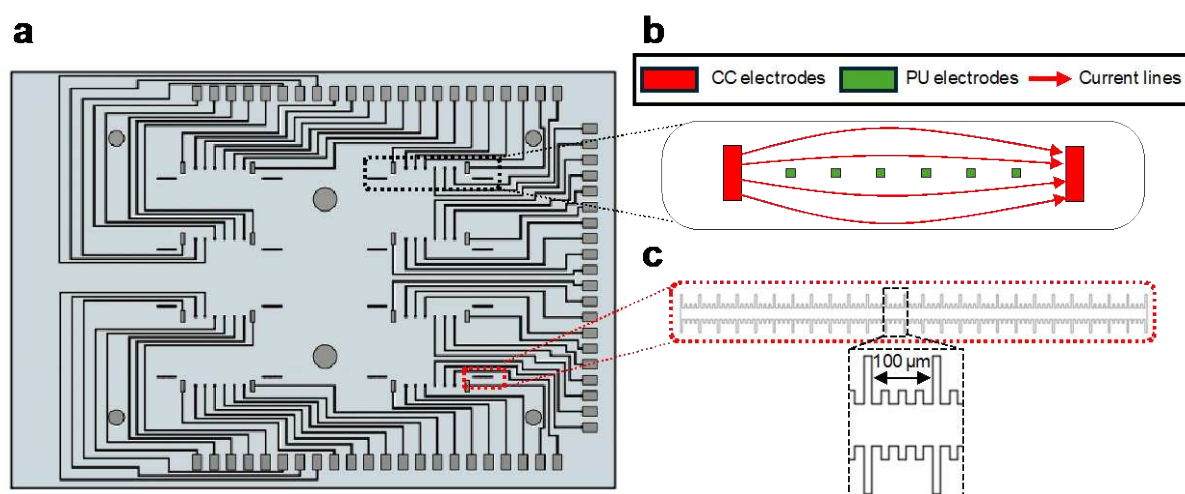


Figure 50. Microelectrode biosensor design. (a) The microelectrode array features 64 individual electrodes, attributing (b) eight electrodes to each individual culture chamber in order to increase the flexibility of the measurement. (c) A ruler was added to the design to measure the alignment between the PDMS bottom part and the electrodes.

1.1.2.1. Electrode material

The first parameter to consider for electrode fabrication is which material to fabricate them with. Given that the objective is to perform bioimpedance measurements on a 3D cell culture, it was crucial to choose a material that was both chemically stable and biocompatible. Furthermore, we restricted ourselves to metals that are routinely used in order to rapidly optimise the fabrication protocol. With this in mind, we considered two commonly used metals: gold and Pt. Although both have high electrical conductivities, with values of 45.2×10^6 and $9.66 \times 10^6\ \text{S.m}^{-1}$, respectively, the use of gold implied a higher fabrication cost than Pt. Hence, we chose Pt as the fabrication material for our electrodes.

1.1.2.2. Electrode fabrication protocol

There are two major techniques to pattern metallic layers on a glass substrate: wet etching, where the metal is patterned by chemically removing unwanted areas, and lift-off, where the metal pattern is defined by the shape of a pre-patterned resist.²²⁴ Both these techniques utilise a photosensitive resin to pattern the metallic layer into the desired shape. However, although wet etching of Pt has previously been realised using Aqua Regia solution (mixture of 3:1 hydrochloric acid and nitric acid (v/)),²²⁵ our first tries were not conclusive. We therefore decided to utilise the lift-off fabrication protocol to create our electrode pattern.

The working principle of lift-off lithography is as follows (**Figure 51.a**): a layer of photoresist is deposited onto the substrate and exposed to UV light according to the desired pattern. The resin is subsequently developed to remove the unwanted resin. The metallic layer is then deposited onto both the substrate and resin alike. The final step is to remove the resin using a solvent bath, leaving only a metallic layer where there were openings in the resin layer. However, we faced fabrication issues using this technique. Indeed, one glaring issue was observed after the lift-off procedure. As the photosensitive resin layer was removed, it creates particles of Pt that fell back down and adhered to the glass substrate (**Figure 51.b**). This was particularly problematic, as these Pt particles connected independent electrodes and connection paths, creating a short-circuit between them, rendering them unusable. Furthermore, when characterising the metallic layer via profilometer measurement, we noticed that the electrode edges were considerably thicker than the intended 120 nm (**Figure 51.c**). This phenomenon is commonly referred to as lift-off wings. It is also problematic as they modified the electrode contact area with the sample and therefore modified the generated electric field.

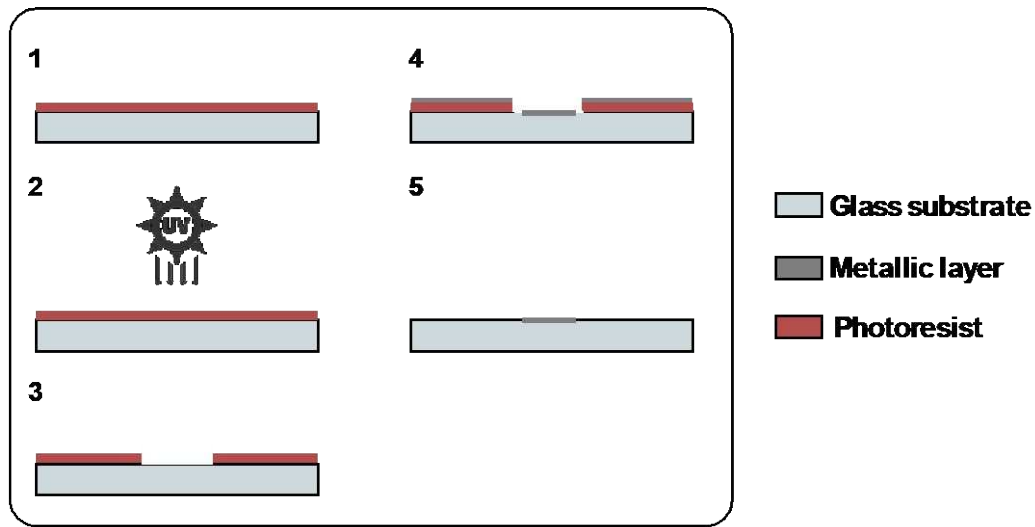
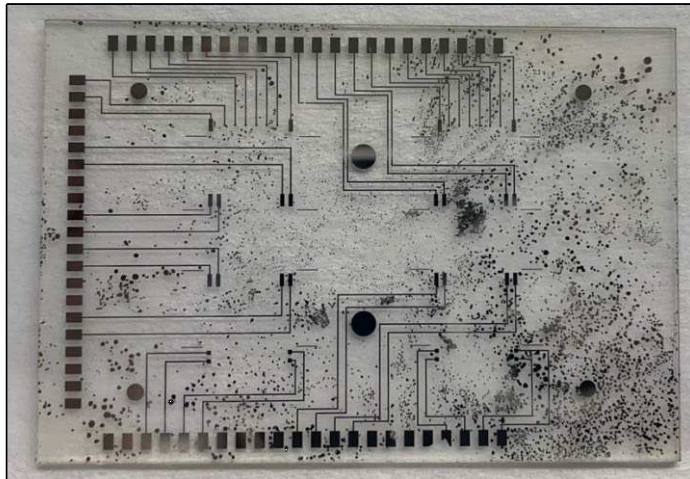
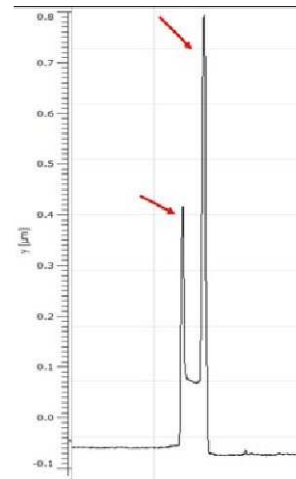
a**b****c**

Figure 51. Lift-off lithography protocol and issues. (a) Lift-off procedure: **1:** Coating of glass substrate with photosensitive resin S1813 **2:** Exposure to UV lights **3:** Resin development **4:** Metallic layers (titanium/platinum) deposition **5:** Lift-off of resin. (b) Photo of platinum particles adhering to the glass substrate during lift-off procedure. (c) Lift-off wings measured by stylus profilometer (red arrows).

Consequently, we changed the fabrication protocol to a bilayer lift-off protocol that is known to reduce both afore mentioned issues (**Figure 52.a**).²²⁶ As its name suggests, this process involved two photosensitive layers instead of one. Briefly, a first layer of LOR-3A resin was deposited onto the substrate, followed by a second layer of S1813 resin. This created a double-layer masking structure which was exposed to UV light and developed. During development, the developer first removed the exposed S1813, then diffused through the LOR-3A layer. This diffusion development created what is known as a cap profile which ensures a

break between the metal layer on the substrate and the one on the resin, thus eliminating the lift-off wings. After development, a 20 nm thick layer of Ti was deposited onto the substrate to serve as an adherent layer to the 100 nm thick Pt layer. The metal was then patterned by the lift-off procedure that removed the excess metal by stripping both photosensitive resin layers. However, as LOR-3A is not dissolvable by acetone, but is by developer, we alternated both baths. This eliminated the adhesion of Pt particles to the glass substrate. This procedure enabled us to successfully fabricate our microelectrode system onto the glass substrate (**Figure 52.b**).

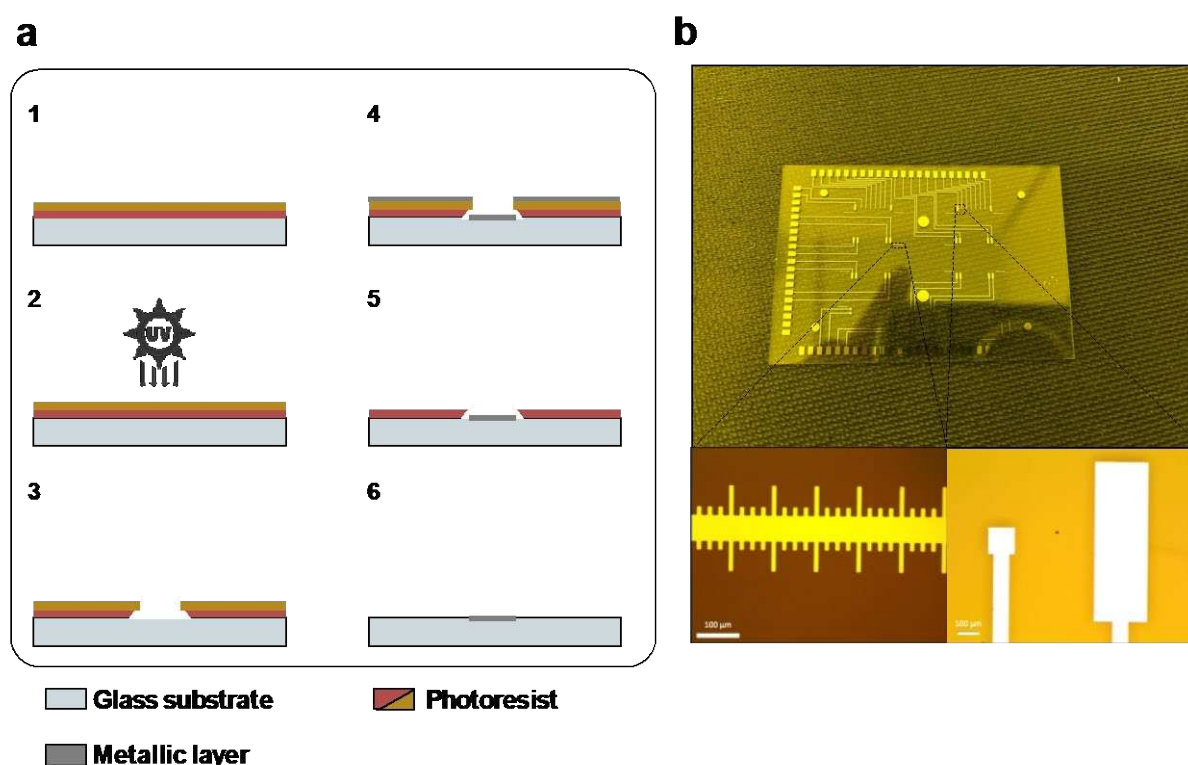


Figure 52. Bilayer lift-off lithography protocol. (a) Bilayer lift-off procedure: **1:** Coating of glass substrate with photosensitive resins bilayer **2:** Exposure to UV lights **3:** Resin development **4:** Metallic layers deposition **5:** Lift-off of outer resin layer **6:** Lift-off of second resin layer. (b) Fabricated electrodes and rulers using bilayer lift-off lithography.

We subsequently measured the metallic layer thickness, as well as the width and spacing of the rulers' digits using a profilometer to validate the fabrication process (**Figure 53**). The measurement baseline is lower than 0, which can be explained by a miscalibration of the instrument. We therefore measured the digits height between their bottom and top. For nine separate microelectrode systems, we measured: an average metallic layer thickness of 117.66 ± 7.42 nm, an average digit width of 12.79 ± 0.26 µm and an average digit spacing of 100.77 ± 0.23 µm. All values are recapitulated in **Table 12**. These represent differences of 1.96 %, 24.48 % and 0.78 %, respectively, with the theoretical design values, validating the precision

of the fabrication protocol. To note, the higher measured difference was for the digit width. This is due to the open cap profile produced during bilayer lift-off procedure: the diffusion of the developer in the bottom resin layer results in an inclined opening that is wider at the bottom than at the top, hence the wider digit width.

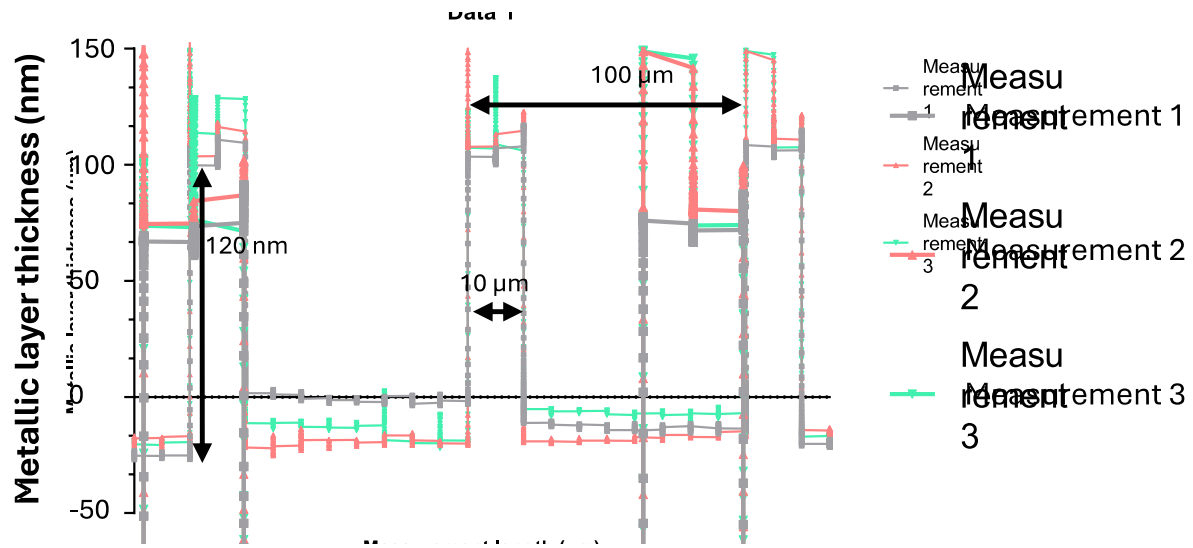


Figure 53. Profilometer measurements performed on a fabricated microelectrode system. Three quantities were analysed: the metal layer thickness, the width of the ruler graduations and their spacing. Their theoretical values are illustrated by the arrows on the graph.

Table 12. Measured parameters to validate the microelectrode system fabrication.

Microelectrode system	Layer thickness (nm)	Digit width (μm)	Digit spacing (μm)
1	125.48	12.43	100.89
2	124.99	12.47	100.59
3	126.23	13.33	101.00
4	120.97	12.84	100.67
5	110.25	12.80	101.00
6	120.97	12.84	100.67
7	110.25	12.80	101.00
8	109.45	12.85	100.78
9	110.35	12.70	100.33

1.1.2.3. Electrode connection

After the fabrication of the electrodes, the next step was to connect them to the impedancemeter to perform measurements. As previously mentioned, we utilised the *CSX-64* impedancemeter manufactured by *Sciospec Scientific Instruments GmbH* to perform bioimpedance measurements. The connection interface of this device was designed to function using a printed circuit board (PCB). Additionally, the use of a PCB offers a stable electrical contact, and a more robust connection compared to other connection methods. We therefore opted to design a custom PCB to connect our electrode system.

The PCB has dimensions of 101.61 x 52 mm and features a notch of dimensions 65 x 43.2 mm (**Figure 54.a**). This notch was designed such that the microfluidic chip could protrude through once the electrodes were connected to the PCB. The PCB is 1.61 mm thick and is composed of six copper layers alternating with five core dielectric layers. Each PCB has 64 connection pads (1.9 x 1.2 mm) enabling each individual electrode to be connected to the impedancemeter. A manufactured PCB can be seen in **Figure 54.b**.

We chose to connect the PCB to the electrodes using a conductive soldering paste. Indeed, as the end use of the system is to analyse a 3D culture model, we needed a robust connection method that could withstand both the incubator's environment as well as repetitive moving. The soldering protocol is shown in **Figure 54.c**. Briefly, the conductive soldering paste is applied on the PCB contact pads using a dedicated stencil. The electrodes are then placed onto the conductive paste. The system is then placed into a reflow oven for 60 s at 250 °C, melting the paste and creating the connection as it cools down.

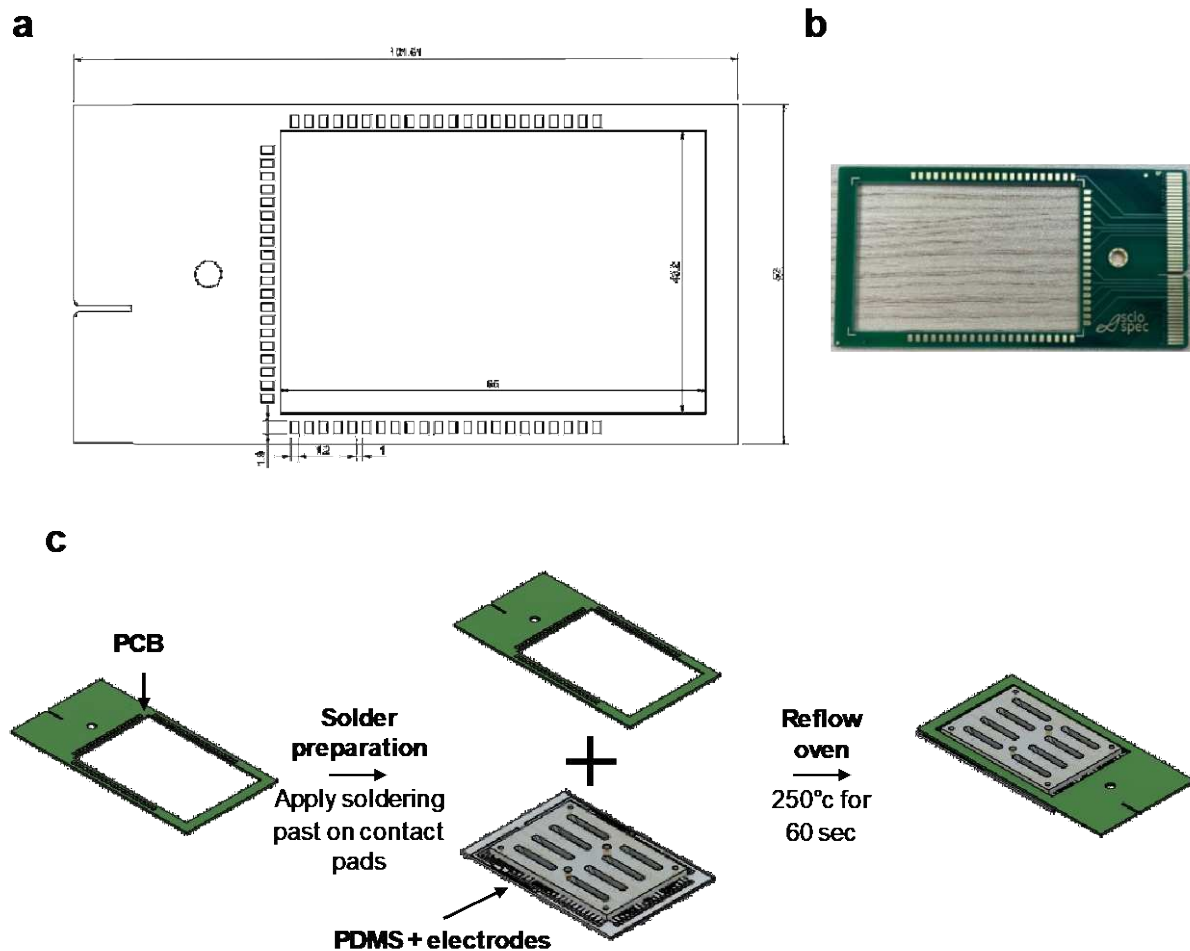


Figure 54. Connection process of the electrodes to a dedicated printed circuit board. (a) The PCB features 64 individual connection pads – one for each electrode and features a notch to allow for the PDMS bottom part to protrude. (b) Photo of the manufactured PCB. (c) Soldering protocol: a conductive soldering paste is applied on the connection pads. As the system is placed in a reflow oven for 60 s at 250°C, the paste melts and creates the connection as it then cools down.

However, as the PCB's length exceeded the glass substrate's, we modified the bottom aluminium closing plate as well as aluminium wedge by opening them up (**Figure 55.a** and **b**). This enabled the PCB to protrude outside of the closing system (**Figure 55.c**), and therefore to be connected to the measuring system (**Figure 55.d**).

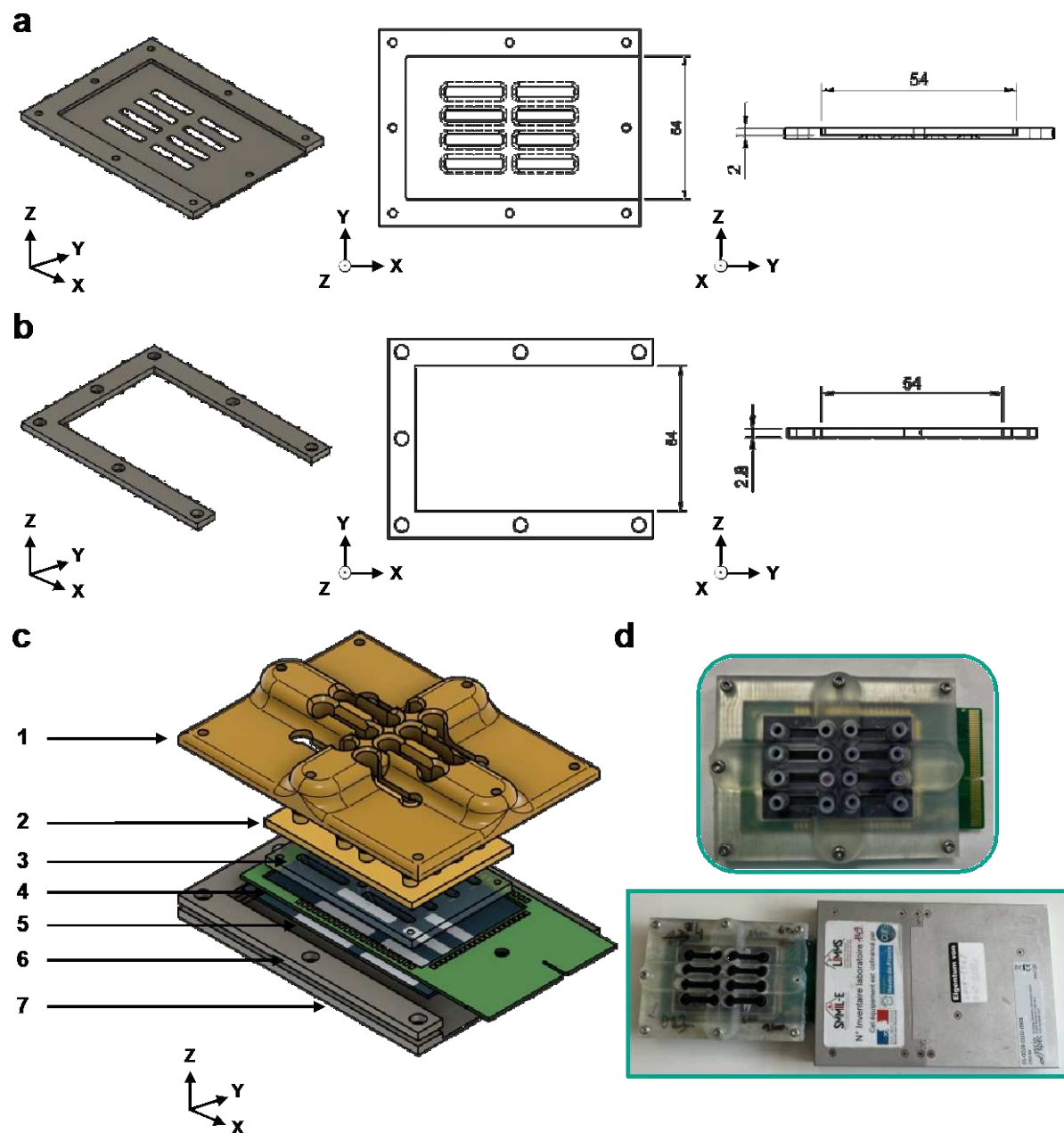


Figure 55. Adapted closing system to accommodate the addition of the PCB. Both (a) bottom aluminium closing plate and (b) aluminium wedge were open to allow the PCB to protrude out of the closing system. (c) Exploded view of the microfluidic chip: 1: 3D-printed reinforced top closing plate 2: 3D-printed cap 3: PDMS bottom 4: PCB 5: glass substrate with electrode pattern 6: aluminium wedge 7: aluminium bottom closing plate. (d) Closed microfluidic chip with protruding PCB and connection to the impedancemeter.

1.1.2.4. Data acquisition and analysis pipeline

After fabricating the device, we developed a dedicated Python data acquisition and analysis pipeline to render both automatic (**Figure 56**). The main objective was to enable the acquisition of successive bioimpedance spectra over time to monitor the temporal evolution of the cell culture and their automated analysis.

To achieve this, a Python script was developed by Aziz Gnewi, an intern at *Sciospec Scientific Instruments GmbH*. It enabled the user to define the measurement parameters:

- The frequency interval of the measurement,
- The number of measurement points,
- The scale of the measurement points (logarithmic or linear),
- The excitation signal amplitude,
- The activated electrodes,
- The time interval between each measurement,
- The time duration of the experiment,
- The folder in which the measurement files are saved.

Furthermore, this Python script enabled all 8 electrode banks to perform a measurement at once, reducing the measurement time by 8. Each measurement generated 48 separate text files (eight per culture chamber, each with six PU electrode pairs). We therefore created additional scripts to extract relevant data (real and imaginary parts of impedance), combine them into a single spreadsheet (CSV file), and calculate key parameters: impedance magnitude and phase angle. Finally, this data was split into individual CSV files for each electrode setup, allowing automated averaging of impedance magnitude under different experimental conditions and plotting of the results. This methodology is particularly powerful as each temporal experiment can generate a considerable amount of data. For example, for a seven-day long experiment, with measurements being taken every hour, we generate 403,200 individual lines of data, which cannot be easily handled in excel files. The sequence of Python scripts is given as an ‘.py’ file in **Appendix 3**.

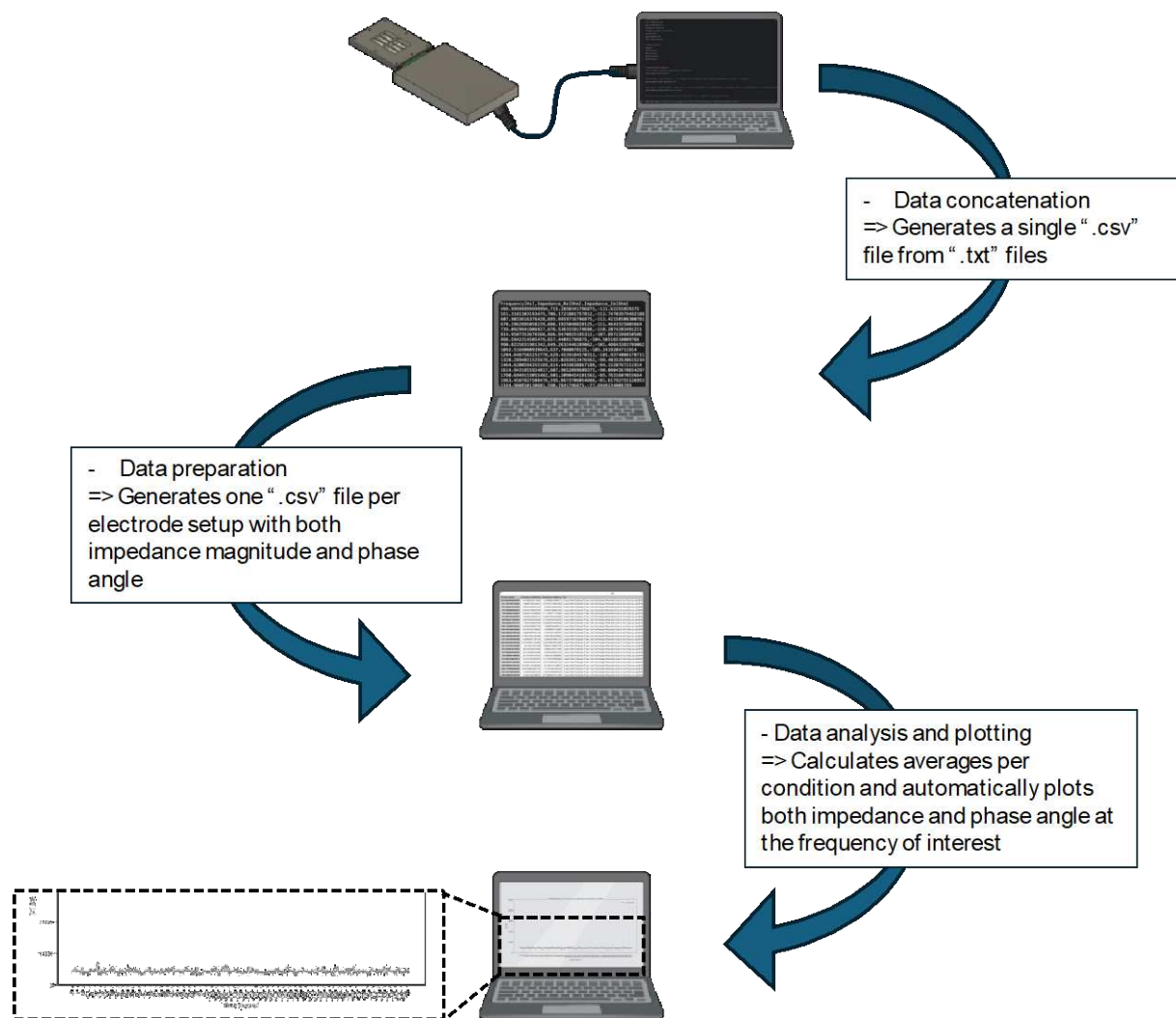


Figure 56. Dedicated Python protocol for automated data acquisition and analysis.

1.2. Microelectrode system characterisation

1.2.1. Conductivity standard impedance measurements

Once the electrodes had been manufactured, we were able to carry out impedance measurements to validate the functionality of the microelectrode system. We initially utilised conductivity standards with different electrical conductivity values (20, 30 and 50 mS.cm⁻¹) as well as culture medium (1X-DMEM) to perform our measurements. To note, we define N as the number of microfluidic chips used for experiments and n the number of individual culture chambers used.

We first measured the electrical conductivities of the standard solutions and found values of 19.11 ± 0.01 , 28.54 ± 0.08 and 48.17 ± 0.10 mS.cm⁻¹ at a room temperature of 22.3°C. We then filled the culture chambers with 60 µL of each solution and applied an excitation signal of 25 mV peak-to-peak amplitude over a frequency range of 500 Hz to 60 kHz. We measured the resulting impedance spectra using the outer most PU electrode pair. We chose to only use the outer PU electrode pair as we assumed a homogeneous filling of the culture chamber and that the electrical conductivity standard were purely resistive in nature at our working frequency interval. We subsequently plotted and compared their impedance spectra (**Figure 57.a**). We observed that all four solutions had non overlapping and constant impedance magnitudes. We observed an inversely proportional relationship between the solution's electrical conductivity and its impedance. The 20 mS.cm⁻¹ had a higher impedance than the 30 mS.cm⁻¹ and the 50 mS.cm⁻¹ with impedance values of $|Z_{20}| = 1480 \pm 108 \Omega$, $|Z_{30}| = 943 \pm 48 \Omega$ and $|Z_{50}| = 594 \pm 47 \Omega$, respectively. As for the culture medium, its impedance was $|Z_{CM}| = 1780 \pm 115 \Omega$. We therefore calculated the cell constant κ from equation (15) and obtained values of 2960, 2829 and 2970 m⁻¹ for each electrical conductivity standard, respectively, giving an average cell constant of 2920 ± 79 m⁻¹. We then extracted the electrical conductivity of the culture medium using this cell constant: $\sigma_{CM} = 16.40$ mS.cm⁻¹, which is in accordance with reported values in the literature.²²⁷

To validate our experimental measurements, we calculated the impedance of the three electrical conductivity standards using the electrical conductivities as given by the manufacturer using our numerical **model 1**. An excitation signal of 25 mV was applied using the left CC electrode while the right CC electrode was declared as ground ($V = 0$). The two PU

electrodes were defined as having no current injection ($I_{PU} = 0$). The volume impedance between the two PU electrode was subsequently calculated as:

$$|Z| = \left| \frac{V_{PU1} - V_{PU2}}{I_{CC2}} \right| \quad (28)$$

with V_{PU1} the voltage at the left PU electrode, V_{PU2} the voltage at the right PU electrode and I_{CC2} the current at the grounded CC electrode. The sequence of COMSOL commands for this modelling is given as an ‘.mph’ file in **Appendix 3**.

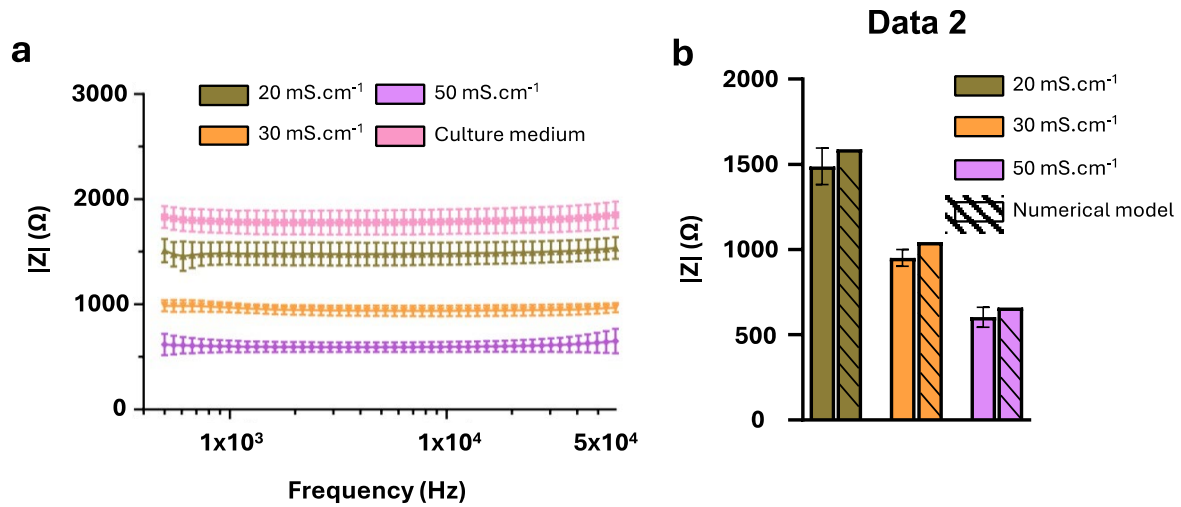


Figure 57. Impedance measurements on different solutions. (a) Impedance spectra of three electrical conductivity standard solutions (20, 30 and 50 mS.cm⁻¹) and culture medium (1X-DMEM). Impedance measurements were performed at a room temperature of 22.3°C (b) Comparison of average experimental and numerical model impedance values of the frequency spectra. The experimental measurements correspond to the average $|Z|$ over the frequency interval. Error bars denote SD. (Number of chips=3, number of channels=8).

We obtained values of 1589 Ω , 1043 Ω and 659 Ω , respectively (**Figure 57.b**). These values are higher than the experimental ones, with an average difference of 9 %. This can be explained by a difference in the electrical conductivity indicated by the manufacturer and the conductivities of the solutions at the moment of the experimental measurements. This difference in electrical conductivity could be explained by either a temperature change or a contamination of the conductivity standards by air molecules. Nonetheless, as the average difference was below 10 %, these measurements enabled us to validate the functionality of our impedance measurement system. As mentioned, our measurement frequency interval was set between 500 Hz and 60 kHz. Although we observed a constant impedance magnitude, we also observed variations at lower (<1kHz) and higher (>30 kHz) frequencies. Furthermore, previous studies highlighted that 10 kHz was the frequency with the strongest distinction between cell-

free and cell/gel mixture.¹⁹¹ Hence, we decided to perform measurements over the entire frequency spectra but for temporal studies, we focused our work at 10 kHz.

1.2.2. Characterisation of external factors' effect on the impedance measurement

After completing the design and fabrication, we validated the system's functionality by measuring the impedance of electrical conductivity standards and comparing the results to a numerical model. While the observed difference between experimental and simulated values allowed us to validate the functionality of the microelectrode system, it also prompted us to investigate whether system parameters influencing the electrical measurement could account for the discrepancy:

1. The geometry of the system, i.e. the culture chamber height and alignment with the electrodes,
2. The environment in which the system is placed in, i.e. the incubator (37 °C, 5 % CO₂).

1.2.2.1 Culture chamber height

As previously discussed, we set the targeted culture chamber height to 600 μm . However, due to observed fabrication variability, we wanted to quantify the impact of this fabrication error on the impedance measurement. We used our previously described numerical **model 1**, and obtained impedance values of 2618, 2095, 1745 and 1496 Ω for culture heights of 400, 500, 600 and 700 μm , respectively. (**Figure 58.b**). The sequence of COMSOL commands for this modelling is given as an '.mph' file in **Appendix 3**. This revealed an inverse relationship between culture chamber height and impedance, which can be explained by a reduction of the culture chamber cross section as we reduce its height, consequently increasing the volume's resistance. From these, we calculated the biosensor's theoretical sensitivity in impedance due to a change in culture chamber height: 3.74 $\Omega.\mu\text{m}^{-1}$.

To validate this result, we fabricated a 3D-printed cap that produced four distinct chamber heights (400 – 500 – 600 – 700 μm) on a single chip (**Figure 58.a**). We measured culture chamber heights of 439 ± 6 , 539 ± 13 , 671 ± 14 and 780 ± 4 μm . This enabled us to calculate the theoretical impedance values for each of the experimentally measured culture chamber height using the biosensor's sensitivity to height: 2476, 1949, 1516 and 1196 Ω . We then filled them with 60 μL of an electrical conductivity standard (20 mS.cm^{-1}) and measured the resulting impedance ($T=20.8$ °C). We obtained impedance magnitudes of 2722 ± 23 , 2085

± 65 , 1451 ± 6 and $1277 \pm 8 \Omega$, respectively, (**Figure 58.b**). These represented an average relative difference of 6.5 % between the numerical and experimental values, thus validating the height sensitivity value obtained ($3.74 \Omega \cdot \mu\text{m}^{-1}$).

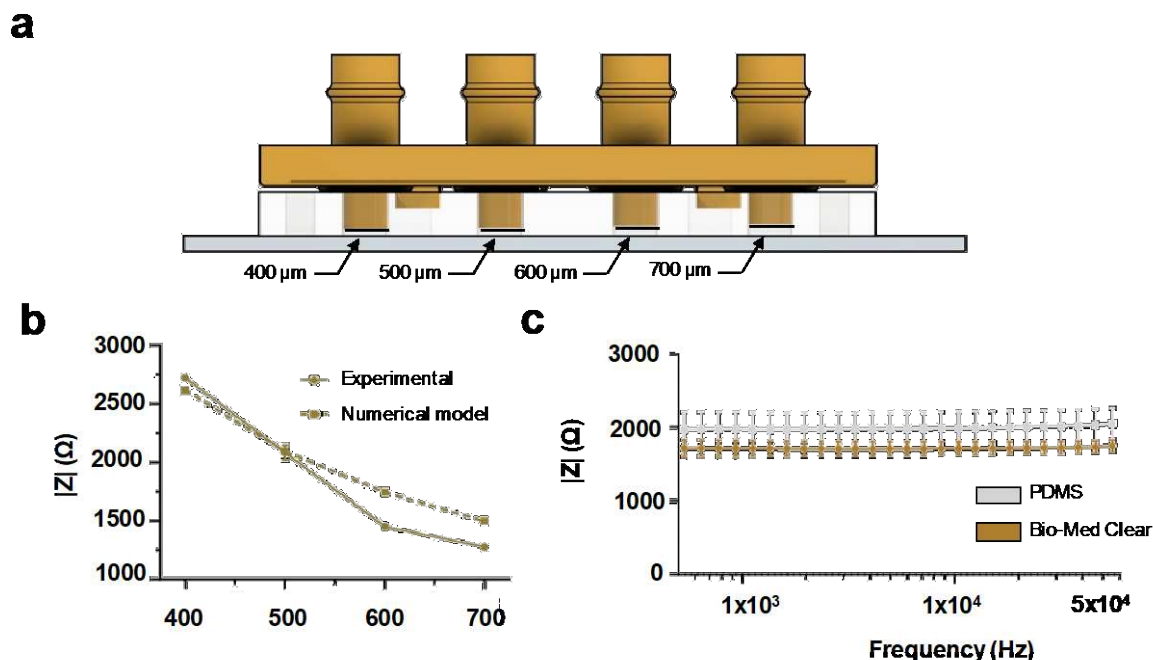


Figure 58. Effect of culture chamber height on the impedance measurement. (a) Schematic of experimental setup. One cap enables the production of four different culture chamber heights (400 – 500 – 600 – 700 μm) on one single microfluidic system. (b) Experimental and numerical modelling of $|Z|$ for different culture chamber heights at a frequency of 10 kHz ($N=2$, $n=4$). (c) Impedance magnitude of 1X PBS solution with a PDMS (grey) and 3D-printed Bio-Med Clear cap (brown) at a room temperature of 20.9°C (Number of chips=3, number of channels=6). Error bars denote SD.

As we previously saw, 3D-printed cap resulted in a more uniform chamber height than a PDMS one ($606.15 \pm 14.60 \mu\text{m}$ vs $646.15 \pm 66.40 \mu\text{m}$). We therefore wanted to evaluate the influence of changing the cap's material on the electrical measurement. We therefore closed our chip with both PDMS and 3D-printed caps, filled them with 60 μL of phosphate buffered saline solution (1X-PBS) and measured the resulting impedance spectra (**Figure 58.c**). We observed a decrease in average impedance magnitude over the frequency measurement interval as we changed the cap's material from PDMS to 3D printing (1983 to 1713 Ω). We did not expect this as 3D printing reduced the average culture chamber height compared to a PDMS cap. This could be explained by the flexible nature of PDMS bottom which may be stretched away when inserting the resin cap.

We observed a noticeable difference when comparing the standard deviations of both measurements: 281 Ω to 94 Ω. This improvement can be directly linked to the reduction in

height variability. Using the previously calculated height sensitivity of $3.74 \Omega \cdot \mu\text{m}^{-1}$, a standard deviation of $66.40 \mu\text{m}$ (PDMS cap) would lead to an expected impedance variation of approximately 248Ω , whereas the 3D-printed cap's reduced height variability ($14.60 \mu\text{m}$) corresponds to only 55Ω . These values are in close agreement with the experimentally measured impedance standard deviations.

To conclude, we characterised the influence of culture chamber height on impedance using both a numerical model and experimental measurements. We found that the biosensor's sensitivity to a change in culture chamber height was $3.74 \Omega \cdot \mu\text{m}^{-1}$. Furthermore, we validated the benefit of switching the cap's material from PDMS to 3D-printing resin with a reduction of the impedance's standard deviation from 281Ω to 94Ω . However, this change in cap material also reduced the impedance magnitude from 1983 to 1713Ω .

1.2.2.2. PDMS/electrode alignment

It is important to ensure that the sample volume probed with the electrical signal remains consistent between different microfluidic chips. This is influenced by the culture chamber height, but also by the microelectrode system's alignment with the PDMS bottom. Variations in both x- and y-directions will shift the position of the culture chamber relative to the electrodes, which can have an impact on the impedance measurement. It is therefore important to ensure a reproducible alignment during PDMS bonding, as well as investigating this alignment's influence on the impedance measurement.

Our first bonding method was to manually place the PDMS onto the glass substrate after O_2 plasma. Although we did not quantitatively measure the alignment error with the microelectrode system, we identified the need to improve this step to reduce a potential misalignment. We therefore developed a dedicated alignment tool for that purpose. The aligner holds both PDMS and glass substrate in place to ensure consistent positioning and is closed to bond both parts after an O_2 plasma (**Figure 59.a**). The alignment error can then be measured in both x- and y-directions using the dedicated ruler patterned as part of the electrode pattern (**Figure 59.b**).

The theoretical placement of the ruler was designed so that it was placed $500 \mu\text{m}$ from the PDMS wall in the y-direction, and $1950 \mu\text{m}$ inside the culture chamber from the inlet and outlet in the x-direction (**Figure 59.b**). We then measured the x-alignment error using the digits

of the dedicated ruler. For the y-alignment error, we imported a photograph in Microsoft PowerPoint and used a proportional scaling method. A reference rectangle was drawn between two ruler digits (corresponding to a known 100 μm distance) to establish a scale. We then drew a second rectangle between the ruler and the PDMS wall and measured its length to extract the distance of the ruler to the PDMS wall. For example, if the first rectangle had a length of 0.31 cm, and the second rectangle had a length of 2.45 cm, then the distance between the ruler and the PDMS wall was 790 μm .

Measurements were performed on 64 individual channels (8 individual microfluidic chips). The absolute x-alignment error was on average 308 with a maximum and minimum error of 830 and -630 μm , respectively. The absolute y-alignment error was on average 158 μm with a maximum and minimum error of 477 and -368 μm , respectively (**Figure 59.c**). This represented an average alignment error of 3 % in the x-direction and 8 % in the y-direction, respectively, relative to the theoretical alignment. Average measurements for all eight microfluidic chips are recapitulated in **Table 13**.

Table 13. Average measured alignment and resulting error between PDMS and electrodes in both x- and y-directions.

Microfluidic chip	x-distance (μm)	y-distance (μm)	x-alignment error (μm)	y-alignment error (μm)
1	1982 $\pm$ 368	563 $\pm$ 229	32 $\pm$ 368	63 $\pm$ 229
2	1962 $\pm$ 271	556 $\pm$ 78	12 $\pm$ 271	56 $\pm$ 78
3	2044 $\pm$ 503	559 $\pm$ 166	94 $\pm$ 503	59 $\pm$ 166
4	2054 $\pm$ 477	526 $\pm$ 242	104 $\pm$ 477	26 $\pm$ 242
5	2003 $\pm$ 212	559 $\pm$ 300	53 $\pm$ 212	59 $\pm$ 300
6	1973 $\pm$ 336	543 $\pm$ 53	23 $\pm$ 336	43 $\pm$ 63

To study the effects of these alignment errors, we incorporated the experimentally measured alignment errors in our previously described numerical **model 1**. The sequence of COMSOL commands for this modelling is given as an ‘.mph’ file in **Appendix 3**. The PDMS bottom alignment with the microelectrodes was set to vary based on the experimental measurements. The volume impedance between the two PU electrodes was subsequently calculated using **equation 28** and represented as a heatmap for each experimentally measured pair of x- and y-alignment errors represented as blue dots (**Figure 59.d**).

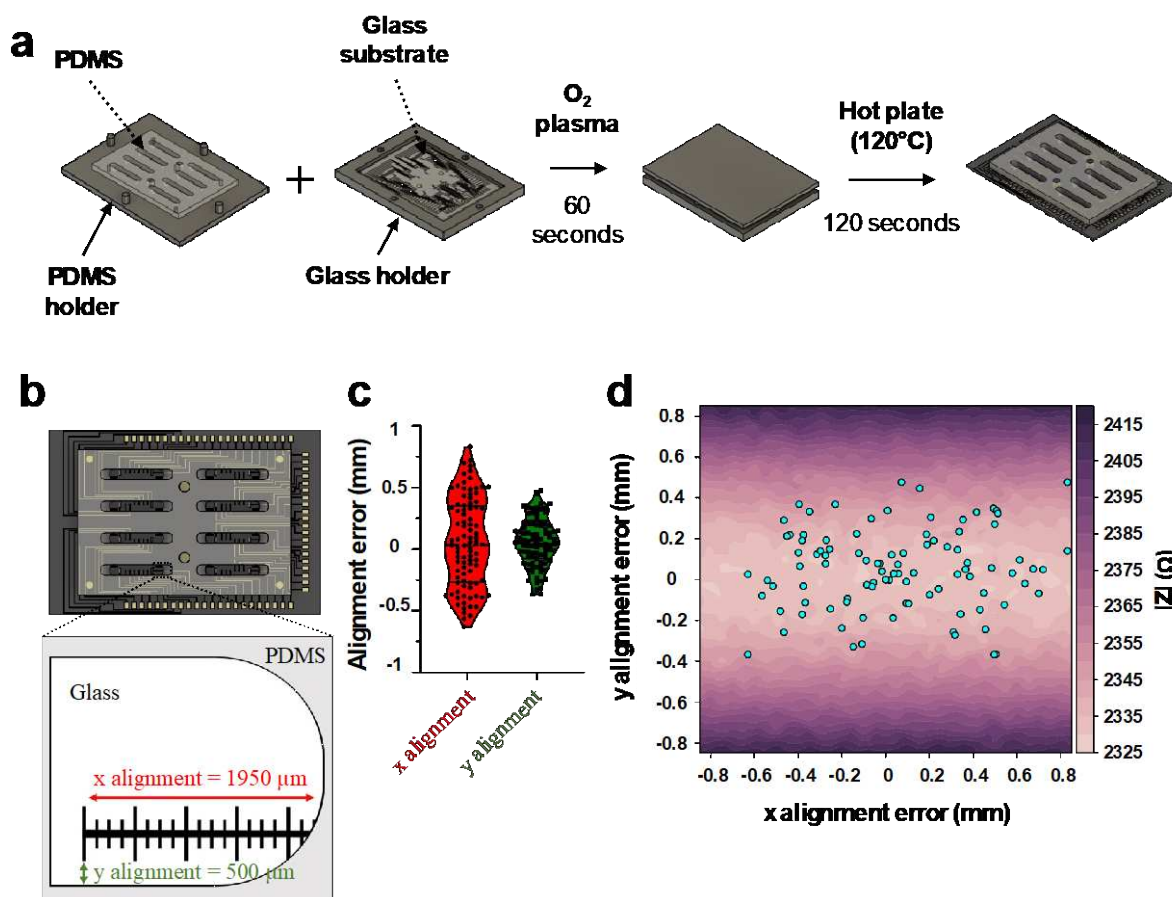


Figure 59. PDMS bonding alignment protocol and electrical characterisation. (a) A custom-made alignment system was fabricated by aluminium milling. Both glass substrate and PDMS bottom were placed in their respective holder and placed in a plasma cleaner to be exposed to an oxygen plasma for 60 s. The alignment was subsequently closed to bond the PDMS and the glass substrate. (b) An alignment measuring tool was incorporated in the electrode pattern which was used to measure the alignment error (c) in both x and y error based on its theoretical placement in the culture chamber (Number of chips=8, number of channels=64). (d) Heatmap of impedance spectra calculated in a numerical model using the experimentally measured x- and y-alignment errors, which are represented by the blue dots.

We observed that the x-alignment error did not result in a change in impedance. This is because x-direction misalignment shifts the PDMS relative to the electrodes without altering the probed volume, resulting in no change in impedance (Figure 60.a). The biosensor sensitivity to x-alignment error was calculated to be $0.0017\ \Omega\cdot\mu\text{m}^{-1}$. On the contrary, we observed that the y-alignment error did affect the impedance (Figure 60.b). We measured on average a $9\ \Omega$ increase per $50\ \mu\text{m}$ for a biosensor sensitivity to y-alignment error of $0.18\ \Omega\cdot\mu\text{m}^{-1}$. We observed a maximum impedance difference of $80\ \Omega$ due to y-alignment error. Furthermore, we observed that as the electrodes are moved away from the PDMS (decrease in y-alignment error), the impedance decreases. On the contrary, as the electrodes are moved closer to the PDMS (increase in y-alignment error), we observed that the measured impedance

increases. This can be explained by the fact that as we move in the y-direction, the PDMS channel wall is brought closer to the electrodes. As PDMS is an insulator, the more it is probed by the electrical measurement, the higher the measured impedance is. However, the maximum difference between two individual culture chambers is less than $100\ \Omega$ ($\sim 1.5\%$).

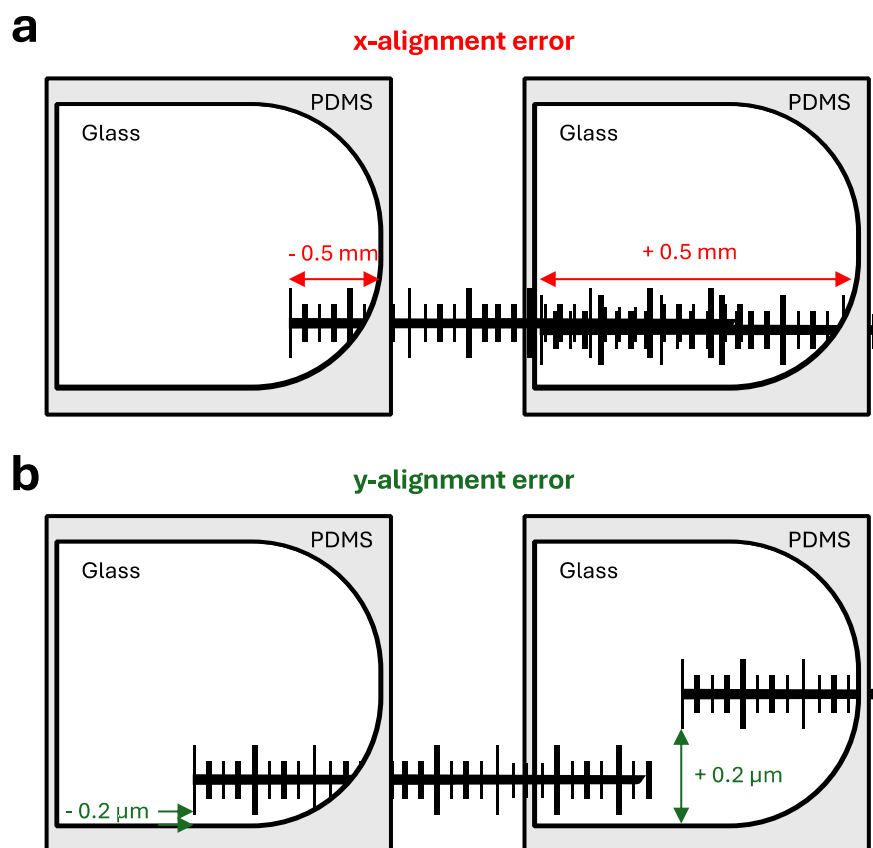


Figure 60. Visual representation of misalignment. (a) Negative and positive alignment error in x-direction. (b) Negative and positive alignment in y-direction.

1.2.2.3. Temperature change

We investigated the effect of the temperature change on the impedance measurement. As the temperature of an ionic solution (here the culture medium) increases, so does its electrical conductivity. This is because the viscosity of the solution decreases, in turn increasing the ion mobility²²⁸. This will be directly translated into a decrease in impedance and needs to be characterised in order to not influence the measurement's interpretation. As for the effect of temperature on the relative permittivity of electrolyte solutions, it has been found to be negligible.²²⁹

Our first step was to measure the conductivity of 1X-DMEM at different temperatures to extract its temperature coefficient of conductivity α_σ [$^{\circ}\text{C}^{-1}$]. This coefficient is the measure

of how much the material's electrical conductivity varies with temperature.²³⁰ It can be calculated after measurement of electrical conductivities at different temperature points with the formula:

$$\alpha_{\sigma} = \frac{1}{\sigma} \frac{d\sigma}{dT} = \frac{\sigma_{T_2} - \sigma_{T_1}}{(T_2 - T_1) * \sigma_{T_1}} \quad (29)$$

with T_1 and T_2 the two temperature measuring points ($^{\circ}\text{C}$) and σ_{T_1} and σ_{T_2} the respective measured electrical conductivities (S.m^{-1}).

We measured the electrical conductivities of 1X-DMEM at four temperature points (25, 30, 35 and 40 $^{\circ}\text{C}$) using a bain-Marie and calculated its thermal coefficient of conductivity. We also performed these measurements on 1.413 and 12.88 mS.cm^{-1} conductivity standards, which have known conductivities at different temperatures, to validate our measurements. With this, we were able to calculate an $\alpha_{\sigma} = 0.0179$ for 1X-DMEM (**Figure 61.a**). Measurements are recapitulated in **Table 14**.

Table 14. Conductivity measurements at different temperature points.

Solution	Conductivity (mS.cm^{-1})				α_{σ} ($^{\circ}\text{C}^{-1}$)
	25 $^{\circ}\text{C}$	30 $^{\circ}\text{C}$	35 $^{\circ}\text{C}$	40 $^{\circ}\text{C}$	
1X-DMEM	15.17	16.859	18.128	19.850	0.0179
12.88 mS.cm^{-1}	12.591	13.731	14.827	15.995	0.0166
1.413 mS.cm^{-1}	1.402	1.509	1.647	1.789	0.0169

We then wanted to model the influence of temperature change on the impedance measurement using this α_{σ} value. For that, we had to derive the temperature coefficient of resistivity α_{ρ} . As $\sigma = \frac{1}{\rho}$, we can derive $\alpha_{\rho} = -\alpha_{\sigma}$ from **equation 29**. In our case, this gave us $\alpha_{\rho} = -0.0179$ $^{\circ}\text{C}^{-1}$. We then plugged α_{ρ} in the numerical model using the linearised resistivity equation (dynamical model of conductivity changes with respect to temperature) using the electric currents interface of the AC/DC module:

$$\sigma = \frac{1}{\rho_{ref} * (1 + \alpha_{\rho} * (T - T_{ref}))} \quad (30)$$

with ρ_{ref} ($\Omega\cdot\text{m}$) the resistivity at the reference temperature T_{ref} ($^{\circ}\text{C}$) and α_{ρ} the temperature coefficient of resistivity. As $\sigma = \frac{1}{\rho}$, we can derive $\alpha_{\rho} = -\alpha_{\sigma}$ from **equation 29**. In our case, this gave us $\alpha_{\rho} = -0.0179\text{ }^{\circ}\text{C}^{-1}$.

To model the change of temperature of the culture chamber, we utilised the ‘Heat transfer in solids (ht)’ interface from the Heat Transfer module of COMSOL Multiphysics under stationary conditions:

$$\rho C_p \vec{u} \cdot \nabla T + \nabla \cdot \vec{q} = Q + Q_{\text{ted}} \quad (31)$$

and

$$\vec{q} = -k \nabla T \quad (32)$$

with ρ the material density ($\text{kg}\cdot\text{m}^{-3}$), C_p the specific heat capacity at constant pressure ($\text{J}\cdot\text{kg}^{-1}\cdot\text{K}^{-1}$), $\vec{u}$ the velocity field ($\text{m}\cdot\text{s}^{-1}$), T the temperature (K), $\vec{q}$ the conductive heat flux vector ($\text{W}\cdot\text{m}^{-2}$), k the thermal conductivity ($\text{W}\cdot\text{m}^{-1}\cdot\text{K}^{-1}$), Q the volumetric heat source ($\text{W}\cdot\text{m}^{-3}$), and Q_{ted} the thermoelectric damping term ($\text{W}\cdot\text{m}^{-3}$). The initial temperature of the culture chamber was set to 293.15 K (20°C) while the outside temperature was set to 310.15 K (37°C) to represent the incubator’s temperature. The heat flux was then defined as a convective heat flux:

$$q_0 = h(T_{\text{ex}} - T) \quad (33)$$

with h the heat transfer coefficient of the material. The sequence of COMSOL commands for this modelling is given as an ‘.mph’ file in **Appendix 3**.

We extracted the resulting impedance as we did in our previously described numerical **model 1**, with the volume impedance between the two PU electrodes being calculated using **equation 28**. The parameters used in the simulation are recapitulated in

Table 15.

As for the experimental measurements, we filled the culture chambers with 60 μL of 1X-DMEM and placed the microfluidic chip in an incubator (37°C , 5% CO_2). We then measured the impedance over a 2 h period with measurement intervals of 3 min and plotted the impedance spectra at a frequency of 10 kHz to compare it to the numerical simulation (**Figure 61.b**).

We observed similar trends in the impedance decreases in both numerical and experimental measurements. For both, the impedance decreased until it reached a plateau after 60 min. This indicated that the first 60 min of measurement after incubation should not be utilised for interpretation as they are still variable. However, we observed a difference in the impedance magnitude, with the numerical model impedance being higher than the experimentally measured impedance.

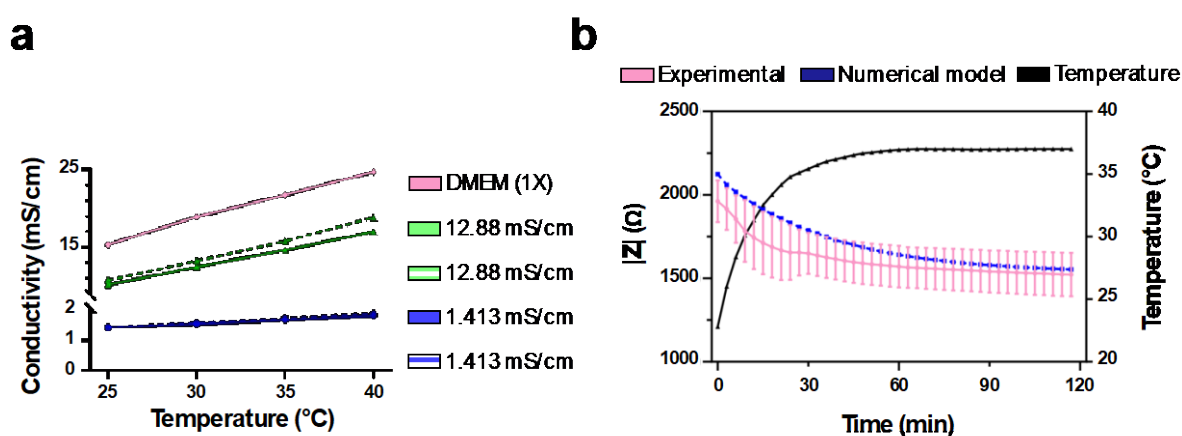


Figure 61. Characterisation of the effects of temperature on the impedance measurements. (a) Conductivity measurements of 1X-DMEM (pink), 12.88 $\text{mS}\cdot\text{cm}^{-1}$ conductivity standard (green) and 1.413 $\text{mS}\cdot\text{cm}^{-1}$ conductivity standard (blue). Dotted lines represent the theoretical change in electrical conductivity values as given by the manufacturer ($n=3$). **(b)** Impedance measurement as the temperature changes (black) of 1X-DMEM (pink) in the chip once placed in the incubator (full line) compared to numerical model (blue dotted line) (Number of chips=2, number of channels=8). Impedance measurement shown was taken at frequency = 10 kHz. Error bars denote SD.

Table 15. Parameters used for numerical simulation of temperature dependence of impedance.

Parameter		Symbol	Unit	Value
Reference resistivity		ρ_0	$\Omega.m$	0.659
Reference temperature		T_{ref}	K	298.15
Coefficient of resistivity		α_ρ	K^{-1}	-0.0179
Thermal conductivity	PDMS	k_{PDMS}	$W.(m.K)^{-1}$	0.16
	Culture medium	k_{CM}	$W.(m.K)^{-1}$	0.6
	Glass	k_{glass}	$W.(m.K)^{-1}$	1.4
Density	PDMS	ρ_{PDMS}	$kg.m^{-3}$	970
	Culture medium	ρ_{CM}	$kg.m^{-3}$	1000
	Glass	ρ_{glass}	$kg.m^{-3}$	2210
Heat capacity at constant pressure	PDMS	$C_{p,PDMS}$	$J.(kg.K)^{-1}$	1460
	Culture medium	$C_{p,CM}$	$J.(kg.K)^{-1}$	4184
	Glass	$C_{p,glass}$	$J.(kg.K)^{-1}$	730
Heat transfer coefficient	PDMS	h_{PDMS}	$W.(m^2.K)^{-1}$	0
	Culture medium	h_{CM}	$W.(m^2.K)^{-1}$	4
	Glass	h_{glass}	$W.(m^2.K)^{-1}$	50

We investigated the influence of parameters such as culture chamber height and electrode alignment on the impedance measurement and calculated the sensitivity of the biosensor with respect to these parameters. This enabled us to calculate the expected error on the impedance measurements due to the fabrication errors we had previously characterised (

Table 16). This highlighted that the culture chamber was the critical parameter to control to reduce the variability of the impedance measurement. We observed that using a 3D-printed cap instead of PDMS reduced the standard deviation of the impedance measurement from 281 Ω to 94 Ω .

Table 16. Influence of geometrical parameters on the impedance measurement.

Parameter	Expected variations	Sensitivity	Expected error on $ Z $ measurement (Ω)
Temperature	16°C	48.8 $\Omega \cdot ^\circ\text{C}^{-1}$	-750
Culture chamber height	15 μm	3.74 $\Omega \cdot \mu\text{m}^{-1}$	± 56
y-alignment	158 μm	0.0017 $\Omega \cdot \mu\text{m}^{-1}$	± 0.1
x-alignment	308 μm	0.18 $\Omega \cdot \mu\text{m}^{-1}$	± 7

We also investigated influence of temperature change when placing the microfluidic chip in the incubator and we concluded that the first 60 min of measurement after incubation should not be considered for interpretation as it takes 60 min for the impedance measurement to stabilise.

1.3. Solid tumour bioimpedance characterisation

1.3.1. Dynamic 2D *in vitro* solid tumour model

We first performed bioimpedance measurements on our 2D dynamic monoculture model using two different culture chamber heights (600 and 2400 μm). As we had previously observed (**Figure 41**), fluorescence microscopy revealed that HPAC-eGFP cells cultured in the 2400 μm high culture chamber covered more of the culture chamber surface than in the 600 μm high chamber (**Figure 63.a**) which we had attributed to the difference in flow velocities between both culture chamber geometries.

As previously, 100 μL of a solution with a concentration of HPAC-eGFP cells of 5×10^5 cells.mL⁻¹ were seeded and allowed to adhere to the glass substrate for 24 h before initiating the dynamic perfusion (3.53 $\mu\text{L} \cdot \text{min}^{-1}$). During this time, we observed that the measured impedance was higher in the 600 μm high culture chamber than in the 2400 μm high culture chamber, with average values of 1267 Ω and 548 Ω , respectively (**Figure 62**). This was consistent with our previous findings on the influence of the culture chamber height on the measured impedance (**Figure 58**). However, we did not observe a change in impedance after

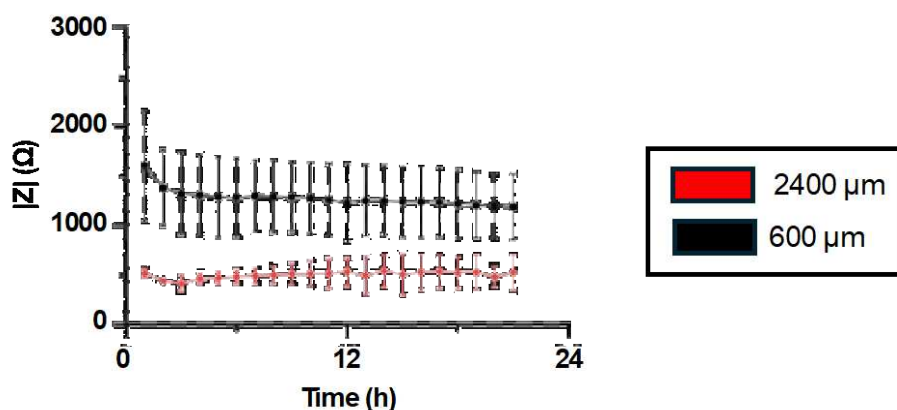


Figure 62. Impedance measurements after 2D cell culture seeding in the microfluidic system before activation of dynamic perfusion. Measurements were taken at a frequency of 10 kHz. (Number of chips=1, number of channels=2). Error bars denote SD.

24h in either culture chamber as there were too few cells to cover the electrodes and change the measured impedance.

After the initial 24 h post cell seeding, we applied a $3.53 \mu\text{L} \cdot \text{min}^{-1}$ flow rate and waited 24 h before measuring the impedance spectra to let the electrical measurement stabilise. We then measured the impedance for both conditions over the following 6 days of culture at a frequency of 10 kHz and observed two distinct periods using the outer PU electrodes (**Figure 63.b**). The first period (24-72 h post perfusion) was characterised with a decrease in the impedance for the 600 μm high culture chamber and a stagnation of the impedance for the 2400 μm high culture chamber: $1814 \pm 1026 \Omega$ to $879 \pm 408 \Omega$ and $1169 \pm 405 \Omega$ to $1261 \pm 666 \Omega$, respectively. The second period we observed was characterised by a steady increase of the measured impedance in both culture chamber heights. The 600 μm culture chamber showed an increase in impedance to 2968 Ω after 96 h, 6856 Ω after 120 h, 8371 Ω after 144 h and 9453 Ω after 154 h, while the 2400 μm culture chamber showed an increase to 2716 Ω at 96 h, 4125 Ω after 120 h, 5288 Ω after 144 h and 5661 Ω after 154 h (**Figure 63.c**). We observed that after 96 h, the measured impedance was higher in the 600 μm compared to the 2400 μm .

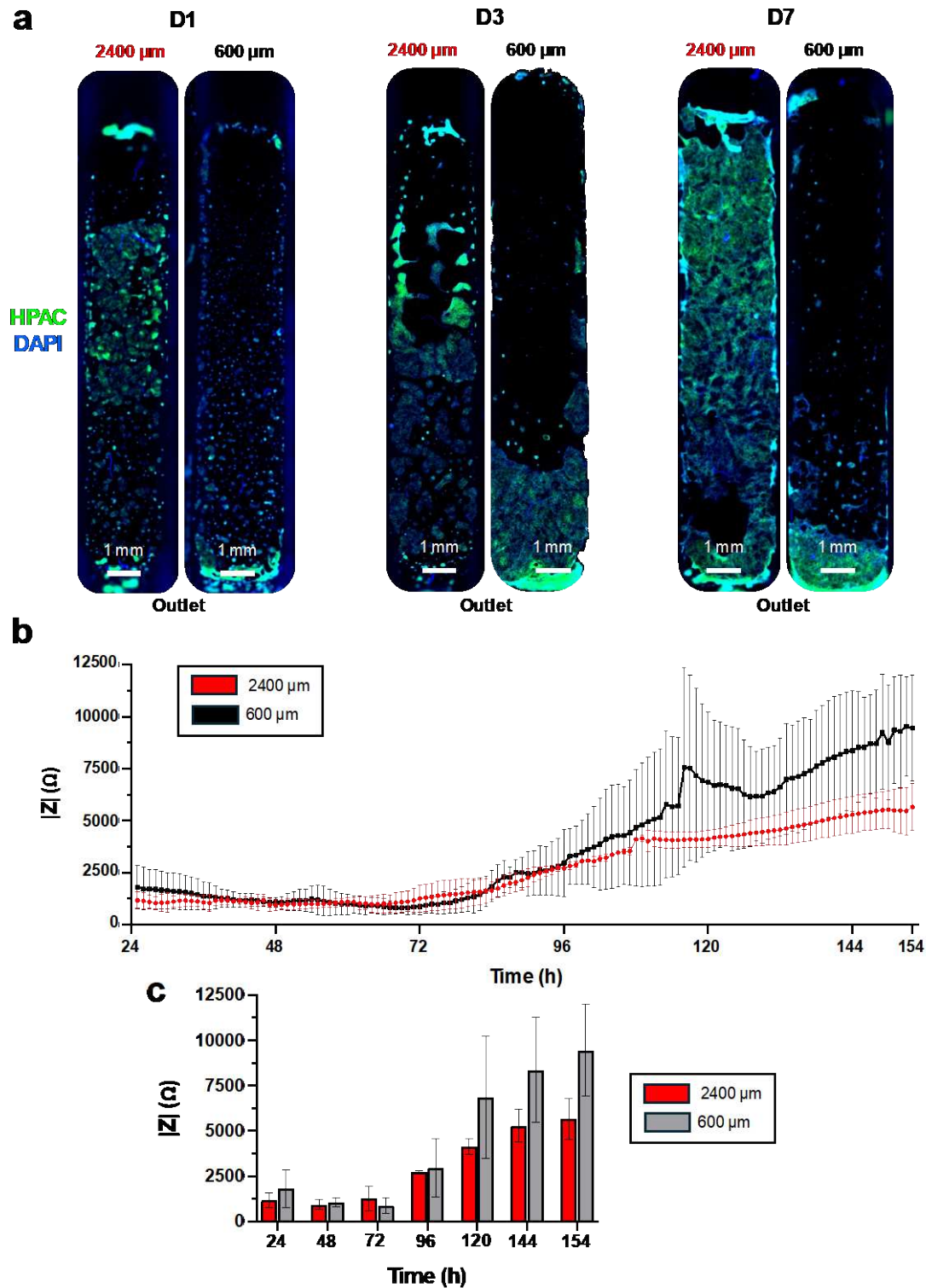


Figure 63. Bioimpedance measurements on 2D HPAC-eGFP culture in the microfluidic system with two channel heights. (a) Full channel imaging of HPAC-eGFP cells (green) grown in a 2D monoculture for seven days with the two culture chamber heights (600 and 2400 μm) after DAPI staining (blue). (b) Time traces of impedance measurement of 2D monolayer using outer PU electrodes at a frequency of 10 kHz. Measurements started 24 h after the activation of the dynamic perfusion to let the electrical measurement stabilise (Number of chips=1, number of channels=2). (c) Histogram of both culture chamber heights impedance at different time points for a frequency of 10 kHz (Number of chips=1, number of channels=2). Error bars denote SD.

We measured the electrical conductivity and pH of the culture medium in a static 2D HPAC-eGFP culture. We seeded 10 mL of 1X-DMEM with 1.4×10^4 cells.mL⁻¹ of HPAC-eGFP cells in ventilated T25 culture flasks. This cell number was chosen to keep the same cell number to culture medium ratio used in the 2D dynamic culture (1.4×10^4 cells.mL⁻¹ of culture medium). We filled a culture flask with only culture medium as a control. Electrical conductivity and pH were measured on days 1, 3, 7, and 9, with no medium changes. For pH, we observed an increase for both conditions after 24 h (**Figure 64.a**). We then observed a decrease in the presence of HPAC-eGFP cells but not for culture medium alone. For the electrical conductivity, we observed a sharp increase after 24 h in both conditions which we attributed to the temperature change (**Figure 64.b**). Using the conductivity values before and 24 h after placement in the incubator and using the thermal coefficient of conductivity α_σ ($=0.0179$), we calculated that the conductivity change represented a temperature change of 16.95°C , which is consistent with the temperature difference between room temperature and an incubator. We then observed a steady increase of the electrical conductivity of both conditions over time.

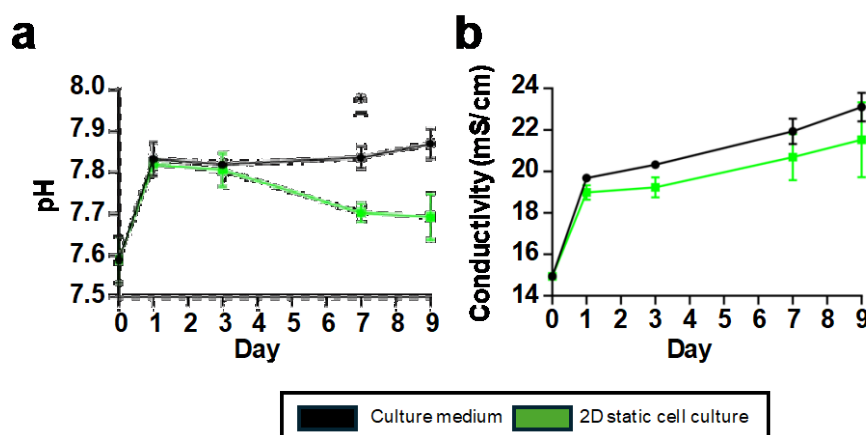


Figure 64. pH and electrical conductivity of HPAC-eGFP cell conditioned culture medium. (a) pH measurement of 1X-DMEM with and without cells over time (n=3). (b) Electrical conductivity measurements of culture medium with and without cells over time (n=3). Error bars denote SD.

To leverage the spatial resolution of our microelectrode system, we analysed the impedance spectra from the different PU electrode pairs that segment the culture chamber into five sub-volumes as presented in **Figure 47**: left, left middle, middle, right middle and right (**Figure 65.a**). We then measured the impedance spectra over time for each sub-volume to extract their respective impedance traces over the culture period (**Figure 65.b, c, d, e and f**). For both culture chamber heights (2400 and 600 μm), we observed that the PU electrode pair

that measured the highest increase in impedance compared to the others were the left and left middle PU electrode pairs. Both these electrode pairs are the PU electrode pairs closest to the culture chamber's outlet.

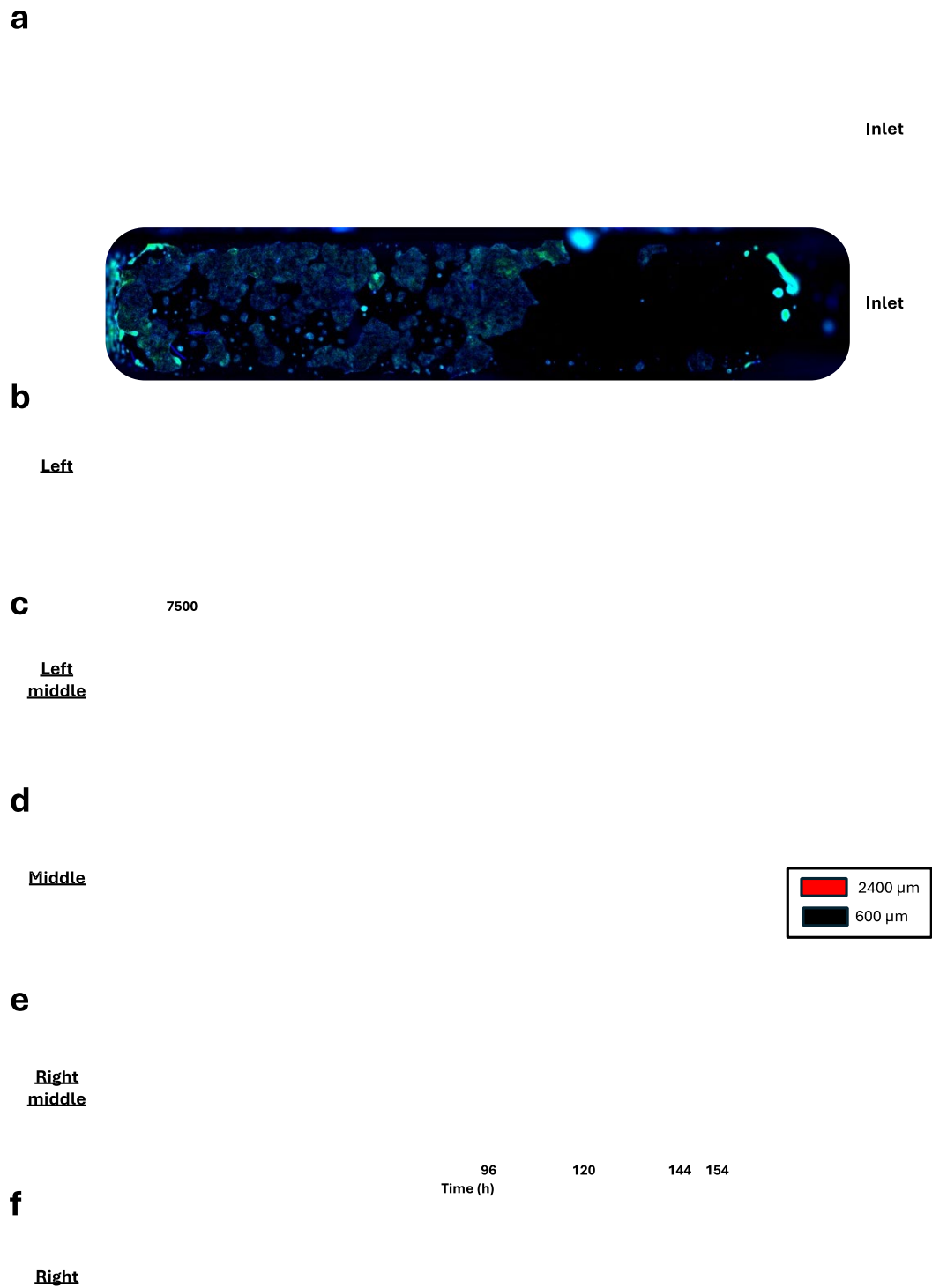


Figure 65. Impedance measurements on 2D HPAC-eGFP dynamic monoculture over time based on PU electrode pairs. (a) Culture chamber separation based on electrode pair shown on fluorescence imaging at day three of the culture. Resulting time traces of impedance measurement using (b) left, (c) left middle, (d) middle, (e) right middle and (f) right PU electrode pairs at a frequency of 10 kHz (Number of chips=1, number of channels=2). Error bars denote SD.

1.3.2. Impedance measurements on 3D *in vitro* PDAC model

1.3.2.1. Collagen 1 ECM electrical characterisation

After performing impedance measurements on a 2D dynamic PDAC cell culture, we aimed to use the microelectrode system on a 3D PDAC *in vitro* model. The first step was to perform measurements on a static 3D model. As previously mentioned, our 3D *in vitro* PDAC model utilises a collagen 1 based hydrogel as an ECM. Our first characterisation compared a 75% (v/v) collagen 1/1X-DMEM solution to 1X-DMEM alone. We chose this ratio as it is the one commonly used in our team for 3D cell culture. 60 μ L of each solution was loaded into the culture chambers, and the chip was placed in an incubator. After 2 h, we observed a significant difference between both solutions, with the collagen 1/1X-DMEM solution having a higher average impedance than 1X-DMEM alone over the frequency interval: $4877 \pm 72 \Omega$ vs $1710 \pm 174 \Omega$, respectively (**Figure 66.a**). This was expected since hydrogel solidification restricts ion mobility compared to electrolytic solutions such as culture medium.¹⁷³

As this solidification of the gel occurs at 37°C inside an incubator, we also characterised the electrical response of both solutions over time when placed in an incubator. We therefore measured their impedance spectra over a 2 h period with measurement intervals of 3 min at a frequency of 10 kHz. We observed a similar difference in impedance values, with the collagen 1/1X-DMEM solution having a higher impedance than culture medium alone over the experiment duration (**Figure 66.b**). Interestingly, both solutions' impedances experienced a similar impedance drop before stabilising 60 min after incubation: 6685 ± 598 to 4836 ± 293 for collagen 1/1X-DMEM solution, and 1999 ± 126 to 1329 ± 94 for 1X-DMEM, which represent a 32 % and 40 % decrease in impedance, respectively. This aligned with our previous characterisation of the effects of temperature change on impedance.

As we wanted to be able to use our microelectrode systems multiple times, we investigated electrode reusability by measuring the impedance of a conductivity standard (20 mS.cm⁻¹) before and after collagen 1 seeding on three separate microfluidic chips (n=12). We therefore developed an electrode cleaning protocol to remove collagen 1 from the culture chamber. This protocol consisted of successive washing of 1X-PBS, 0.25 % trypsin solution and a 0.1% (w/v) acetic acid buffer with a pH of 3.4 to dissolve collagen 1. We found no significant differences between average impedance magnitude before and after cleaning over the frequency measurement interval: $1539 \pm 61 \Omega$ before collagen 1 seeding and cleaning,

while it was $1560 \pm 174 \Omega$ after cleaning (**Figure 66.c**). This validated the cleaning protocol and reusability of the electrode system.

We then wanted to optimise the seeding volume of the collagen 1/1X-DMEM solution as well as the collagen 1 concentration to minimise the impedance measurement variability (or standard deviation). We first investigated the influence of the seeding volume on the measurement by loading the culture chambers with varying volumes of a 75% (v/v) collagen 1/1X-DMEM solution (60, 70, 80 and 100 μL) and measured their respective impedance spectra (**Figure 66.d**). We observed a linear increase in impedance as the loaded volume increases with average values of $3509 \pm 140 \Omega$, $4327 \pm 243 \Omega$, $4821 \pm 337 \Omega$ and $5602 \pm 113 \Omega$, respectively (**Figure 66.e**).

Our second step was therefore to characterise the influence of collagen 1 concentration on the impedance measurement by measuring the impedance spectra of four solutions with different % (v/v) of collagen 1: 25 %, 50 %, 75 % and 100 %. We observed an exponential increase in impedance magnitude as the concentration in collagen 1 increases, with values of $2117 \pm 158 \Omega$, $2699 \pm 54 \Omega$, $4301 \pm 27 \Omega$ and $23,951 \pm 348 \Omega$, respectively (**Figure 66.f and g**).

Overall, we observed that both 50% and 75% collagen 1 concentrations resulted in lower impedance variability. Similarly, we observed that we obtained a lower impedance variability when seeding a smaller volume into the culture chamber. Hence, we chose to use a final volume of 50 μL of 50 % collagen 1/1X-DMEM (v/v) for cell culture.

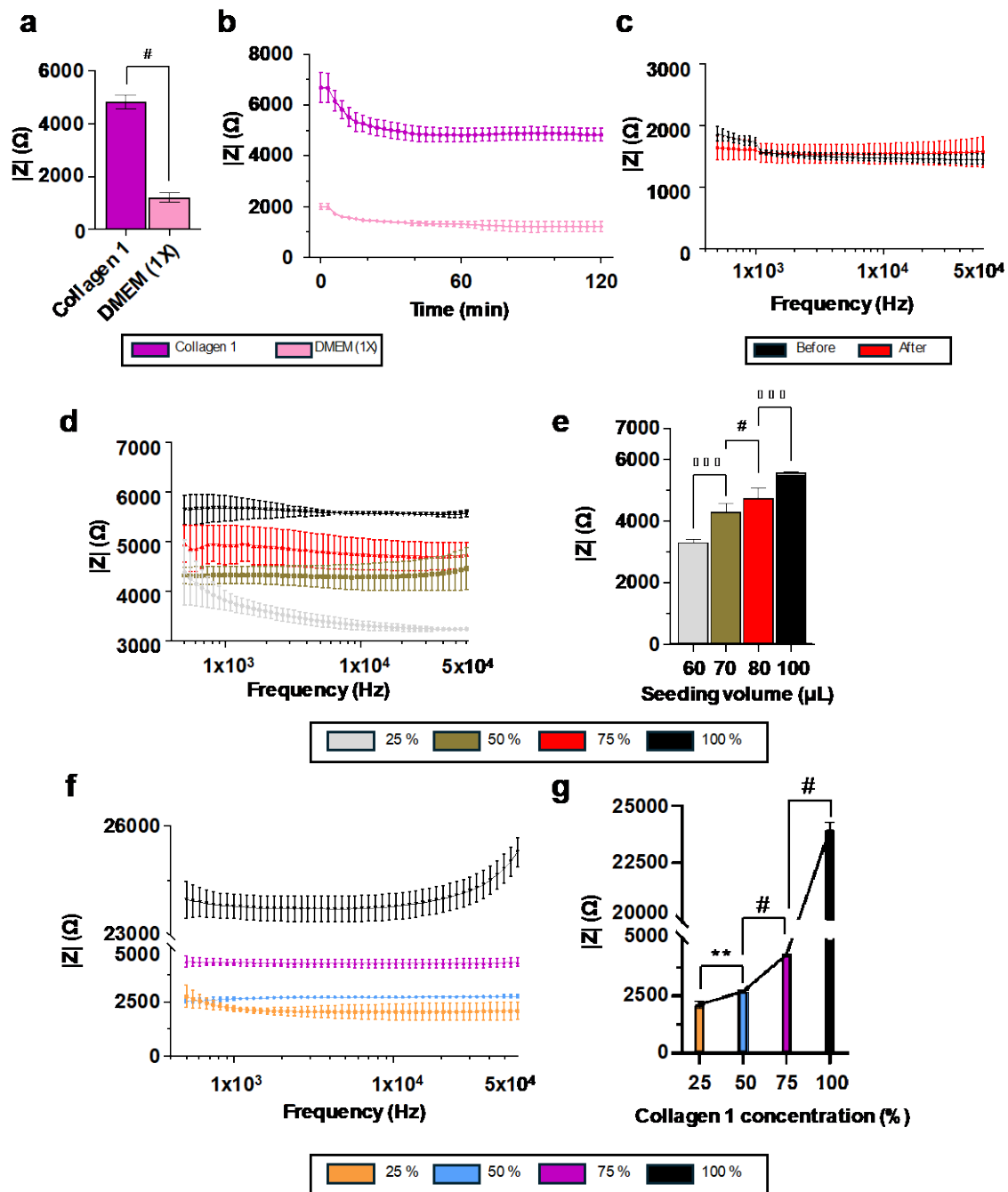


Figure 66. Collagen 1 hydrogel electrical characterisation. (a) Comparison of the average impedance magnitude over the frequency interval between 75% collagen 1/1X-DMEM solution (v/v) and culture medium 2 h after incubation (Number of chips=3, number of channels=12). (b) Temporal comparison of 75% collagen 1/1X-DMEM solution (v/v) and 1X-DMEM after placement in incubator at a frequency of 10 kHz (Number of chips=3, number of channels=12). (c) Comparison of 20 mS.cm⁻¹ conductivity standard impedance before and after filling the culture chambers with collagen and performing electrode cleaning (Number of chips=3, number of channels=12). (d) Impedance spectrum of different volumes of a 75% collagen 1/1X-DMEM solution (v/v) (Number of chips=3, number of channels=6). (e) Extraction of mathematical relationship between seeding volume and impedance at 10 kHz ((Number of chips=3, number of channels=6). (f) Impedance spectrum of solutions with different collagen 1 concentrations (Number of chips=3, number of channels=6). (g) Extraction of mathematical relationship between collagen 1 concentration and impedance at 10 kHz (Number of chips=3, number of channels=6). Error bars denote SD. ** for $p < 0.01$, *** for $p < 0.001$ and '#' for $p < 0.0001$.

1.3.2.2. Impedance measurements on static 3D in vitro PDAC model

After choosing a final seeding volume of 50 μL and a concentration of 50 % collagen 1/1X-DMEM (v/v), we seeded PDAC cells in our hydrogel and performed impedance measurements in static conditions. 50 μL of 50% (v/v) collagen 1/1X-DMEM solution with a cell concentration of 1×10^6 HPAC-eGFP cells were seeded in the culture chamber and compared to cell-free collagen 1/1X-DMEM solution (**Figure 67.a**). We performed bioimpedance measurements 2 and 24 h after incubation. We first compared the impedance of the cell-free gel at 2 and 24 h and found no statistically significant differences, with average values over the frequency spectrum of $3411 \pm 891 \Omega$ and $3103 \pm 993 \Omega$, respectively (**Figure 67.b**).

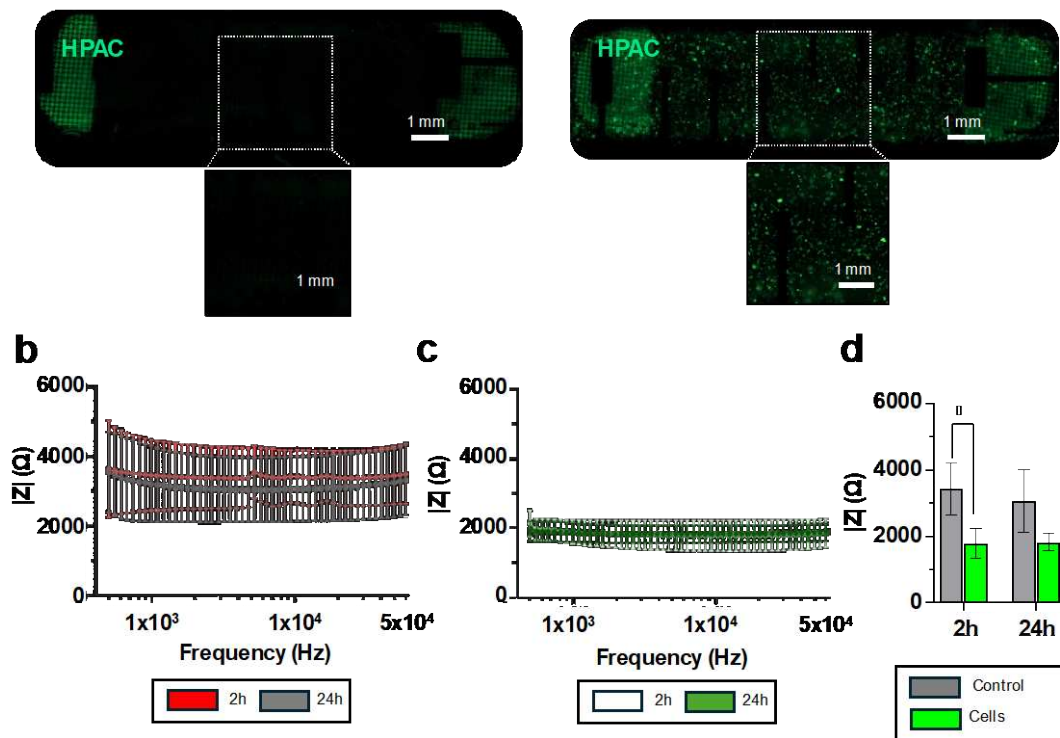


Figure 67. Sensing of 50 % collagen/1X-DMEM (v/v) solution containing HPAC eGFP cells. (a) Fluorescence microscopy of both cell-free collagen and HPAC-eGFP/collagen mixture 2h after seeding and incubation. **(b)** Impedance measurements of cell-free collagen 1 hydrogel after 2 and 24 h incubation (Number of chips=3, number of channels=6). **(c)** Impedance measurements of HPAC-eGFP cells seeded in collagen 1/1X-DMEM solution after 2 and 24h incubation (Number of chips=3, number of channels=6). **(d)** Histogram of impedance magnitude for both conditions at frequency = 10 kHz (Number of chips=3, number of channels=6). Error bars denote SD. * for $p < 0.05$.

Similarly, the cell/gel mixture impedances did not statistically differ between 2 and 24 h, with values of $1806 \pm 418 \Omega$ and $1851 \pm 259 \Omega$, respectively (**Figure 67.c**). However, we

found a significant difference between cell-free collagen 1 and cell/gel mixture 2 h after cell seeding at a frequency of 10 kHz: $3430 \pm 782 \Omega$ and $1776 \pm 455 \Omega$, respectively (**Figure 67.d**). We observed a similar difference in impedance magnitude after 24 h with values of $3066 \pm 937 \Omega$ and $1825 \pm 249 \Omega$, respectively.

1.3.2.3. Impedance measurements on dynamic 3D in vitro PDAC model

50 μL of 50% (v/v) collagen 1/1X-DMEM solution with a cell concentration of 1×10^6 HPAC-eGFP cells were seeded in the culture chamber and cultured for 7 days. After 7 days of culture, 1 mL of a 5 % (v/v) 100X-Triton/1X-DMEM (5X-Triton) solution was perfused to permeabilise and kill the cells, mimicking the effect of a chemotherapy on cancer cells. The culture was then extended by two additional days to measure the effects of the 5X-Triton on the cells via bioimpedance measurements. Prior to 5X-Triton perfusion, we measured its conductivity to be 14.00 mS.cm^{-1} at a room temperature of 21°C . This corresponds to a conductivity of 15.00 mS.cm^{-1} which is similar to that of 1X-DMEM alone.

We observed the formation and growth of HPAC-eGFP cells over the first 7 days of culture (**Figure 68.a and b**). Furthermore, we observed a high rate of cell death after the perfusion of 5X-Triton. Indeed, as seen in the fluorescence microscopy (both full channel and zoom), there were no cells left after two days of Triton 5X perfusion.

However, when we investigated the impedance spectra of both conditions using the outer PU electrodes, we did not see any changes over time (**Figure 68.c**). At first, we observed a difference in impedance magnitude between cell/gel mixture and cell-free gel, with values of $618 \pm 601 \Omega$ and $1181 \pm 504 \Omega$, respectively at a frequency of 10 kHz and 2 h after the start of the dynamic perfusion.

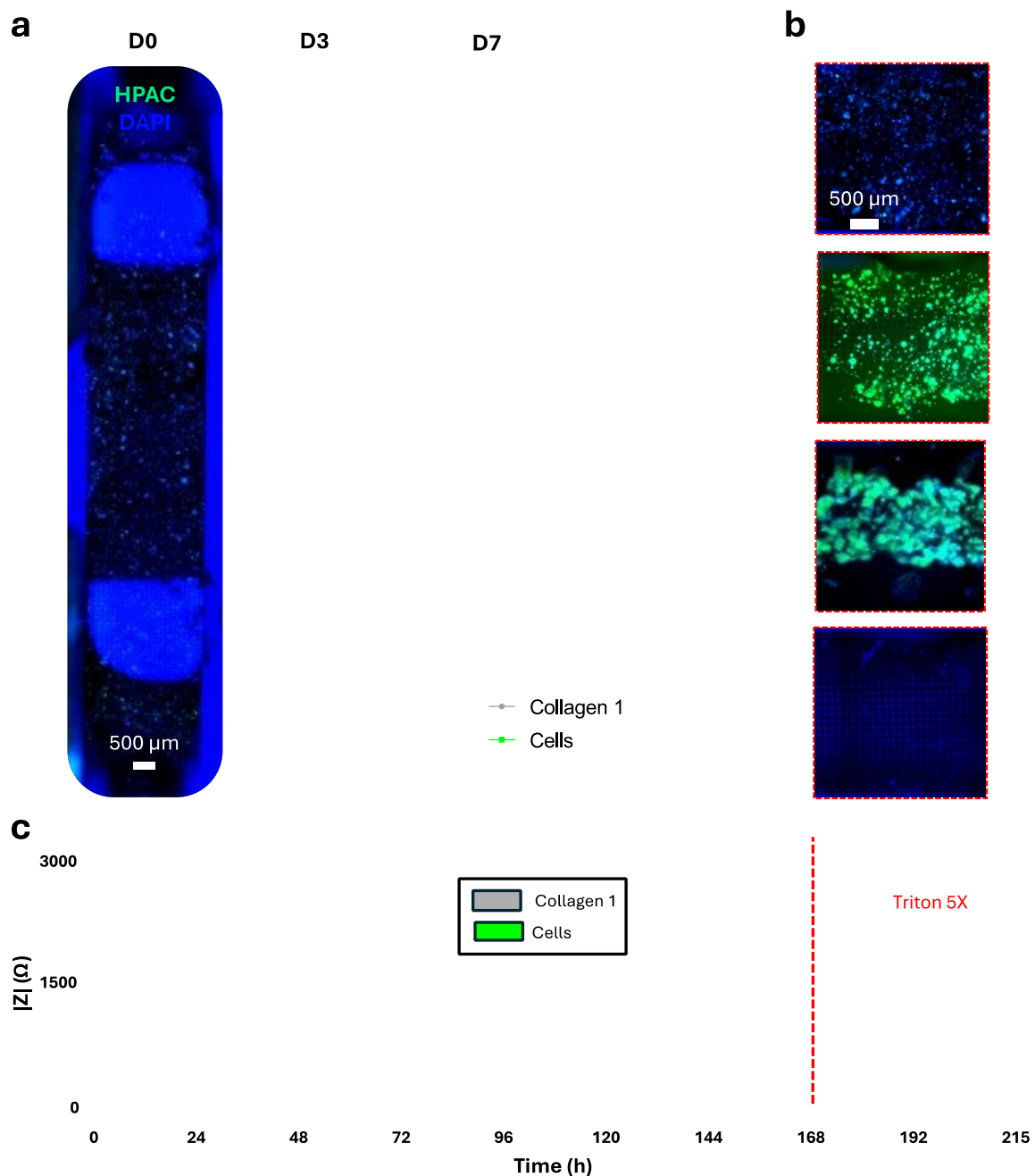


Figure 68. Bioimpedance measurements on 3D HPAC-eGFP culture in the microfluidic system. (a) Full channel imaging of HPAC-eGFP cells grown in a 3D collagen 1/DMEM for seven days and exposed to 5% (v/v) 100X-Triton for two days at $z = 400 \mu\text{m}$. (b) Channel zooms reveal a growth in HPAC-eGFP spheroids as well as the 3D nature of the culture for the whole duration of the culture. (c) Time traces of impedance measurement of 3D culture using outer PU electrodes at a frequency of 10 kHz (Number of chips=1, number of channels=2). Error bars denote SD.

1.4. Discussion

In this second chapter of results, we designed and fabricated a microelectrode array within the culture chambers of the microfluidic system. We first optimised the electrode geometry by calculating the sensitivity field of three potential patterns and selecting the 8-electrode configuration. We then utilised the bilayer lift-off lithography fabrication technique to fabricate the electrode array using Ti and Pt and subsequently connected them to the impedancemeter using a custom-made printed circuit board. Afterwards, we validated the functionality of the electrode system using electrical conductivity standards and thereafter characterised the effects of external parameters on the electrical measurement. Finally, we utilised the electrode array to perform impedance measurements on *in vitro* 2D and 3D hydrogel-based 3D PDAC models.

We designed our coplanar microelectrodes with smaller PU electrodes than CC electrodes to maximise their sensitivity to the evolution of cell density. Indeed, smaller electrodes have been found by Zhang et al. to have a larger impedance response to cell monolayer.²³¹ Similarly, using a 2-point electrode configuration, X. Zhang et al. systematically investigated the impact of electrode dimensions on a 2D cell monolayer proliferation sensitivity, demonstrating that smaller working electrode significantly enhance impedance responses to variations in cell density.²³² Specifically, when reducing the electrode radius from 150 μm to 100 μm , the impedance shift in response to an increase in cell density as cells proliferate on the working electrode from 90,000 to 100,000 cells. cm^{-2} improved from 350 Ω to 597 Ω . Similarly, for a cell density increase from 90,000 to 110,000 cells. cm^{-2} , the impedance shift rose from 880 Ω to 1336 Ω . However, drastically reducing the electrodes size has been found to increase electrode polarisation.²³³ Wang et al. studied the effects of electrode diameter on the impedance values of Pt electrodes in phosphate-buffered saline solution. They found that reducing the electrode diameter from 1000 μm to 2.5 μm increased the measured impedance at 1 kHz from 1.9 k Ω to 9.0 M Ω .²³⁴ Although this could prove to be problematic, as this electrode polarisation can mask the sample's impedance at lower frequencies, our microelectrode array uses the four-electrode configuration as it has been previously described to significantly reduce the effects of electrode polarisation on the measured impedance compared to other electrode configurations (**Figure 69.a**).²³⁵ This enabled us to obtain a “flatter” impedance spectra, as can be seen when we performed impedance measurements on a 1X PBS solution using both two- and four-electrode configurations (**Figure 69.b**).

Furthermore, this configuration eliminated the impedance of the electrodes as well as the electrode connection paths, hence the lower impedance magnitude for the 4-point electrode configuration.

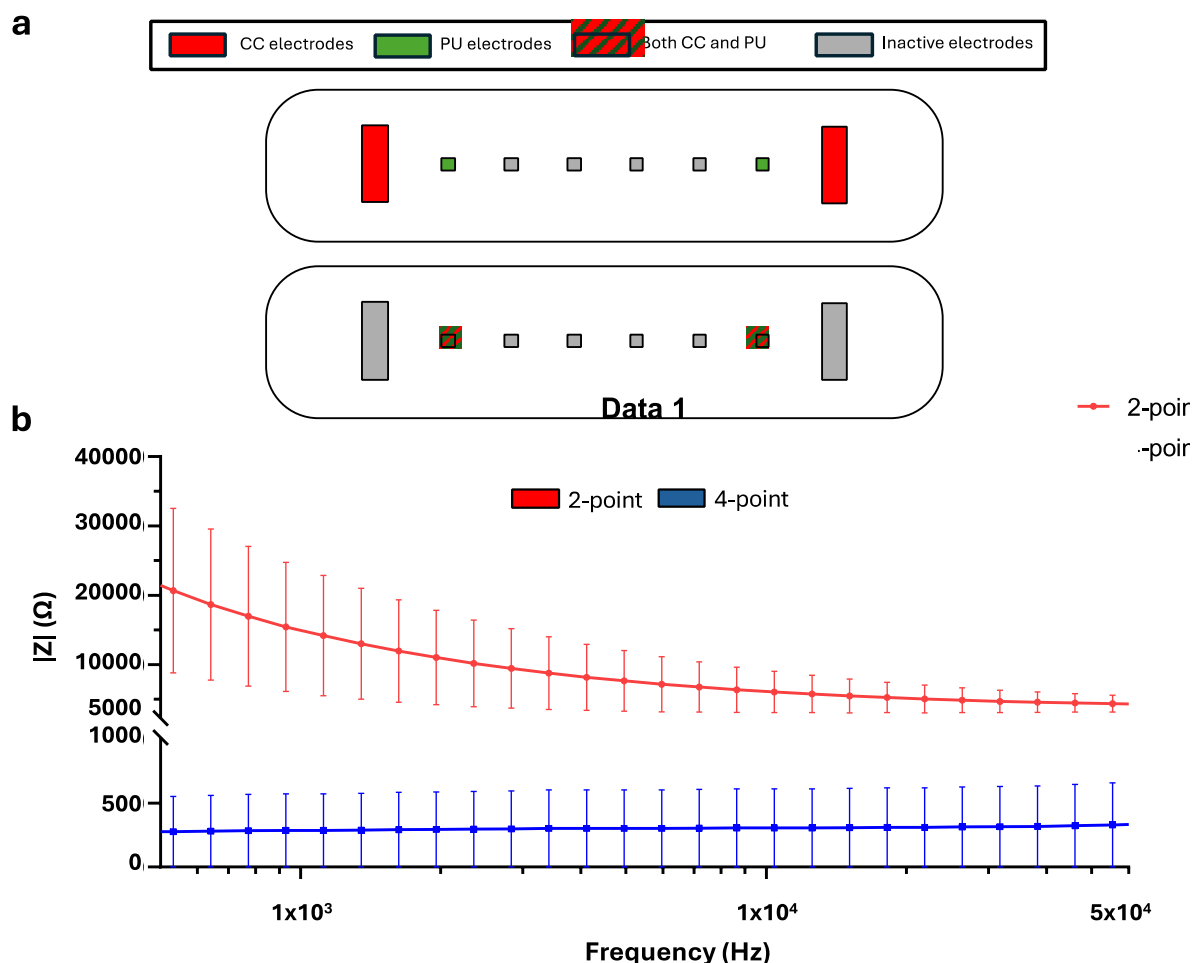


Figure 69. Comparison of impedance spectra of phosphate-buffered saline solution using 2- and 4-electrode configurations. (a) Electrode configurations used to measure the impedance spectra of 1X PBS in the culture chamber (top: 4-point measurement, bottom: two-point measurement). (b) Impedance spectra of 1X PBS using both 2- and 4-point electrode configurations (Number of chips=2, number of channels=4). Error bars denote SD.

We then chose the 8-electrode geometry by basing our choice on maximising the sensitivity field of the electrodes. We calculated this sensitivity field using the electromagnetic field theory and reciprocity theorem. Bötttrich et al. were one of the first studies to employ this for the design of electrodes for cell applications.²²³ As the culture chamber is rectangular, we decided to place the PU electrodes along the same axis across the culture chamber length. We did not however, study the influence of the placement or spacing of these PU electrodes on the sensitivity field.

As our aim was to study a 3D culture, sensitivity along the height of the chamber was a critical parameter. Although the 8-electrode geometry resulted in a higher sensitivity along the z-axis, we observed a sharp decrease as we moved up the culture chamber: 39 % from 10 to 250 μm , and 18 % from 250 to 500 μm . This limitation in achieving uniform sensitivity along the z-axis has previously been reported for coplanar electrodes. They have been described to struggle in driving the electric field into the culture volume, which hampers their translation from 2D to 3D cell culture monitoring.²³⁶ Other teams have explored alternative electrode geometries such as parallel electrodes or 3D microelectrodes to improve sensitivity along the z-axis. Lei et al. monitored the impedance changes of human hepatoma cancer cells in a hydrogel using parallel electrodes to drive the electrical field through the 3D cell culture (Figure 70.a).¹⁸⁸

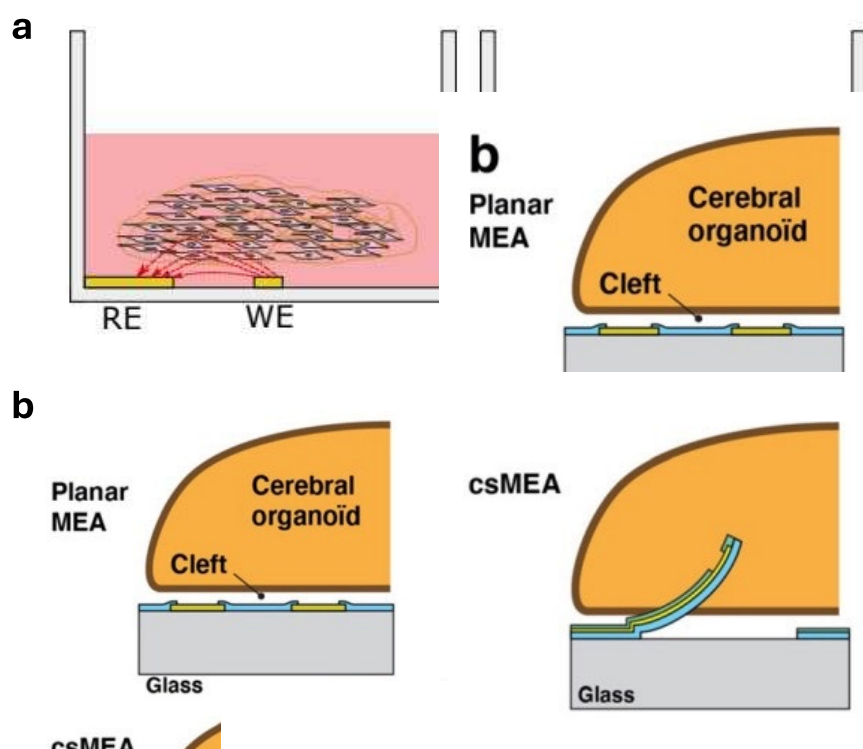


Figure 70. Alternative electrode configurations for bioimpedance measurements on 3D cell culture. (a) Comparison of coplanar electrodes and parallel electrodes to perform bioimpedance measurements on 3D cell culture. From [188] (b) Schematic of the placement of a cerebral organoid on top of a conventional planar microelectrode array (MEA) showing the cleft layer between cells and electrodes and a schematic representation of an organoid inserted on a curvy and spiky microelectrode array (csMEA). From [237].

This configuration has been implemented in microfluidic systems for microfluidic impedance cytometry using 2-point electrode configuration.²³⁷ However, adapting this to a four-point configuration would require more complex connections. Moreover, our use of a 3D-printed cap and dual closing plates makes implementing this geometry technologically

challenging with our current manufacturing methods. Phouphetlinhing et al. developed a protruding cantilever microelectrode array designed specifically to penetrate thick, spheroid-shaped cerebral organoids (**Figure 70.b**).²³⁸ Their fabrication process relies on internal stress relaxation of layered materials deposited over sacrificial layers, creating vertically oriented cantilever electrodes protruding over 200 μm to access internal organoid regions. This configuration could more easily be integrated into our microfluidic system, as the electrodes are fabricated directly onto the glass substrate. We could therefore modify our fabrication protocol to manufacture them and connect them using our custom-made PCB. To illustrate the potential benefit of using 3D electrodes, we carried out the same sensitivity study as in **Figure 48** and **Figure 49**, but this time, we increased the thickness of the electrodes to 250 μm . When we compare the heatmaps of the sensitivity fields, there is no apparent differences between 3D and coplanar electrodes (**Figure 71.a, b, c and d**). However, if we compare the average sensitivity of the cross section S1 at each z-height (10, 250 and 500 μm) for the outer and middle PU electrode pairs, we obtained values of: 3.27×10^{11} , 3.26×10^{11} and 3.14×10^{11} and 3.84×10^{11} , 3.76×10^{11} and 3.01×10^{11} , respectively (**Table 10**). These are similar for the outer PU electrodes but interestingly, the average sensitivity is lower at 10 μm for the middle PU electrodes, similar at 250 μm and higher at 500 μm , when using 3D electrodes compared to coplanar ones. Thus, using 3D electrodes may offer a beneficial trade-off: a slight decrease in sensitivity near the electrodes in exchange for enhanced detection capabilities further up the culture chamber, potentially improving the impedance measurement accuracy for thicker or multilayered cell cultures.

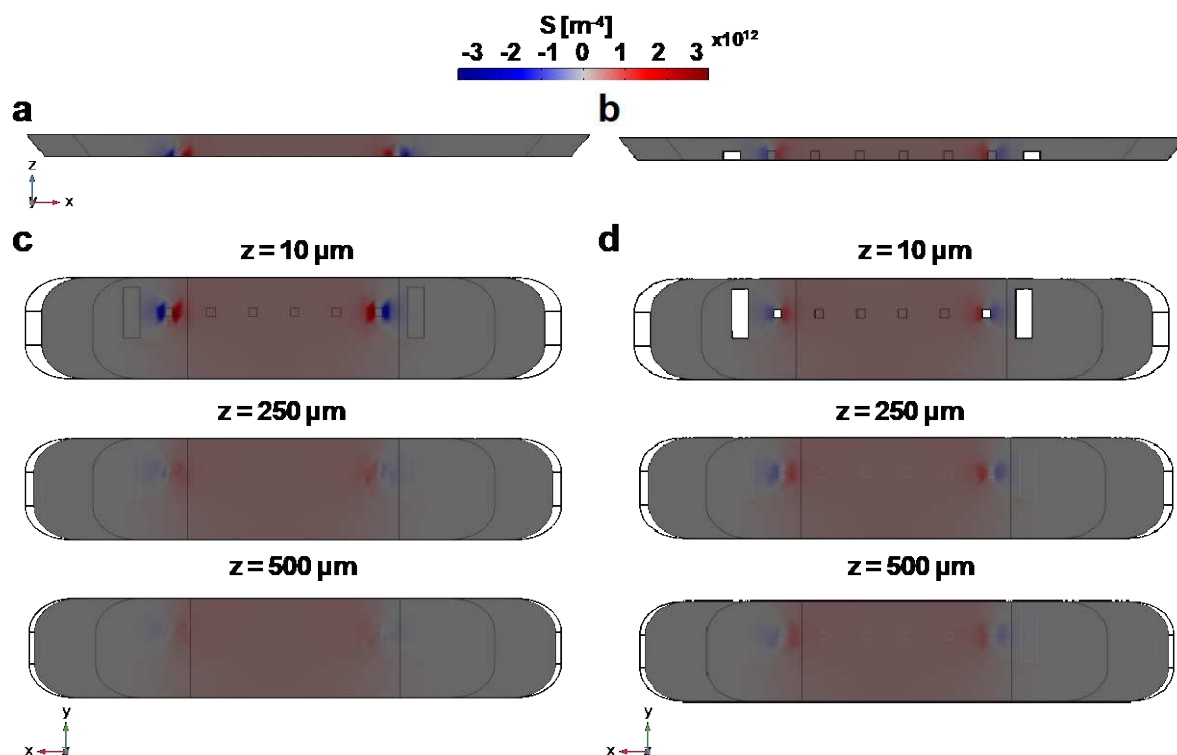


Figure 71. Sensitivity field comparison between coplanar and 3D electrodes. Sensitivity [m^{-4}] field heatmap in the xz-direction for the coplanar (a) and 3D electrodes (b). Sensitivity [m^{-4}] heatmap in the xy-direction for three z-positions (10, 250 and 500 μm) for the coplanar (c) and 3D electrodes (d).

We utilised a bilayer photolithography lift-off fabrication protocol based on the thermal evaporation of metal to fabricate our electrode system using a Ti / Pt bilayer. Although we did not consider indium tin oxide as we did not have the necessary manufacturing tools, it has been previously used for electrode fabrication,²³⁴ mainly due to its transparency, meaning that indium tin oxide electrodes do not obstruct the optical path during microscopy.²³⁹ Using this, Lewis et al. performed impedance measurement on an endothelial cell 2D monolayer to study barrier function under stimuli in a microfluidic system.²⁴⁰ They were then capable of correlating a change in the normalised impedance with a change in morphology after the introduction of shear stress. However, as we discussed previously, manufacturing in a clean room environment requires access to specialised equipment and is both time-consuming and expensive. To circumvent these issues, screen-printing and additive manufacturing have recently been utilised to fabricate electrodes and both show promise for inexpensive and rapid integration of biosensors.²⁴¹ Screen-printing, which involves depositing inks through a patterned mesh, has been employed to produce electrodes on various substrates, including glass and polymers.²⁴² It can produce electrodes using various materials on a wide range of substrate such as glass and polymers. Kawakita et al. leveraged this by incorporating carbon-based

screen-printed electrodes in a microfluidic system to study the formation of confluent monolayers of blood-brain barrier cells via trans-endothelial electrical resistance (**Figure 72.a and b**).²⁴³ However, a key limitation of this method is its inability to produce features smaller than several tens of micrometres.²⁴⁴ This is not an issue for our electrode system as the smallest active structures are the connexion paths, which are 150 μm wide. Furthermore, variations in ink composition, substrate properties and printing conditions can lead to inconsistencies in sensor performance and reproducibility.

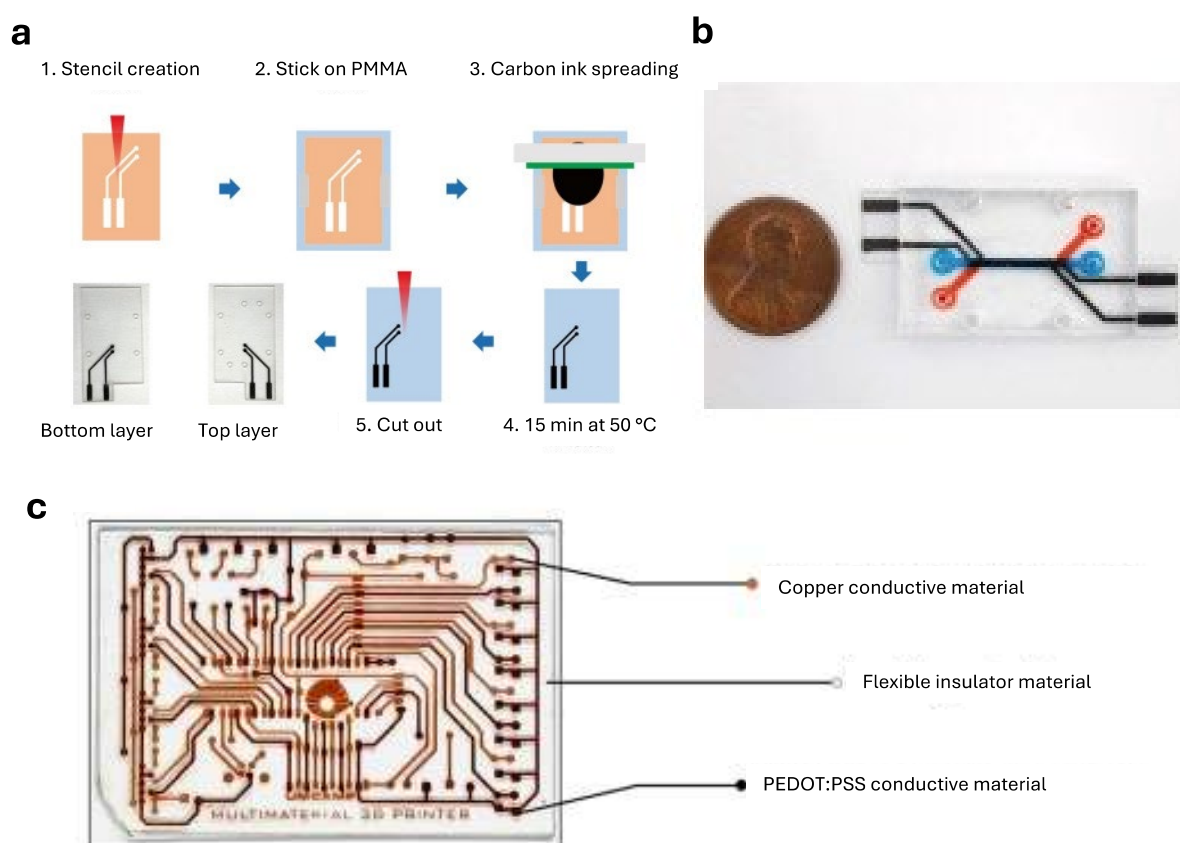


Figure 72. Alternative microelectrode fabrication methods. (a) Screen-printing protocol developed by Kawakita et al. **1:** The stencil is cut out in a paper **2:** It is then stuck onto the wanted substrate **3:** The ink is spread across the stencil **4:** It is then cured at 50°C for 15 min to solidify **5:** The electrodes are then cut out. (b) Photo of the actual chip. From [240]. (c) A printed circuit board was built in flexible resin (insulator) and PEDOT-based and CuNP-based conductive resins. From [246].

Additive manufacturing, on the other hand, employs 3D printing to fabricate electrodes using thermoplastic such as polylactic acid or acrylonitrile butadiene styrene, that have been modified to improve their conductive properties. Quero et al. prepared a conductive-photopolymerisable resin based on PEDOT:PSS dispersed in clear resin GEM 1.²⁴⁶ They then printed a PCB using a flexible resin as an insulator and the PEDOT:PSS modified resin for the conductive paths (**Figure 72.c**). However, additive manufacturing for electrode fabrication

suffers from drawbacks such as the limited availability of commercial conductive material. Nevertheless, both techniques could have allowed the use of thinner substrates by allowing for different materials such as PMMA that cannot be used using thermal evaporation of metals due to the high deposition temperature. This could therefore be advantageous for microscopy.

Regarding the electrode connexion to the impedancemeter, we opted to use a PCB and to solder it directly onto the electrode connexion pads. This seemed as the most reliable and robust way to connect the electrodes compared to other methods such as gold wire bonding, which was an important requirement since the system is placed for long period of times in a humid environment (incubator). Although this enabled us to reuse our system multiple times with no impact on the electrical measurement, this forced us to only perform measurements on one microfluidic chip at a time. To increase the number of connected chips, we could consider using an automated multiplexed PCB that could automatically switch between the microfluidic chips in-between impedance measurements. This was notably done in a study conducted by Bürgel et al. in which they developed an automated multiplexed EIS platform and simultaneously monitored up to 15 culture chambers.²⁴⁷ While such multiplexing would significantly increase experimental throughput, it would also introduce measurement delay. In our case, impedance measurements across one microfluidic chip's eight culture chambers for a frequency interval between 500 Hz and 60 kHz require approximately 2 min 30 s. That would total in a measurement time in 10 min if four microfluidic chips were multiplexed. This temporal resolution would be sufficient for monitoring biological events such as drug induced apoptosis as they occur hours after drug administration.²⁴⁸

As our main objective was to monitor the evolution of a dynamic cell culture (2D and/or 3D) using our electrode system, we first needed to validate its functionality, functionality being the capability of inserting a sample into the culture chamber and to measure its impedance. We first measured the impedance of electrical conductivity standards. By plotting the impedance against the inverse of conductivity, we observed that the electrode system was linear (**Figure 73.a**). This indicates that the system produces consistent and predictable measurements under controlled conditions.²⁴⁹ However, while this supported the system's functionality, the linearity of an impedance system can be more robustly confirmed with a Lissajous plot, where the applied AC voltage is plotted against the resulting AC current. A system with a linear response would result in an elliptical plot (**Figure 73.b**), while a system with a non-linear response would result in a distorted plot (**Figure 73.c**).²⁵⁰ Additionally, this experiment enabled us to

extract the resolution of our biosensor, which is the smallest change in impedance that can be reliably distinguished from background measurement noise. This value, also known as the limit of detection (LOD), is defined to be equal to 3 times the standard deviation value.²⁵¹ In our case, if we take the average standard deviation to be $68\ \Omega$ as calculated from the electrical conductivity standards impedance measurements (**Figure 57.a**), we calculated LOD of $203\ \Omega$. This means to be monitorable by our electrode system, biological events such as cell proliferation or death need to generate a change in impedance bigger than $203\ \Omega$.

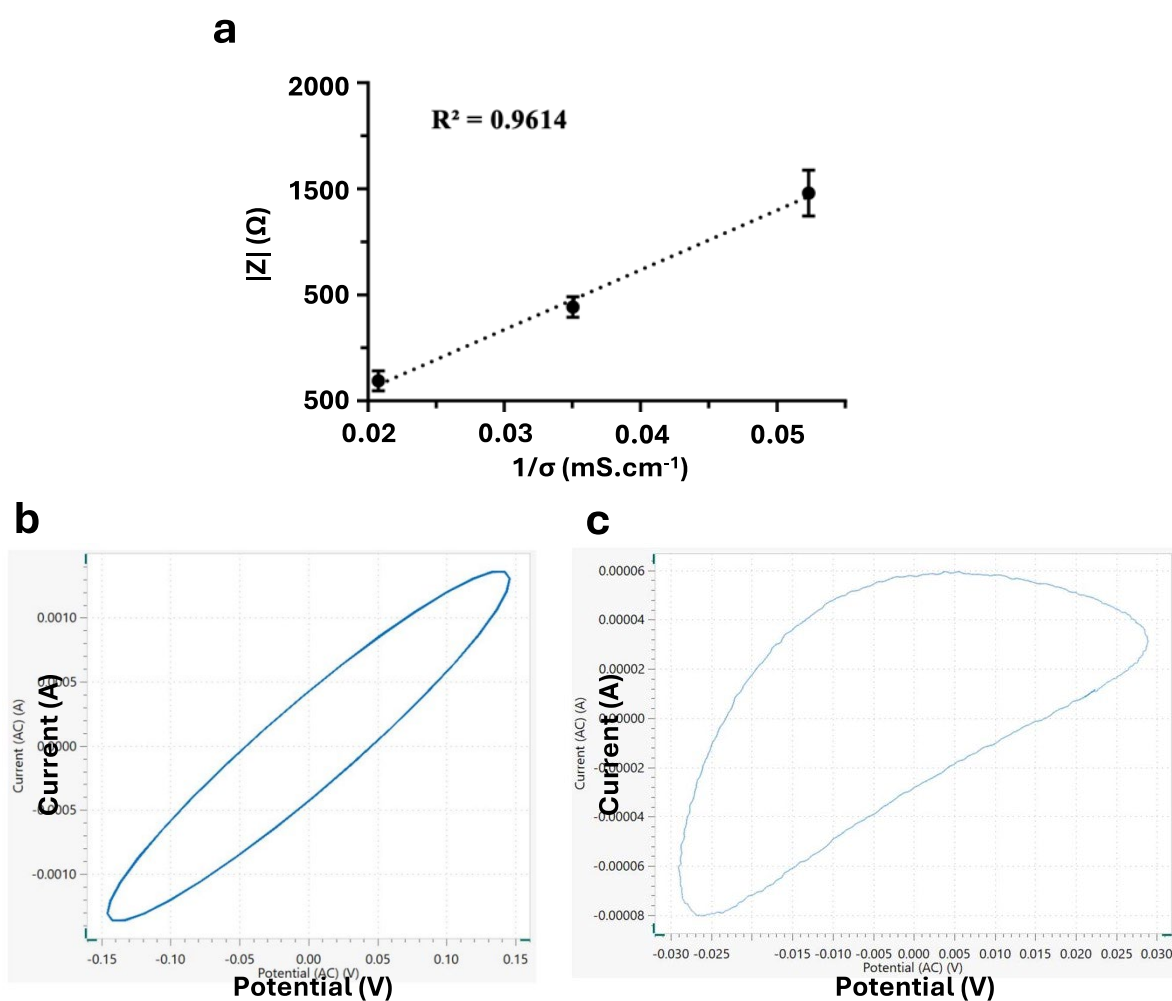


Figure 73. Linearity determination of impedance measurement system. Impedance magnitude with respect to the inverse of conductivity using our electrode system. (Number of chips=3, number of channels=8). (b) Lissajoux plot of linear system. (c) Lissajoux plot of a non-linear system. From [250].

When we compared experimental measurements to a numerical model, we observed that experimental impedance values were, on average, 9% lower than simulated ones (**Figure 57.b**). This is not the system's precision, as we modelled an ideal culture chamber and electrode

system (600 μm chamber height, no alignment error), but it indicated that parameters of the system influenced the impedance measurement. It thus highlights the influence of physical and fabrication parameters on impedance measurements. We first investigated the influence of the culture chamber height on the impedance measurement and confirmed, both experimentally and numerically, an inverse relationship between culture chamber height and impedance (**Figure 58**). This is consistent with other studies such as the one by Rapier et al.²⁵² who investigated the effects of culture chamber width, or Zitzmann et al.²⁵³ who studied the effects of culture chamber height. In our case, we obtained a sensitivity of the system to culture chamber height of $3.74 \Omega \cdot \mu\text{m}^{-1}$, which if multiplied by the fabrication error we had measured, would give a 55Ω variation of the impedance measurement. This would partially explain the difference we obtained between numerical model and the experimental measurement of the conductivity standards' impedances. Importantly, this variation remains below the previously LOD, suggesting that it is unlikely to compromise the system's ability to resolve biologically relevant impedance changes under typical conditions. Furthermore, the switch to a 3D printed cap reduced the variability of the impedance measurement, with a reduction of the standard deviation from 281Ω to 94Ω . This correlates well with the reduction in culture chamber height variation we had previously investigated. However, this change in the cap's material also reduced the impedance magnitude from 1983 to 1713Ω . We did not expect this, but it could be explained by the flexible nature of PDMS, meaning that the PDMS bottom may be stretched when inserting the resin cap, which could locally reduce the width of the channel. This would require further investigating.

Additionally, we investigated the influence of PDMS alignment with the electrode system, but we did not find that it affected the electrical measurement, with sensitivity to alignment error in x- and y-directions of $0.0017 \Omega \cdot \mu\text{m}^{-1}$ and $0.18 \Omega \cdot \mu\text{m}^{-1}$, respectively (**Figure 59**). When combining the average error obtained in the y-direction ($39 \mu\text{m}$) with its estimated effect on the impedance (7Ω) and summing this with the contribution from the culture chamber height variation (55Ω), the resulting total variation (62Ω) approached the average standard deviation observed in our conductivity standard measurements. This suggests that a combination of fabrication errors could plausibly explain most of the experimental variability observed in our system.

We also investigated the influence of temperature, specifically to assess the influence of placing the microfluidic system in and out of the incubator. We confirmed both

experimentally and numerically that the measured impedance decreased as the temperature of the system increased (**Figure 61.b**). This was consistent with other studies,²²⁷ and enabled us to establish a 60 min period during which we would not interpret the measurements. However, we observed a difference in impedance magnitude between the experimental and numerical measurements (1570 ± 125 vs 1641Ω). Additionally, we noticed that the standard deviation of the culture medium measured impedance was higher than previously measured ($\pm 115 \Omega$, **Figure 57.a** vs $\pm 130 \Omega$, **Figure 61.b**). This indicated that a parameter other than temperature alone influenced the electrical measurement after placing the system in the incubator. We further confirmed this when we measured the electrical conductivity of culture medium placed in the incubator over 9 days. We were able to correlate the initial increase to the change of temperature after placement in the incubator, but we also observed a constant increase of the conductivity over the following days (**Figure 64**). Although we did not experimentally find an explanation, this was also reported by other research teams, who attributed this to CO_2 dissolution in the culture medium,^{254,255} which would increase the ionic strength of the culture medium, thus increasing its electrical conductivity.

When monitoring a dynamic 2D PDAC cell monolayer using our electrode system in culture chambers of two heights ($600 \mu\text{m}$ and $2400 \mu\text{m}$), we observed an increase in impedance over time for both conditions (**Figure 63**). Fluorescence microscopy confirmed this trend corresponded to an increase in cell density. This correlation is consistent with previous studies reporting that impedance increases with cell confluence.^{254,256} Interestingly, no impedance increase was detected until 72 h after the start of perfusion. This is consistent with observations by Meissner et al. who described three phases during dynamic cell culture: an initial lag phase during the first 72 h of culture followed by an exponential growth phase and finally a stationary phase as confluency was reached.²⁵⁷ We did not observe the stationary phase in our experiment as we did not reach confluency after 7 days of culture. While a lag phase was observed in the $2400 \mu\text{m}$ chamber, the $600 \mu\text{m}$ chamber instead showed a decrease in impedance during the first 72 h. We attributed this to the higher flow velocity generated in the $600 \mu\text{m}$ culture chamber being detrimental to the HPAC-eGFP cells and detaching cells in during the first 3 days of culture. This was reinforced when we segmented the culture chamber with the other PU electrode pairs and noticed that a higher impedance was measured near the culture chamber's outlet compared to the other PU electrode pairs.

After 96 h, we observed that the measured impedance was higher in the 600 μm compared to the 2400 μm . We had two hypotheses to explain this. First, the difference in volume of the chambers resulted in a lower baseline impedance in the 2400 μm chamber. Second, cell proliferation in the larger chamber may have increased the medium's conductivity, reducing the impedance rise caused by the HPAC-eGFP cells coverage of the electrodes. However, as we did not observe a difference between a control and a static 2D cell culture, we could not attribute the change of conductivity to the metabolic activity of cells. Since the cells' metabolic activity did not lead to an increase in the electrical conductivity compared to cell-free medium, we concluded that the lower impedance measured in the 2400 μm -high culture chamber, despite a higher number of cells, was primarily due to the increased chamber height.

One notable limitation was the high variability in impedance data for the 600 μm chamber, as reflected in larger standard deviations. This may be due to both biological heterogeneity and limited experimental replication: we conducted this test only once, with two culture chambers per condition. Additionally, the higher shear stress in the 600 μm chamber likely contributed to a less uniform monolayer compared to the 2400 μm chamber, further increasing measurement variability.

Before monitoring a 3D hydrogel-based PDAC cell culture, we measured the impedance magnitude of a collagen 1 / 1X-DMEM solution and compared it to culture medium after placing the microfluidic system in the incubator (**Figure 66.a**). We measured a 32% and 40% decrease in impedance, respectively (**Figure 66.b**). This aligned with our previous characterisation of the effects of temperature change on impedance. However, we did not see an increase in the hydrogel's impedance as we would expect as it solidifies.²⁵⁸ We hypothesised that we did not see this solidification of the hydrogel as it is likely masked by the rise in temperature after placement in the incubator. Indeed, the reduction in impedance is lower in the case of the hydrogel (32%) than for the culture medium alone (40%). The two effects are therefore likely counterbalancing themselves with respect to the impedance measurement, i.e. the solidification of the gel increases the impedance while the increase in temperature reduces it.

We then evaluated how collagen I concentration and seeding volume affected impedance measurements (**Figure 66.d, e, f and g**). We found that increasing the collagen 1 concentration in the collagen 1 / 1X-DMEM solution led to an increase in impedance. As we

had previously calculated the cell constant to be 2920 m^{-1} , we calculated the equivalent complex conductivity. This revealed an inverse linear relationship between collagen 1 concentration and electrical conductivity (**Figure 74.a**), which is consistent with other studies.²⁵⁸

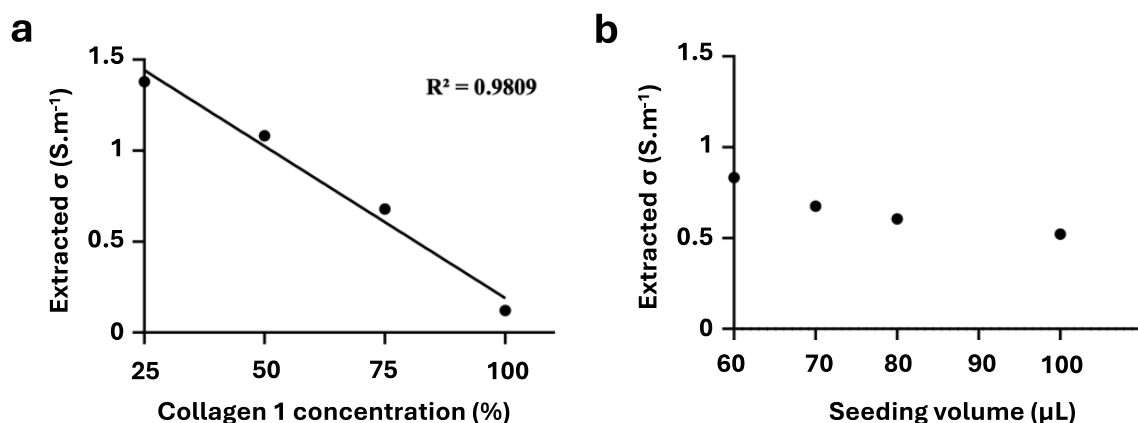


Figure 74. Complex conductivity extraction using electrode system cell constant. (a) Extraction of the electrical conductivity using the measured impedance for different collagen 1 concentrations. (b) Extraction of the electrical conductivity using the measured impedance for different seeding volumes.

Interestingly, seeding volume also exhibited an inverse relationship with extracted conductivity, though the effect was non-linear and less pronounced than that of concentration (**Figure 74.b**). We did not expect this as our previous results demonstrated that an increase in the measured volume resulted in a decrease of the measured impedance (**Figure 58**). We hypothesised that there are two possible explanations, both being related to an increase in compression of the collagen 1/1X-DMEM solution as its volumes increased. First, compression may increase the effective collagen I concentration within the hydrogel. This was demonstrated by Ajalloueian et al. who specifically measured the concentration of collagen 1 varied when compressed.²⁵⁹ For example, they found that after compression with a 120 g weight, the hydrogel saw its collagen 1 concentration increase from 1.64 mg.mL^{-1} to 328 mg.mL^{-1} . This increase in collagen 1 concentration could therefore explain the increase in impedance we observed. Second, compression may expel the aqueous phase from the hydrogel, thereby reducing its effective conductivity. This was notably shown in a study by Brown et al. where they observed that a 10 g load placed for 10 sec on a collagen 1 hydrogel resulted in almost 60 % decrease of the gel wet-mass.²⁶⁰

Finally, we seeded HPAC-eGFP cells within the hydrogel and measured the cell/gel mixture impedance 2 and 24 h after cell seeding in static conditions (**Figure 67**). We observed

a significant difference between the cell/gel mixture and cell-free hydrogel which was consistent with previous studies.¹⁹¹ However, no significant change in impedance was detected after 24 h of static culture in either condition. It is worth noting that we used the impedance magnitude to compare conditions, rather than a cell index as done in previous studies.¹⁹¹ That is because there is no general consensus on the formula for this cell index, complicating comparison across studies. Furthermore, this normalisation of the raw data can be misleading, as it may mask meaningful components of the measurement. For instance, applying the formula used by Pan et al. to our static culture data yields cell index values of 0.48 and 0.40 at 2 and 24 hours, respectively.¹⁸³ This difference would have been misinterpreted as a change due to cells whereas in reality, we observed that this difference was caused by a decrease in the hydrogel impedance, most likely due to the culture medium hydrating the hydrogel, thus increasing its conductivity.²⁶¹ Nonetheless, this result showed that the microelectrode system could detect HPAC-eGFP cells seeding in a collagen 1-based hydrogel under static conditions. Furthermore, as we did not observe changes after 24 h of static culture, and because there was not enough culture medium to extend this experiment past 24 h, we measured the impedance of HPAC-eGFP cells embedded in our collagen 1 hydrogel and cultured in dynamic conditions within the microfluidic system.

We therefore monitored a dynamic 3D PDAC cell culture over 9 days, including a 2-day perfusion of 5X-Triton to induce cell death (**Figure 68**). We wanted to determine if we were able to quantify cell proliferation and detect cell death under dynamic 3D culture conditions. Similar to the static 3D culture, we initially observed a lower impedance in the cell/gel mixture compared to cell-free hydrogel. However, the measured values were much lower than those in the static culture. We attributed this to the addition of the microfluidic circuit, which increased the conductive volume and thus reduced overall measured impedance. Although we observed cell proliferation with fluorescence microscopy, we did not measure any changes during the dynamic culture. Similarly, we did not measure any changes after the perfusion of 5X-Triton. De Leon et al. studied both 2D and 3D cell culture using coplanar electrodes, and although their experiments were in static conditions, they had the same difficulty of translating measurements performed on a 2D cell monolayer to a 3D hydrogel-based culture.²³⁶ They observed an increase in impedance in wells containing cells whereas impedance was stable in cell-free wells. In contrast, they did not measure a difference between collagen 1 seeded with cells and cell-free collagen 1 over a 10-day culture. They suggested that

this was because of the coplanar electrodes' limitation in achieving uniform sensitivity along the z-axis. Another possible explanation could be that while cell proliferated, they might not have formed strong enough gap junctions to influence the impedance measurements. Indeed, impedance measurements on 3D cell culture relies not only on cell density but also on the gap junctions formed between individual cells.^{183,262} In our case, the dynamic nature of the culture could also be a reason for the fact that we did not measure changes in time. Although we managed to reduce mechanical degradation by using a cell strainer to act as a protective barrier, we did not completely suppress it. We did not investigate this but this slow mechanical degradation could change the composition of the hydrogel through increased hydration.²⁶¹ Another possibility would be to use a higher initial cell seeding concentration at the start of the experiment to increase their effect on the impedance measurement.

Conclusion and Perspectives

This thesis work led to the development of an instrumented microfluidic system for the study of solid biological tissues. This was in response to a need among researchers to evolve conventional culture models (static *in vitro* 2D and 3D and *in vivo*) that suffer from limitations such as simplified tissue representation and interspecies incompatibility. The second need addressed by this work was the need to be able to carry out non-destructive viability measurements in order to assess the efficacy of therapeutic candidates. In response to both of these needs, our microfluidic system was designed taking into account *in vivo* flow velocity data available in the literature and was combined with an impedance biosensor in order to measure in real time the evolution of cell density in 2D monolayer culture and in 3D culture.

In order to facilitate the use of our device, and increase its potential reach, we designed it to be compatible with the SBS plate format by centring the culture chambers with the wells of a 96-well plate. In particular, this allowed us to easily use the device with automated microscopes. In addition, we separated the device into two distinct parts to allow biological material recovery at the end of the culture. However, this forced us to use dimensions that prevented us from fabricating it using traditional methods. We therefore explored the use of 3D printing as an alternative manufacturing approach. We compared two 3D printing techniques (SLA and LCD) as well as the printing orientation's influence on the printing precision. This enabled us to choose the LCD-based 3D printer for the fabrication of our PDMS moulds. In addition, in order to obtain a uniform culture chamber height, we used 3D printing to manufacture the chip's caps. After studying the cytotoxicity and auto-fluorescence of the Bio-Med Clear (BMC) resin, we functionalised and validated it for cell culture with an immersion in a Sudan Black B (SBB) solution followed by a Parylene C coating. Finally, we validated the use of this microfluidic device for both 2D and 3D cell culture. This enabled us to perform a study on the effects of flow velocities on a 2D culture PDAC cells, highlighting its influence on cell proliferation. Furthermore, we demonstrated the ease of adaptability of our fabrication protocol by adapting the cap and validating its use by utilising it to perform a 3D culture of PDAC in a collagen 1-based hydrogel over a seven-day period.

Following this, we integrated an impedance biosensor in the chambers of the microfluidic system. We defined its geometry using numerical modelling and studied the distribution of its sensitivity field. As we faced fabrication error when using the lift-off lithography method, we manufactured it using the bilayer lift-off technique to pattern a Ti / Pt metallic bilayer. We then validated its functionality by measuring the impedance spectra of

Conclusion and Perspectives

three electrical conductivity standards and compared them to a numerical method. However, we measured a 9% difference between both experimental and numerical measurements. We attributed this to fabrication errors of the microfluidic system, hence, we characterised and quantified the impact of geometry and temperature on the electrical measurement. We then validated the electrode system by measuring the change in cell density in a 2D dynamic cell culture. Finally, we quantified the relationship between collagen 1 concentration and impedance. This enabled us to choose the collagen 1 concentration for our 3D cell culture. We then measured the effects of PDAC cell seeding in the collagen 1 / 1X-DMEM construct in both 3D static and 3D dynamic culture conditions. We utilised this biosensor to measure the increase in impedance as cells proliferated in a 2D dynamic cell culture. It enabled us to measure the presence of PDAC cells in a collagen 1 hydrogel in a static culture, but we did not measure a change of impedance over time in dynamic culture conditions.

The development of this instrumented microfluidic system and the integration of a bioimpedance microelectrode system provide a wide range of perspectives:

Microfluidic system

We developed a parallelised and openable microfluidic system to both increase the number of possible conditions and facilitate the recovery of biological material. However, further adapting the microfluidic system to be fully compatible with the 96 well plate SBS format. As one microfluidic chip is equal to a quarter of a 96 well plate, we could significantly improve the experimental throughput by multiplying the system's size by 4 and having the number of culture chambers increase to 32 per microfluidic chip. This would enable us to carry out more conditions at a time but would require a redesign of the microfluidic system as a whole, most notably the replacement of the peristaltic pumps as flow generators. This could be achieved by directly integrating micropumps into the microfluidic system. Another aspect to be improved is the microfluidic circuit, more specifically the tubing and culture medium reservoirs. As of today, the fabrication of the circuit requires the use of long lengths of expensive commercial tubing, which prevented replacement between experiments. Furthermore, the use of long lengths of tubing requires a large volume of culture medium (3.5 mL per microfluidic loop) which dilutes cell secretions. This is a limitation, particularly when comparing the microfluidic system to a static control.

Conclusion and Perspectives

We fully utilised 3D printing's potential by creating both PDMS moulds and constitutive parts of the system, but there is still room for improvements. Eliminating the 3D printing resin functionalisation protocol would both shorten the fabrication process as well as simplify certain steps such as sterilisation of the microfluidic system. Indeed, since Parylene C has a glass temperature of 80 °C, its use stopped us from autoclaving the system prior to cell culture. We therefore used successive 1X PBS and 70 °C baths to clean and sterilise the microfluidic circuits, which was both time-consuming and yielded inconsistent results. It would therefore be interesting to either try other commercially available resins or manufacture our own, as other research teams have done. We would then be able to resolve both autofluorescence and cytotoxicity issues while eliminating the need for post-printing procedures.

We drastically reduced the number of leaks occurring during dynamic culture by combining the addition of leak-proof cordons around the culture chambers and a dedicated closing system. The number of leaks could be further reduced by minimising fabrication variability in the PDMS moulds and 3D printed cap. This could potentially be done by applying a correction to the 3D printing designs based on characterised dimensional deviations. Another improvement could be to switch the substrate material to a plastic such as PMMA. This would enable a tighter screwing of the closing system without breaking the substrate. Furthermore, it would enable us to use a thinner substrate which, although sufficient in our current setup, would be beneficial for microscopy imaging, especially for 3D cell culture. It would however require a modification of the biosensor fabrication protocol to be compatible with plastic-based substrates.

Bioimpedance biosensor

Our microelectrode system enabled us to measure the impedance of 8 individual culture chambers in parallel in a robust and reliable manner thanks to the use of a PCB to connect it to the impedancemeter. Although this allows for multiple conditions to be studied at once, there is room for improvement to further increase the experimental throughput. For example, the addition of a multiplexer, combined with a modification of the PCB, could allow the connection of more microfluidic systems to the impedancemeter.

We quantified the influence of fabrication errors such as the culture chamber height and the PDMS alignment on the impedance measurements. This quantification showed that the

variation of the measurement could be partially explained by errors in the fabrication of the system. However, we did not quantify the influence of dynamic changes on the measurement. Most notably, the influence of the dynamic perfusion on the electrical measurement needs to be investigated. Furthermore, this could be coupled with a quantification of the collagen 1 / 1X-DMEM construct degradation as a result of the dynamic perfusion. Another parameter that would be interesting to quantify would be the effect of CO₂ on the measurement. Indeed, we quantified the effect of temperature, but noticed a difference between experimental and numerical measurements, which could be explained by other factors such as CO₂. This could be performed by placing the system in an incubator and by manually changing the CO₂ concentration and measuring the resulting impedance spectrum.

Finally, it would be interesting to explore different electrode geometries, such as a 3D electrode geometry, to investigate whether they are more sensitive to 3D cell cultures. One example of such a geometry would be protruding electrodes, as they directly penetrate within the tissue. Furthermore, in our case, the fabrication process of such electrodes is feasible as we have access to the required tools. Another electrode configuration, more precisely parallel electrodes, would also be interesting but more challenging to produce due to the two-component nature of our microfluidic system, as well as the fact that we utilise a 4-point electrode configuration.

Biological model

We observed differences in cell proliferation in the microfluidic system when we cultured PDAC cells (HPAC-eGFP) as a 2D monolayer at two different flow velocities. It would be interesting to repeat this experiment using additional PDAC cell lines, such as Panc-1 (highly mesenchymal-like) or MiaPaCa-2 (moderately mesenchymal-like), which represent different stages of PDAC development. This was notably studied in breast cancer by Tchafa et al.²¹⁹ and it could allow us to better understand how cells at distinct stages respond to interstitial flow, potentially offering insights into its role in PDAC progression and resistance to therapy. We could couple this experiment with the impedance biosensor and quantify the differences in cell densities using the biosensor. Furthermore, it would be interesting to expose both 2D and 3D PDAC cell cultures to a chemotherapy and to quantify and compare their respective responses using the bioimpedance biosensor, to demonstrate the effect of 3D cell culture on the drug response of PDAC. Although we measured a decrease in impedance as we embedded

Conclusion and Perspectives

cells in a collagen 1 hydrogel, we only performed measurements at one single cell density. It would be interesting to measure the impedance with increasing cell densities. This could also lead to an increase of their effect on the impedance measurement and potentially enabling their sensing in dynamic culture conditions.

One aspect to improve would be the ECM we used for the 3D cell culture. We utilised a collagen I-based hydrogel as the company producing the porous hydroscaffold we intended to use ceased operations. While this alternative allowed us to culture a 3D PDAC model under dynamic conditions, it suffered from drawbacks such as a lack of direct flow perfusion and uncontrolled mechanical degradation. We could circumvent this by fabricating our own porous hydroscaffold using conductive materials such as PEDOT:PSS, as demonstrated by other research teams.^{263,264} Such a scaffold would be more stable than a hydrogel under dynamic conditions, and would also facilitate the transmission of the electrical signal. Thus, the measured impedance would be more directly influenced by cell resistance.

One thing to note is that the microfluidic system we developed, as well as the bioimpedance biosensor, can be used on any solid tissue model. It would therefore be valuable to study other biological models in the system. For instance, hepatic solid tissue would be particularly interesting since liver models are already widely used in microfluidics to evaluate unwanted toxicity. Moreover, given that most therapeutic molecules are metabolised by the liver, using hepatic models in our system could give important insights into hepatic cytotoxicity and drug-induced liver injury, making it highly relevant for pharmaceutical applications.

A longer-term perspective would be to design and use caps to induce a mechanical compression on the studied biological model, and to combine this with therapeutic molecules. This would highlight the combined effects of both compression and shear stresses on the drug response of solid tumours.

Appendices

Appendix 1: Electrode configuration sensitivity

Table 17. Average sensitivity for all electrode configurations in both centred and offset geometries.

Electrode configuration		z-height (μm)	Sensitivity (m^{-4})		Difference (%)
			centred	offset	
4-square		10	3.11E+11	3.14E+11	0.83%
		250	2.74E+11	2.80E+11	2.11%
		500	2.74E+11	2.80E+11	2.11%
4-rectangle		10	2.39E+11	2.44E+11	2.02%
		250	2.24E+11	2.27E+11	1.47%
		500	2.19E+11	2.22E+11	1.39%
8-electrode	Outer	10	3.44E+11	3.44E+11	0.02%
		250	3.21E+11	3.18E+11	0.85%
		500	3.11E+11	3.09E+11	0.82%
	Left	10	5.13E+11	5.15E+11	0.31%
		250	3.49E+11	3.47E+11	0.39%
		500	2.94E+11	2.88E+11	2.09%
	Left middle	10	4.97E+11	5.02E+11	1.04%
		250	3.44E+11	3.38E+11	1.76%
		500	2.83E+11	2.87E+11	1.23%
	Middle	10	5.09E+11	5.15E+11	1.11%
		250	3.40E+11	3.45E+11	1.37%
		500	2.86E+11	2.80E+11	2.33%
	Right middle	10	5.04E+11	4.94E+11	1.93%
		250	3.42E+11	3.42E+11	0.07%
		500	2.87E+11	2.87E+11	0.12%
	Right	10	5.26E+11	5.20E+11	1.27%
		250	3.49E+11	3.43E+11	1.64%
		500	2.92E+11	2.88E+11	1.34%

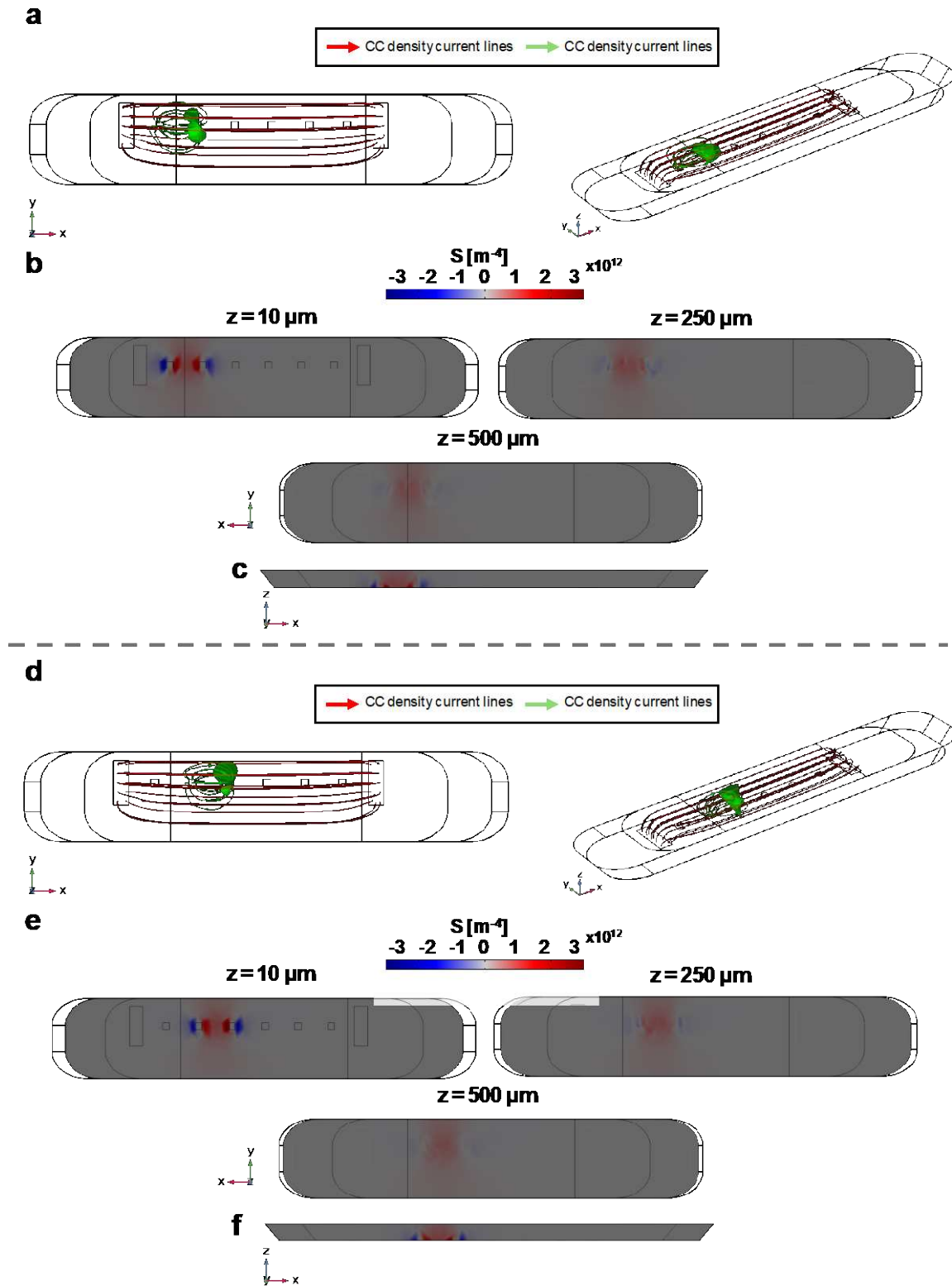


Figure 75. Sensitivity field analysis of 8-electrode geometry left and left middle PU electrode pairs. We visualised the current density field lines both xy- and 3D-view of the channel for both left (**a**) and left middle (**d**) PU electrodes (red lines represent current density of CC electrodes and green of PU electrodes). Similarly, we visualised the sensitivity [m^{-4}] heatmap in the xy-direction for three z-positions (10, 250 and 500 μm) for both pairs (**b** and **e**) as well as in the (**c** and **f**) z-direction.

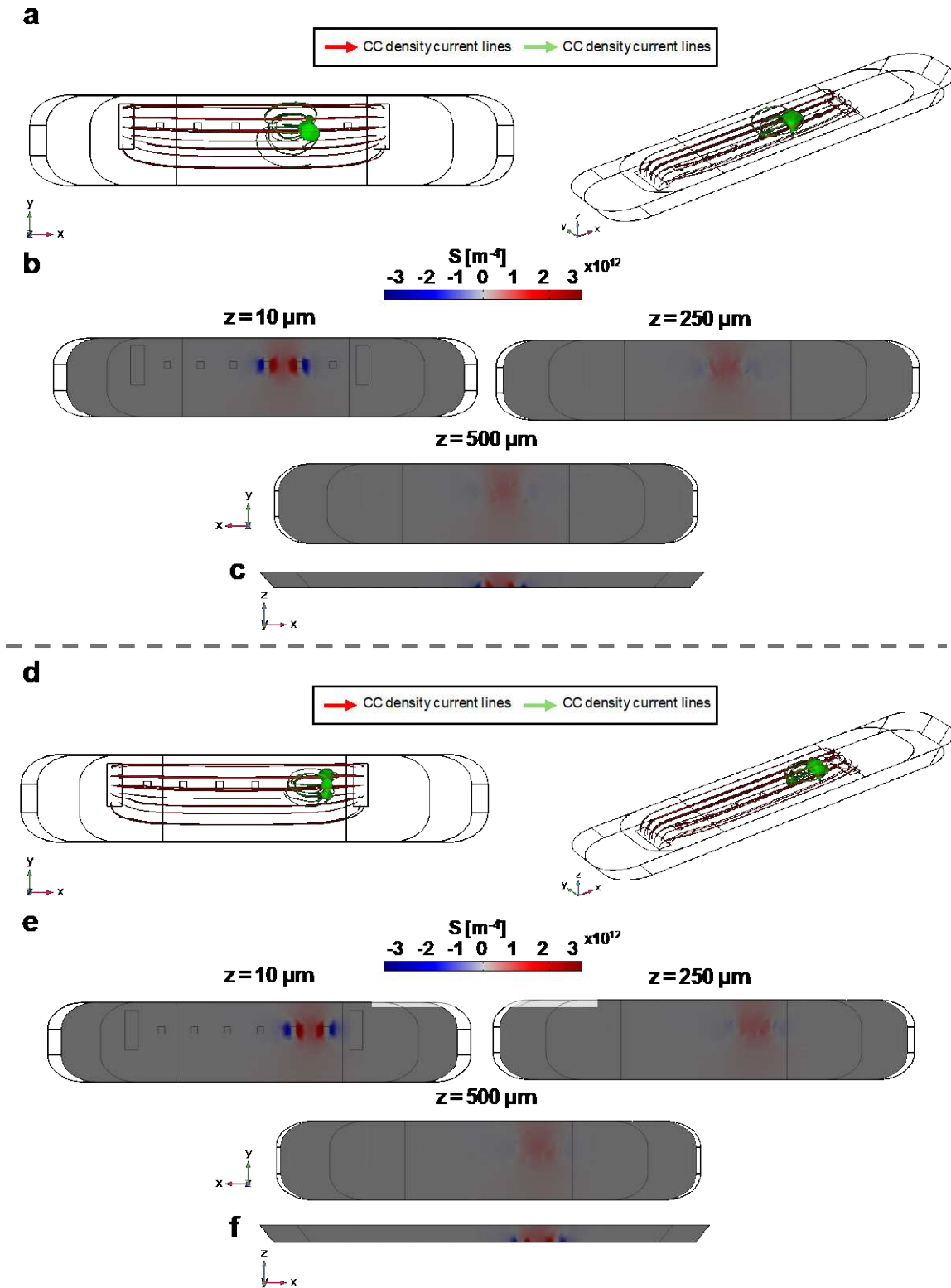


Figure 76. Sensitivity field analysis of 8-electrode geometry right middle and right PU electrode pairs. We visualised the current density field lines both xy- and 3D-view of the channel for both right middle (**a**) and right (**d**) PU electrodes (red lines represent current density of CC electrodes and green of PU electrodes). Similarly, we visualised the sensitivity [m^{-4}] heatmap in the xy-direction for three z-positions (10, 250 and 500 μm) for both pairs (**b and e**) as well as in the (**c and f**) z-direction.

Appendix 2: Material and Methods

Numerical simulation

All numerical simulations were performed in the COMSOL Multiphysics® software (Version 5.6). All COMSOL ‘.mph’ files can be found in **Appendix 3**.

Laminar flow simulation

The channel geometry was exported from Fusion 360 and imported in COMSOL MULTIPHYSICS. It was then meshed using tetrahedral elements with an element size set to finer. This resulted in a mesh of 3,752,042 elements with an average quality of 0.67. The laminar flow was implemented using the laminar flow module according to the Stokes equations:

$$\mu \nabla^2 \vec{u} = \nabla p - \vec{f}$$

with μ the dynamic viscosity of the fluid, $\vec{u}$ its velocity, p its pressure, ρ its mass density and $\vec{f}$ the mass forces being applied to the fluid. The parameters used in the simulation are recapitulated in **Table 3**.

Fluorescein flow simulation

The channel geometry was exported from Fusion 360 and imported in COMSOL. It was then meshed using tetrahedral voxels with an element size set to extra-fine. This resulted in a mesh of 28,195 elements with an average quality of 0.62. The mass transport of fluorescein was implemented using a model described in the literature by Baydoun et al:¹⁹⁷

$$0 = -u \nabla c_i + \beta D_i^a \nabla^2 c_i$$

with u the flow velocity, D_i^a the diffusion coefficient of the species i in the liquid phase a , β an empirical factor taking into account the retardation effect observed in the diffusion of molecules (equal to 1 in the culture medium) and c_i the fluorescein concentration of the injected bolus. The parameters values utilised in both simulations are recapitulated in **Table 8**.

Two simulations were done, one only taking advection of fluorescein into account. Therefore, we set the variable D_i^a to 0, whereas the second one implemented convection

(advection and diffusion) of the fluorescein by setting D_i^a equal to the fluorescein diffusion coefficient.

Electrode sensitivity distribution

The channel geometry and the three considered electrode patterns in a numerical model (**model 1**) using the AC/DC module of COMSOL MULTIPHYSICS using the ‘electric current (ec)’ interface. It was then meshed using tetrahedral elements with an element size set to fine for the three electrode geometries (4-square, 4-rectangle and 8-electrode). This resulted in meshes of 26,412; 26,336 and 27,260 elements, respectively, with average qualities of 0.64, 0.64 and 0.64, respectively. Two electric currents interface were utilised, one for each electrode pairs. One electrode set to ground ($V = 0$), while the other was utilised to apply an electrical potential of 25 mV for each electric currents interface. Electrical insulation was applied to the culture chamber walls and the initial potential in the culture simulation volume was set to 0 V. The simulation of the sensitivity fields was carried out according to the current conservation equation for conductive media in the frequency domain:

$$\nabla \vec{J} = Q_{j,v}$$

$$\vec{J} = \sigma \vec{E} + j\omega \vec{D} + \vec{J}_e$$

$$\vec{E} = -\nabla V$$

$$\vec{D} = \varepsilon_0 \varepsilon_r \vec{E}$$

with $\vec{J}$ the total current density ($A.m^{-2}$), $Q_{j,v}$ the volumetric current source ($A.m^{-3}$), σ the electrical conductivity ($S.m^{-1}$), $\vec{E}$ the electric field ($V.m^{-1}$), $\vec{D}$ the electric displacement field ($C.m^{-2}$), $\vec{J}_e$ the externally applied current density ($A.m^{-2}$) and V the electric potential (V). The model then computed the sensitivity field S according to equation (22) assuming the culture chamber was filled with an electrical conductivity standard ($\sigma = 20 \text{ mS.cm}^{-1}$ and $\varepsilon_r = 80$)

Culture chamber height effect on impedance measurement

The channel and electrode geometries were implemented in COMSOL MULTIPHYSICS. It was then meshed using tetrahedral elements with an element size set to fine. This resulted in a mesh of 27,260 elements with an average quality of 0.64. The impedance

spectrum was then calculated using the electrical currents interface of the AC/DC module according to the current conservation equation for conductive media in the frequency domain as previously described. The volume impedance, assuming the culture chamber was filled with an electrical conductivity standard ($\sigma = 20 \text{ mS.cm}^{-1}$ and $\epsilon_r = 80$), between the two PU electrode was subsequently calculated as:

$$|Z| = \left| \frac{V_{PU1} - V_{PU2}}{I_{CC2}} \right|$$

with V_{PU1} the voltage at the left PU electrode, V_{PU2} the voltage at the right PU electrode and I_{CC2} the current at the grounded CC electrode. The culture chamber height was set to vary between 400 and 700 μm at intervals of 50 μm .

Electrode / PDMS alignment effect on impedance measurement

The influence of PDMS alignment on the impedance measurement was investigated with the same numerical model as the influence of culture chamber height and the resulting impedance was calculated using equation (28). However, the culture chamber height was fixed to 600 μm and the PDMS was subsequently moved according to the x- and y-alignment errors ranging from -1 to +1 mm in both directions at intervals of 50 μm .

Temperature effect on impedance measurement

We implemented the channel and electrode geometry in the numerical model. It was then meshed using tetrahedral elements with an element size set to fine. This resulted in a mesh of 27,260 elements with an average quality of 0.64. The modelling of the temperature dependence of impedance over a 2 h period was carried out according to the linearised resistivity equation (dynamical model of conductivity changes with respect to temperature) using the electric currents interface of the AC/DC module:

$$\sigma = \frac{1}{\rho_{ref} * (1 + \alpha_\rho * (T - T_{ref}))}$$

with ρ_{ref} ($\Omega.m$) the resistivity at the reference temperature T_{ref} ($^{\circ}\text{C}$) and α_ρ the temperature coefficient of resistivity. As $\sigma = \frac{1}{\rho}$, we can derive $\alpha_\rho = -\alpha_\sigma$ from equation (29).

To model the change of temperature of the culture chamber, we utilised the ‘Heat transfer in solids (ht)’ interface from the Heat Transfer module of COMSOL Multiphysics under stationary conditions:

$$\rho C_p \vec{u} \cdot \nabla T + \nabla \cdot \vec{q} = Q + Q_{ted}$$

and

$$\vec{q} = -k \nabla T$$

with ρ the material density ($\text{kg} \cdot \text{m}^{-3}$), C_p the specific heat capacity at constant pressure ($\text{J} \cdot \text{kg}^{-1} \cdot \text{K}^{-1}$), $\vec{u}$ the velocity field ($\text{m} \cdot \text{s}^{-1}$), T the temperature (K), $\vec{q}$ the conductive heat flux vector ($\text{W} \cdot \text{m}^{-2}$), k the thermal conductivity ($\text{W} \cdot \text{m}^{-1} \cdot \text{K}^{-1}$), Q the volumetric heat source ($\text{W} \cdot \text{m}^{-3}$), and Q_{ted} the thermoelectric damping term ($\text{W} \cdot \text{m}^{-3}$). The initial temperature of the culture chamber was set to 293.15 K (20 °C) while the outside temperature was set to 310.15 K (37 °C) to represent the incubator’s temperature. The heat flux was then defined as a convective heat flux:

$$q_0 = h(T_{ex} - T)$$

with h the heat transfer coefficient of the material.

We extracted the resulting impedance as we did in our previously described our numerical **model 1**, with the volume impedance between the two PU electrodes being calculated using equation (28). The parameters used in the simulation are recapitulated in **Table 15**.

Design and printing process:

All the components were conceptualised using Autodesk Fusion 360 Computer-Aided Design (CAD) software (version 2.0.20256 x86_64). The 3D files were then exported in STereoLithography (STL) format and prepared for printing in the CHITUBOX Basic software (version 2.1). Specifically, the parts to be printed were virtually positioned on the printing plate and a raft was placed as a base to facilitate detachment. Furthermore, pillars were placed as a support to ensure the print of all the parts’ details. The printing parameters were then chosen depending on the resin used (**Table 5** and

Table 6) and a '.fictor and .nauta' and '.ctb' file was created and transferred to the printer, based either on SLA (DWS, Thiene, Italy, XFAB 2000) or LCD (Phrozen, Hsinchu, Taiwan, Sonic Mini 8K) technology.

Once printing was complete, the parts were lifted off the printing plate and separated from the construction pillars. The post-printing process depended on the resin used. Briefly, the parts were cleaned with isopropyl alcohol (IPA) (VWR, West Chester, Pennsylvania, United States, 20922.368) in a propeller bath (Formlabs, Somerville, Massachusetts, United States, Form Wash) or ultrasound cleaner (Vevor, France, 020), followed by drying with compressed air. The parts were then left to dry for about 30 min, before being put in a UV oven (Formlabs, Form Cure) to reticulate the resin. To ensure the manufacturer's properties of each resin, including biocompatibility, one single vat was associated to each resin, and IPA used for cleaning was not mixed between resins. Both temperature and hygrometry were recorded regularly in the printing room over a 3-month period using a digital thermometer and hygrometer (Atome 3D, Toulouse, France, AFAI1040). The obtained temperature was stable over time with an average of 20.82 °C as the laboratory is heat-regulated, with only local variations mainly due to day/night cycle and current weather. The hydrometry increased over time due to the seasonal weather changes, with an average of 44.97%.

Characterisation platform design:

A characterisation platform (CP) was designed and produced both in horizontal and vertical orientations to assess the dimensional precision of the printer. Protrusions and cavities were designed to assess the printing precision both in the positive and negative directions, ranging from 50 to 5000 µm in height for 2 mm constant width (for 10 mm base length). Cylindrical and punch-shaped features were incorporated to assess the integrity of pillars and holes, as well as the precision in diameter dimensions, ranging from 50 to 5000 µm in diameter for 2 mm constant height. The platform also included a designated flat surface area without any specific pattern for conducting average surface roughness measurements. The platforms were printed in triplicates, for each printer and resin (DWS: Precisa 780; Phrozen: TR300 Ultra-High-Temp).

Characterisation of fabrication precision using a digital microscope:

The dimensions of the produced CPs were measured using a digital microscope (Keyence, Osaka, Japan, VHX-7000) also allowing 3D profiles of the imaged object. Protrusions, cavities and angles were imaged as 3D acquisitions, using a 100X magnification, with complete annular, complete coaxial, and lower annular illumination. The cylinders and punches were imaged as 2D acquisitions using a 50X magnification with complete annular illumination. For all the acquisitions, the whole range of a pattern type was imaged all at once by assembling images along a 40 mm linear track. From the raw pattern measurements, the absolute error compared to the design was calculated for all pattern sizes. The measurements of the protrusions and cavities heights were considered characteristic of the printers and resins precision in both Z-axis (height) and XY-axis (surface pattern), for horizontal and vertical printing orientations, respectively. Protrusions were considered positive patterns and cavities negative. Following the characterisation of the dimensions of the produced CPs, the microfluidic devices were assembled and closed using the closing plates. The culture chambers were then imaged as 3D acquisitions using a 100X magnification with complete annular illumination to extract their resulting height.

Profilometer surface roughness evaluation:

Surface roughness of CPs was evaluated using a stylus profiler (KLA Instruments, Milpitas, California, United States, Alpha-Step® D-600 Stylus) with a tip diameter of 2 μm . The measurements were realised with a stylus force of 15 mg, over a length of 1 mm and at a speed of 0.05 mm/s. For each platform, six measurements (three horizontal and three vertical measurements) were carried out. All the profiles were then determined and analysed using a custom-made Python script to extract the average roughness Ra based on the definition by The American Society of Mechanical Engineers.²⁶⁵

Sudan Black B and Parylene C device treatment:

0.3 % (w/v) Sudan Black B (SBB) (Sigma-Aldrich, St. Louis, Missouri, United States, 199664-25G) solution was prepared as described.²⁰⁴ The 3D-printed objects were exposed to O₂ plasma for 60 s and immersed in the SBB solution overnight at 4 °C. After staining, the objects were rinsed in 1X Phosphate-Buffered Saline (PBS) (Gibco, New York, United States, 14190-144) three times, then sterilised by UV exposure for 30 min. Thin film Parylene C

coating was done by depositing a 2 μm thick Parylene C (Specialty Coating Systems, Indianapolis, Indiana, United States 28804-46-8) film using the SCS Labcoter 2 Parylene C deposition system (Specialty Coating Systems, PDS 2010). Briefly, 4g of DPX-C was placed in a vaporizer under vacuum. The process began at a base chamber pressure of 7 mTorr, and the furnace was heated to a temperature of 690 $^{\circ}\text{C}$, after which the vaporizer was ramped up to 175 $^{\circ}\text{C}$ to sublime the dimer which is then deposited onto the component.

Resin cytotoxicity assessment using resazurin assay:

To assess the cytotoxicity of the treated and untreated 3D printed components, wells based on the size of a 96-well plate (Falcon, 353072) made from 3D printing resins were created. 3D-printed inserts of each condition (BMC, BMC + SBB, BMC + SBB + Parylene C) were produced to fit into a 48-well plate (Falcon, 353078) to replicate a 96-well plate well's dimensions. The dimensions of the insert were as follows: 9.70- and 10.00-mm bottom and top outer diameters; 6.35 and 6.85 mm top and bottom inner diameters, respectively. The inserts were bonded to a 48-well plate using PDMS. 1.7×10^4 HPAC-eGFP cells (see **Human pancreatic cancer cell line** section) were seeded and cultured for 48 h. Follow-up imaging was carried out at 24 and 48 h. After 48 h, a resazurin viability assay was performed and a double absorbance reading at 595 and 570 nm was taken after 16 h. The 570 nm reading was subtracted from the 595 nm reading and the values were compared with the control, which consisted of cells cultured with no 3D-printed components. A 10 % (v/v) resazurin solution (150 mg/mL resazurin (Sigma-Aldrich, #R7017), 25 mg/mL methylene blue (Sigma-Aldrich, #MB-1), 329 mg/mL potassium hexacyanoferrate (III) (Sigma-Aldrich, #P8131) and 422 mg/mL potassium hexacyanoferrate (II) trihydrate (Sigma-Aldrich, #P9387)) was used.

Resin fluorescence assessment:

To assess the fluorescence of the treated and untreated 3D printed components, 3D printed pellets of each condition (BMC, BMC + SBB, BMC + SBB + Parylene C) were produced with dimensions as follows: 8 mm in diameter and 2 mm thick. Subsequent imaging was performed using an automated fluorescence microscope (ZEISS, Oberkochen, Germany, Cell Discover 7) using three laser wavelengths (388, 469 and 567 nm) and brightfield imaging to measure the mean pixel intensity.

Microfluidic system fabrication process:

Aluminium components were made by machining aluminium using a Computer Numerical Control (CNC) milling machine (Datron, Annecy, France, Datron Neo). The aluminium plate (RS, 188-321) was placed in the milling machine and held in place with a vacuum plate. The desired patterns were then produced according to the previously created Fusion 360 design files using micro-end mills (Datron, 0068020E). The upper closing plate was 3D printed in BMC resin. The lower part of the microfluidic chip was fabricated in polymerised PDMS by mixing 10:1 (w/w) ratio Sylgard 184 Silicone Elastomer with the corresponding curing agent. The solution was then mixed and degassed for 30 min. The solution was then poured into the master moulds, which were closed using a set of 3D printed rings that held a 200 µm thick PVC sheet (Fellowes brands, Itasca, Illinois, United States, CRC53761), ensuring a smooth surface after demoulding. The mould was then degassed for an additional 20 min, then placed in an oven (Virtus GmbH, Hamm, Germany, 5695TD) at 70°C for 3 h. Once cured, the PDMS was demoulded using a scalpel and tweezers, and washed for 3 min in IPA using an ultrasonic cleaner. To complete the process, the PDMS chip was bonded to a glass substrate after exposure to an O₂ plasma (Harrick Plasma, Ithaca, New-York, United States, PDC-002-CE) for 60 s and subsequently placed on a hot plate at 120°C for 2 min.

Microfluidic setup:

The microfluidic device and circuits were prepared, washed and sterilised with 70% ethanol (v/v) prior to each experiment. One circuit consisted of a 16-in-1 connector for connecting the eight pairs of fluidic inputs and outputs of the device, a glass culture medium reservoir (MOD'VERRE, Grasse, France, custom-made), 1.6 mm tubes (Ibidi®, Gräfelfing, Germany, 10842), 0.5 to 1.6 mm adaptors (Ibidi®, 10829), all of which are coated with a 2 µm thick Parylene C coating, and the peristaltic pump (Ismatec™, Glattbrugg, Switzerland, ISM930A). The circuits were closed without the devices at first and connected to the running peristaltic pump (Ismatec™, IPC-N8 ISM936) to fill the tubing with culture medium. Then, the device was carefully closed and constrained using the screwed closing plates. After cell, the reservoirs were filled with 3.5 mL of culture medium. The circuits were then connected to the device and the peristaltic pump (0.5 mm tubing, Ibidi®, 10840) for flow actuation.

Peristaltic pumps flow rate assay:

Flow meters (Fluigent, Flow sensor M) were fitted in the microfluidic circuit after the outlet of the microfluidic chip, and the circuit was subsequently filled with 3 mL of water. Flow was established using both peristaltic pumps (Ismatec, 8- and 24-channels) at four indicated flow rates (3.74, 10, 25 and 50 $\mu\text{L}\cdot\text{min}^{-1}$). The actual resulting flow rates were then measured using the flow meters.

Fluorescein flow rate assay:

An in-line injection port (Ibidi®, Germany) was fitted in the microfluidic circuit at the inlet of the 16-in-1 connector, and the circuit was subsequently filled with 3 mL of DI water. A solution of DI water containing fluorescein powder (Sigma-Aldrich, USA) was prepared at $4.26 \times 10^{-10} \text{ mol}\cdot\text{m}^{-3}$. The solution was aspirated in a 1 mL syringe which was fitted with a needle (Terumo®, Japan) used to pierce the silicone septum to inject a 100 μL bolus of the fluorescein solution. The flow was established by an IPC High precision multichannel pump (Ismatec®, Switzerland) at the prescribed flow rate. Flow was established from 10 min to 1 h depending on the flow rate. Imaging was performed on a DMI8 microscope (Leica Microsystems, Germany). Two zones of interest of similar size were placed at the beginning and end of the channel, allowing their average fluorescence intensities to be measured over time, enabling the average flow velocity to be extracted.

Human pancreatic cancer cell line:

Cell line was cultured at 37°C in a 5 % CO₂ humid atmosphere incubator. The HPAC pancreatic cancer cell line (ATCC® CRL-2119™) was cultured in 1X-DMEM medium (Dulbecco's Modified Eagle Medium, Gibco, 41965-039), supplemented with 10% (v/v) Foetal Bovine Serum (FBS) (Gibco, 10270-106), 2 mM Glutamine (Gibco, 25030-024), and penicillin (100 IU/mL) – streptomycin (100 $\mu\text{g}/\text{mL}$) (Gibco, 15140-122). The HPAC cell line was genetically modified to respectively express the green fluorescent protein (HPAC-eGFP) (Vect'UB vectorology platform, TBM Core, UMS3427, University of Bordeaux). This cell line was selected for its epithelial phenotype, enabling the formation of stable monolayers with well-developed intercellular junctions.

2D culture in the microfluidic device:

To evaluate the proliferation of HPAC-eGFP cells in 2D under different flow velocity profiles, the height of the cap was modified to have chambers with heights of 600 μm and 2400 μm , respectively. After trypsin detachment, 100 μL of 1X-DMEM with a concentration of HPAC-eGFP cells of $5 \times 10^5 \text{ cells.mL}^{-1}$ was seeded in the culture chambers. Cells were incubated for 24 h in an incubator to allow proper attachment to the glass substrate, after which a 3.53 $\mu\text{L/min}$ flow rate was delivered with the peristaltic pump. Cells were subsequently cultured for 7 days. Follow up imaging was carried out at days 1, 3 and 7 using an automated fluorescence microscope (ZEISS, Oberkochen, Germany, Cell Discover 7). Nuclei staining was performed using DAPI staining (DNA-intercalating fluorescent dye) and nuclei were counted using Zeiss Zen Blue 3.5 software and the Image analysis pack. Impedance measurement was started 24 h after delivery of the flow and impedance spectra were recorded with a 25 mV alternating voltage in a frequency range of 500 Hz to 60 kHz.

3D culture in the microfluidic device:

50 μL of 50% (v/v) collagen 1/1X-DMEM solution with a cell concentration of 1×10^6 HPAC-eGFP cells were seeded in the culture chamber resulting in a final collagen 1 (354236, BD Sciences, United States) concentration of 1.86 mg.mL^{-1} . To reduce mechanical degradation of the hydrogel, a 70 μm nylon filter was extracted from cell strainers (Falcon, 352350) and shaped precisely to the channel size using a scalpel. Hollow extensions (600 μm high, 1.7 mm long, 3 mm wide, 200 μm thick) were added on the top part design on each extremity of the channel. A cut filter piece was bound on those extensions' bottom surface using BMC 3D printing resin that was solidified by UV reticulation for 1h and treated with Parylene C for toxicity prevention. The chip was incubated for 2 h to allow gel solidification, after which a 3.53 $\mu\text{L/min}$ flow rate was delivered with the peristaltic pump. Cells were subsequently cultured for 7 days. For dynamic drug response assay, 1mL of 5 % (v/v) Triton X-100 (T-9284, Sigma-Aldrich, Missouri, United States) was added to the appropriate culture medium reservoirs on day 7 to permeabilise the cell membrane to induce cellular death. The culture was subsequently maintained for an additional 2 days. Follow up imaging was carried out at days 1, 3, 7 and 9 using an automated fluorescence microscope. Impedance measurement was started 2 h after incubation and impedance spectra were recorded with a 25 mV alternating voltage in a frequency range of 500 Hz to 60 kHz.

Microelectrode system fabrication process:

The microelectrodes were conceptualised in the CleWin4 software (WieWeb, Netherlands) and exported in Graphic Data System (.gds) format. The electrodes were fabricated following a bilayer lift-off lithography protocol as follows: the glass substrate was placed in a piranha solution (3:1(v/v) sulfuric acid + hydrogen peroxide (15269636 and 10687022, Fisher Scientific, New Hampshire, United States)) for 10 min. The substrate was then washed in deionised water and dehydrated at 240°C for 10 min. The substrate was then placed in an acetone (10652241, Fisher Scientific, New Hampshire, United States) bath and in an ultrasound cleaner for 10 min, followed by washing and dehydrating as previously done. A 220 nm thick layer of LOR 3A (Micro resist technology GmbH, Germany) was deposited using a spin coater (EDC-650-8B, Laurell Technologies Corporation, Pennsylvania, United States) (10 sec at 800 rpm with an acceleration of 1000 followed by 60 sec at 3000 rpm) followed by soft bake of 5 min at 190°C. A second 1.5 µm thick layer of S1813 (Micro resist technology GmbH, Germany) was deposited using the spin coater (10 sec at 800 rpm with an acceleration of 1000 followed by 60 sec at 3000 rpm) followed by a soft bake of 1 min 20 sec at 110°C. The photosensitive resins were then exposed to UV light (390 nm) using a maskless lithography system (µPG 501 Maskless Lithography, Germany) with an exposure time of 80 ms. It was then developed in a 45 s developer bath (MEGAPOSIT MF-21 A Developer, Micro resist technology). The Ti/Pt bilayer (20/100 nm) was then deposited with a Tornado E-Beam deposition system (Tornado 406E E-beam PVD system, Polytechnik, Denmark). Finally, the photosensitive resin layers were removed by alternating three sets of 2 min acetone and developer baths. The substrate was then washed using deionised water and dehydrated at 200°C for 10 min.

The soldering paste (SMD291SNL500T4, Chipquick®, New-York, United States) was applied onto the PCB connexion pads using a soldering stencil and a mechanical stretching frame (ZelFlex QR, LPFK, Germany). The PCB was then placed onto the glass substrate and the whole system was placed into a reflow oven (Asscon Quicky 450 VP, PMTECH, United Kingdom). The system was heated to 150 °C over 90 s and then to 175°C for the next 90 s. It was then heated to 249 °C over 60 s, after which the system was cooled down to let the soldering paste solidify.

Impedance measurements:

Impedance measurements were performed using an ultra-compact flexible multichannel 64-channel impedance measurement system (CSX-64, Sciospec Scientific Instrument GmbH, Germany). Impedance data acquisition was performed using Python scripts executed in PyCharm Community Edition 2023.1.1 (JetBrains, Czech Republic). Scripts were run within a Python virtual environment (provided in an online repository, see **Appendix 3**) to ensure consistent dependencies and data acquisition reproducibility.

Electrical impedance measurements were carried out using an alternating voltage excitation of 25 mV amplitude. Frequency sweeps were performed across 50 logarithmically distributed frequency points ranging from 500 Hz to 60 kHz. Measurement intervals and total measurement durations were user-defined in minutes prior to each experiment. Measurements were conducted sequentially across eight individual electrode setups per culture chamber at each user-defined time interval. Impedance data were saved automatically in ASCII text file format (.txt), with each electrode setup producing a separate file at every measurement point, resulting in eight files per chamber per measurement. Files were systematically named and timestamped according to the electrode configuration and measurement interval to facilitate subsequent analysis.

Data analysis was carried out according to previously established impedance spectroscopy methods (**Figure 56**).

Conductivity standards

The microfluidic chip was closed, and the culture chambers were subsequently filled with 60 μL of electrical conductivity standard solution (20, 30 and 50 $\text{mS}\cdot\text{cm}^{-1}$) and 1X-DMEM using a 100 μL micropipette. Impedance spectra were then measured as described previously at room temperature.

Culture chamber height influence

A 3D-printed cap that generated four distinct chamber heights (400 – 500 – 600 – 700 μm) on one chip was fabricated and subsequently used to close the microfluidic chip. The culture chambers were then filled with 60 μL of an electrical conductivity standard ($\sigma = 20$

mS.cm⁻¹ and $\epsilon_r = 80$). Impedance spectra were then measured as described previously at room temperature.

Temperature influence

The microfluidic chip was closed, and the culture chambers were filled with 60 μ L of 1X-DMEM using a 100 μ L micropipette. It was then placed in an incubator (37 °C, 5 % CO₂), after which impedance spectra were measured as previously described over a 2 h period with measurement intervals of 3 min.

Collagen 1/1X-DMEM characterisation

Rat tail collagen 1 (354236, BD Sciences, United States) solutions were prepared at different concentrations. Briefly, culture medium and collagen 1 solutions were placed on ice to prevent hydrogel solidification. They were then mixed at the appropriate concentrations in a 1.5 mL falcon tube (EP0030125150, Sigma-Aldrich, St. Louis, Missouri, United States). The glass substrate was then placed on a cold plate, and the collagen 1 / 1X-DMEM solution was seeded with the appropriate volume using a 100 μ L micropipette. The chip was then closed and placed in an incubator (37 °C, 5 % CO₂) for 2 h to let the hydrogel solidify. Impedance spectra were then measured as previously described.

Cell seeding in collagen 1 ECM

50 μ L of 50% (v/v) collagen 1/1X-DMEM solution with a cell concentration of 1×10^6 HPAC-eGFP cells were prepared similarly to collagen 1 / 1X-DMEM solutions. After trypsin detachment, cells were counted using a Malassez counting cell grid and recovered in culture medium. The culture medium was then removed and replaced with the 50 μ L of 50% (v/v) collagen 1/1X-DMEM solution. 50 μ L of cell/gel mixture was then seeded into the culture chambers as described for the collagen 1 / 1X-DMEM solution. The chip was then placed in an incubator (37°C, 5% CO₂) for 2 h to let the hydrogel solidify. Impedance spectra were then measured as previously described.

Microelectrode cleaning protocol:

After impedance measurement, the microelectrode arrays in each culture chamber were rinsed with three times with 100 μ L of 1X PBS. They were then filled with 150 μ L of 0.25 %

(v/v) trypsin (25200-056, Gibco, New York, United States) and placed in an incubator (37 °C, 5 % CO₂) for 5 min. They were subsequently rinsed with 100 µL 1X PBS and filled with 150 µL of a 0.01% (v/v) acetic acid (10744361, Fisher Scientific, New Hampshire, United States) buffer of pH 3.8 and left at room temperature for 10 min. They were then rinsed with 100 µL of 1X PBS and dried with compressed air.

Electrical conductivity measurements:

Electrical conductivity measurements were carried out using a benchtop conductivity meter (SevenDirect SD23, Mettler Toledo, Ohio, United States) equipped with a 4-pole graphite conductivity cell with a cell constant of 0.57 cm⁻¹ (InLab 731-ISM, Mettler Toledo, Ohio, United States). The cell was calibrated electrical conductivity standards with electrical conductivities of 1.413 mS.cm⁻¹ and 12.88 mS.cm⁻¹ (51302049 and 51302050, Mettler Toledo, Ohio, United States) prior to each measurement session in accordance with the manufacturer's instructions. Measurements were performed in a bain-marie maintained at the temperature of interest and the compensation function of the instrument was disabled to ensure direct comparison of raw conductivity values. Prior to each measurement, the electrode was rinsed thoroughly with deionised water and gently blotted dry with lint-free tissue to avoid cross-contamination.

pH measurements:

pH measurements were carried out using a benchtop pH meter (SevenDirect SD23, Mettler Toledo, Ohio, United States) equipped with pH electrode (InLab Expert Pro-ISM, Mettler Toledo, Ohio, United States). The electrode was calibrated using pH technical buffer solutions with pH values of 4.01, 7.00 and 9.21 (51302069, 51302047 and 51302070, Mettler Toledo, Ohio, United States) prior to each measurement session in accordance with the manufacturer's instructions. Measurements were performed in a bain-marie maintained at 25°C. Prior to each measurement, the electrode was rinsed thoroughly with deionised water and gently blotted dry with lint-free tissue to avoid cross-contamination.

Statistical analysis:

Statistical analyses were carried out using GraphPad Prism 8.0.2 software. Normality and homoscedasticity for the data were checked for each performed statistical test using

quantile-quantile and residual plots, respectively. Depending on these results, appropriate parametric or non-parametric tests were chosen. Errors for the different printers and resins depending on the pattern size were analysed using two-way ANOVA followed by Tukey's tests. The overall printer and resin precision, average surface roughness and the influence of collagen 1/1X-DMEM volume were analysed using one-way ANOVA followed by Tukey's tests. Average assembled culture chamber heights and HPAC-eGFP cells seeding in collagen 1 hydrogel were analysed using an unpaired T-test. HPAC-eGFP cell viability was analysed using a Brown-Forsythe and Welch one-way ANOVA followed by Games-Howell's tests. A $p < 0.05$ value was considered statistically significant and was denoted with '*' for $p < 0.05$, '**' for $p < 0.01$, '***' for $p < 0.001$ and '#' for $p < 0.0001$. Data are represented as mean $\pm$ standard deviation. The number of replicates is described in each figure legend.

Appendix 3: Python scripts and COMSOL Multiphysics models

All Python scripts and COMSOL Multiphysics models used in this thesis have been deposited in an online Zenodo repository: [10.5281/zenodo.15333191](https://doi.org/10.5281/zenodo.15333191).

Appendix 4: Scientific contributions

Original publications

Comparison and characterisation of 3D-printers and resins for the development of a customisable parallelised multi-component microfluidic system for biological applications

F. Royer*, T. Meynard*, R. Houssier*, O. Bajeux, S. Paget, F.Lahdaoui, A.M. Valdivia, N. Maubon, J. Vicogne, I. Van Seuningen*, V. Senez*, submitted to publication.

Scientific meetings

Oral presentations

- **Lille PhD Days, 2022, Lille, France-** presentation in format "My thesis in 180 seconds" (17th and 18th of March 2022), "Development of an instrumented microfluidic culture system to study tumour-stroma interaction and drug sensitivity of pancreatic adenocarcinoma", **Félix Royer**, Thomas Meynard, Isabelle Van Seuningen, Vincent Senez.
- **Journée André Verbert 2023, Lille, France-** presentation + video in english (13th of November 2023), "Development of an instrumented microfluidic system to study chemosensitivity in pancreatic ductal adenocarcinoma", **Félix Royer**, Thomas Meynard, Orégane Bajeux, Robin Houssier, Ørjan Grøttem Martinsen, Isabelle Van Seuningen, Vincent Senez.
- **Lille PhD Days, 2024, Lille, France** (29th – 30th April 2024), "Development of an electrical biosensor in a 3D microfluidic system: study of the influence of the microenvironment on the electrical measurement", **Félix Royer**, Thomas Meynard, Orégane Bajeux, Robin Houssier, Ørjan Grøttem Martinsen, Isabelle Van Seuningen, Vincent Senez.

Poster presentations

- **ONCOLille Days 2022, Lille, France** (2nd to 4th of November 2022), "Development of a microfluidic system to study tumor-stroma interactions and drug sensitivity", **Félix Royer**, Thomas Meynard, Robin Houssier, Isabelle Van Seuningen, Vincent Senez.
- **MPS World Summit 2023, Berlin, Allemagne** (26th to 30th of June 2023), "Development of an instrumented microfluidic system to study chemoresistance in pancreatic ductal adenocarcinoma", Thomas Meynard, **Félix Royer**, Orégane Bajeux, Robin Houssier, Clemens Wagner, Sophie Müller, Sebastian Wegner, Sonia Paget, Audrey Vincent, Alejandra Mogrovejo, Elodie Vandenhoute, Nathalie Maubon, Ørjan G. Martinsen, Isabelle Van Seuningen, Vincent Senez.

- **Club Français du Pancréas 2023, Lille, France** (21st and 22nd of September 2023), "Développement d'un système microfluidique instrumenté pour l'étude de la chimiosensibilité de l'adénocarcinome pancréatique canalaire", **Félix Royer**, Thomas Meynard, Orégane Bajoux, Robin Houssier, Ørjan Grøttem Martinsen, Isabelle Van Seuningen, Vincent Senez.

Appendix 5: Training courses attended

Category: Aide à l'insertion professionnelle

Coaching via des témoignages de docteurs travaillant en R&D d'entreprise (27th March 2024) – 3 credits

Category: Ethique et intégrité scientifique

Validation de MOOC _ Intégrité scientifique dans les métiers de la recherche (1st of September to 31st of August 2024) – 6 credits

Category: Parcours PEARL

Digital identity management & networking (8th and 22nd of February 2022) – 7 credits

Doctoral Research Methodology (16th and 31st of December 2023 – 7 credits

Intellectual property management workshops (21st and 28th of January 2022) – 7 credits

Organize and succeed in one's thesis while communicating effectively with one's supervisors (29th to 30th of March 2022) – 5.3 credits

Category: Poursuite de carrière

Job market for PhDs and career plan (17th January 2024) – 4 credits

Category: Séminaires

Innovation challenge: lead a team project to serve an innovative start-up (13th to 17th of March 2023) – 25 credits

Communication et Séminaires scientifiques

Journée André Verbert 2023 - Colloque des doctorants (13th of November 2023) Lille medicine faculty - Pôle Recherche – 3 credits

Club Français du Pancréas 2023 (21st to 22nd of September 2023) Club Français du Pancréas, Lille, France – 5 credits

Microphysiological Systems World Summit 2023 (26th to 30th of June 2023) Center for Alternatives to Animal Testing, Berlin, Germany – 9 credits

Appendices

Training courses attended

ONCOLille Days 2022 (2nd to 4th of November 2022) OONCOLille Institute, Lille, France – 8 credits

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Appendix 5: Original paper

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ARTICLE

Design of a microfabrication pipeline combining PDMS soft lithography and vat photopolymerization for the engineering of various microfluidic systems to study tissues and multicellular organisms

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Despite its capability to create much more realistic microenvironments for *in vitro* culturing of animal or human biological models, the spread of microfluidic tools in the world of biology and medicine has still not reached the predicted scale. Major obstacles to widespread their acceptance by end-users are manufacturing cost and operational complexity. 3D printing is a technology that is now widely democratised, thanks to its ease of use and very attractive cost/performance ratio. In particular, photopolymerisation through a liquid crystal screen is experiencing very significant growth. Here, we describe the methodology we developed to evaluate this microfabrication technique and selected a photoprintable resin to manufacture a fluidic microsystem dedicated to tissue or micro-organism culture. The first originality of our approach lies in the architecture of the microsystem, which is made up of an elementary culture chamber in two parts, making it very easy to open after the culture period to carry out *ex situ* biochemical analyses. The second one is the nature of the materials used to make up the culture chamber, which consists of polydimethylsiloxane and a photoprinted resin. This hybrid assembly, combining an elastomer and a rigid plastic material, ensures a better seal and better dimensional control once the assembly is complete. We demonstrate the ability of our microfabrication protocol to flexibly design different culture chambers through three applications: a 2D layer of cell lines, a parasitic worm and a 3D microtissue from a pancreatic cancer patient.

Introduction

The technology of three-dimensional fluidic microsystems makes it possible to manufacture culture chambers with 3D geometries for the dynamic study of living structures:¹ single cells,² assembly of cells in 2D or 3D configurations using a bottom-up approach (i.e. creation of a tissue from cells)³ or a top-down approach (i.e. culture of explants, chorioallantoic membrane of the hen's egg),⁴ or even multicellular organisms (i.e. worms, embryos).⁵ These fluidic microsystems are extremely powerful tools if they possess the several essential attributes: biocompatibility, transparency in visible wavelengths, low autofluorescence, geometric modularity in three dimensions, manufacturing process offering high reproducibility and making it possible to address dimensions of the order of several hundred microns, low dead volume, scale-

up manufacturing process and ease of implementation.

Although they display many of these characteristics, silicon/glass technologies derived from microelectronics are limited to the production of 2.5D structures with thicknesses of less than 100 microns, and their cost is incompatible with the mass production of disposable objects dedicated for biological and medical applications.

Polymer moulding microtechnologies (hot embossing, injection moulding, elastomer casting) extend the range of structural materials, reduce manufacturing costs and enable the production of truly 3D objects.⁶ These methods are based on the replication of a master structure (the geometric inverse of the desired structure). The mould is generally an object made of metal, silicon, glass or even polymer. It can be produced either by mechanical^{7–9} or thermal¹⁰ material removal methods or by chemical¹¹ or 3D printing^{12–14} additive techniques.

The development of new optical methods has led to advances in 3D printing processes based on photopolymerisation.¹⁵ Early micro/nano-stereolithography technologies involved manufacturing an object voxel by voxel and required very long printing times.^{16,17} Today, micromirror matrix (DMD)¹⁸ or liquid crystal display (LCD)¹⁹ devices enable the voxel-by-voxel processing to be parallelized. In the latter case, the LCD screen acts as an optical mask, which minimizes

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optical distortion, but has the disadvantage of absorbing a large proportion of light energy (around 90%).²⁰

New printable polymer materials appear every year. They are distinguished by different characteristics such as their biocompatibility, their optical and mechanical behaviours. These properties depend on their chemical composition but also on the printing conditions: dissolved oxygen level, annealing temperature, exposure time, object size, thickness of the layer to be polymerised, orientation of this layer, post-printing treatment. The American scientific organisation U.S. Pharmacopeia has defined test standards to validate the use of a material in the fields of medicine and life biology.²¹ These standards have been adopted by the International Standards Organisation as the ISO 10993 family of standards.²² However, the specifications provided by manufacturers of biocompatible photoprintable resins do not give the tests that have been carried out to qualify the material.²³ It is therefore essential to carry out these tests when developing a microfabrication process.

In this article, we present our methodology to design an optimised microfabrication pipeline capable of generating a wide variety of device geometries dedicated to the culture of various micro-tissues or multicellular organisms. This work is part of a series of recent studies demonstrating the value of LCD photopolymerisation technology for the micro-engineering of *in vitro* devices to study living objects.^{24–26} We first compared the performance in terms of precision, reproducibility, roughness and flatness of two printers, one using UV laser scanning and the other using LCD display as light sources. We then repeated these tests with LCD technology for four commercially available resins. The biocompatibility and autofluorescence were also assessed. A microfluidic culture platform was finally designed to demonstrate the flexibility of this microfabrication process.

Results

Comparison of LCD- and SLA-based 3D printing technologies

Two commercially available 3D printers were evaluated to determine which produces the most precise pattern dimensions: the DWS XFAB 2000, which utilises SLA technology (Fig. S1.a), and the Phrozen Sonic Mini 8K, which employs LCD technology (Fig. S1.b). Their specifications are defined in Table S1. The aim was to identify the 3D printer most capable of creating components with the highest geometrical accuracy. To this aim, we used a digital microscope to determine the fabrication precision of a characterisation platform (CP) made of geometrically defined positive and negative motifs (Fig. 1.a). We based our analysis on indicators such as printing orientation, pattern type and height (Fig. 1.b).

The averaged measured error in pattern height demonstrated that the two printers could not be significantly discriminated in accuracy for positive patterns: -120.1 ± 5.0 (LCD) and -152.3 ± 20.3 (SLA) μm for Z+; -33.9 ± 0.4 μm (LCD) and -29.6 ± 2.3 μm (SLA) for XY+ (Fig. 1. c and e). However, for negative patterns, the accuracy was significantly better for the LCD-based printer compared to the SLA: -9.4 ± 3.8 μm (LCD) and -52 ± 10.8 μm (SLA) for Z-, respectively; and -11.5 ± 2.2 μm (LCD)

and -16.7 ± 0.7 μm (SLA) for XY- (Fig. 1. d and f). The measured error depending on pattern design height indicated that the LCD printer generated less error in Z-axis patterns, even if only a few points were found to be significantly different. Interestingly, for the precision in Z- indicator, the difference in error increased with design height. However, it was more difficult to determine a tendency for the XY- patterns as the two printers yielded similar accuracy.

The area indicator used to represent the curvature of the CPs was found significantly lower when printed vertically compared to horizontally: 1.74 ± 0.9 mm^2 (vertical) and 8.77 ± 3.0 mm^2 (horizontal) for LCD-based printer; and 2.53 ± 0.2 mm^2 (vertical) and 27.45 ± 4.2 mm^2 (horizontal) for SLA-based printer. In addition, the LCD-based printer yielded a lower curvature than the SLA, with a significant difference for horizontal printing (Fig. 1.g).

The measured average surface roughness was significantly lower for platforms printed horizontally using the LCD printer: 0.33 ± 0.0 μm (horizontal LCD) compared to 1.78 ± 0.1 μm (vertical LCD), 2.06 ± 0.7 μm (horizontal SLA) and 1.88 ± 0.3 μm (horizontal SLA) (Fig. 1.h). No significant differences were observed for the two printers for vertical print, or for the SLA printer for both orientations. On the images of the platform's surface produced with the LCD printer (Fig. S2). It can be seen that the resin layers were visible for the vertical print but not the horizontal one, which is consistent with the printing process.

In conclusion, the LCD-based printer yielded: (i) a better geometrical accuracy, (ii) a lower curvature and (iii) a lower surface roughness. The LCD-based printer was thus selected for the rest of our study.

Comparison of commercial resins

Four types of resins were compared based on their characteristics: TR300, a high-temperature resistant resin; Aqua-Clear, a transparent resin; Bio-Med Clear, a transparent and biocompatible resin; and Precisa 780, a precise printing resin. Their physical characteristics are recapitulated in Table S2. As with the two 3D printers, the four resins were evaluated for dimensional precision.

The results indicate that Precisa 780 resin (red bar) was significantly less accurate than the other three resins. TR300 resin (grey bar) was significantly more accurate, while Aqua-Clear (blue bar) and Bio-Med Clear (beige bar) resins showed very close in-between error averages (Fig. 2.a, c, e and g). The XY+ indicator can be used as an overview of these results, with -33.9 ± 0.4 μm (Precisa 780), -10.3 ± 0.2 μm (TR300), -22.2 ± 1.8 μm (Aqua-Clear) and -24.1 ± 3.4 μm (Bio-Med Clear) average errors, respectively (Fig. 2.e). Z+ printing is a particular case where the Bio-Med Clear resin displayed similar accuracy to the Precisa 780 resin, with -116.7 ± 7.9 μm and -120.1 ± 5.0 μm , respectively (Fig. 2.a). The TR300 resin was significantly less precise than the three others for the unique case of XY- printing with -18.9 ± 1.3 μm (Fig. 2.d).

To compare the precision of all the resins depending on the design height, a square matrix was created for all heights and concatenated for each indicator (Fig. 2. b, d, f, and h). The

elements above the matrix's diagonal represent the difference in absolute error in pattern dimension between the two compared resins. The colour intensity is proportional to that difference; thus, a negative value means that the first resin yields a lower error. The elements under the matrix diagonal represent the calculated statistical significance of that difference, with a colour intensity inversely proportional to the logarithm of the calculated p-value (with $p_{\text{value}} < 0.05 \Leftrightarrow -\log(p_{\text{value}}) > 1.3$). The results confirmed the tendencies observed with the averages, as the TR300 resin yielded a lower error when compared to the other resins, except for the XY-indicator. Precisa 780 showed a higher error, except when compared to the Bio-Med Clear for Z+ and when compared to the Aqua-Clear for Z-. As for the printers, the difference in error, as well as the significance, did not increase proportionally with the design height.

Similarly to the comparison between the two printers, the vertically printed platforms displayed significantly lower curvature than the horizontal ones. When printed horizontally, the curvature was more important for the Aqua-Clear resin with $38.22 \pm 1.9 \text{ mm}^2$ and less important for the Precisa 780 with $8.77 \pm 3.0 \text{ mm}^2$ (Fig. 2.i). No significant differences were found for the curvature when printed vertically. For the average surface roughness, horizontal printing was significantly lower than vertical printing, with no significant differences between the different resins when printed in the same orientation (Fig. 2.j). As it was not more precise and took longer to print (as it was not adapted to the LCD printer due to its high viscosity), we did not use the Precisa 780 resin for the rest of the work.

To consider the long-term use of the resins as constitutive parts of the microfluidic device and circuit, it was necessary to assess their cytotoxicity. For that, 3D-printed inserts were designed and fabricated to reproduce the dimensions of a 96-well plate well when inserted into a 48-well plate (Fig. 3.a and b). Fluorescent HPAC-eGFP and PS-1-TD Tomato cells were seeded into the inserts and cell proliferation and viability were evaluated over 48 h. The results indicate that Aqua-Clear and TR300 resins negatively impacted the growth of both cell lines, with an obvious reduced number of cells when compared to the control in regular 96-well plate (Fig. 3.c and e). This was confirmed by the cell viability measurements with the resazurin assay showing 2-3% and 9-26% of cell viability for Aqua-Clear and TR300, respectively (Fig. 3.d and f, Table S3). HPAC-e and PS-1-TD Tomato cultured in Bio-Med Clear showed similar morphology compared to the control at 24 h (Fig. 3.c and e). Cell viability was 77.8% and 82.0% for HPAC-eGFP and PS-1-TD Tomato compared to control at 48 h, respectively (Fig. 3.d and f, Table S3), which was still unsatisfactory for cell culture purposes (also non-viable parasite culture, data not shown).

In conclusion, the TR300 resin yielded better dimensional accuracy, lower curvature and surface roughness. It shows however an important cytotoxicity and was thus rather selected to produce our polydimethylsiloxane (PDMS) moulds. On the other hand, the Bio-Med Clear resin showed the best results on cell growth and viability and was thus selected to produce 3D printed constitutive parts of the microfluidic device.

Pretreatment of Bio-Med Clear to reduce autofluorescence and cytotoxicity

To reduce residual cytotoxicity and autofluorescence of Bio-Med Clear components (Fig. 3.c), surface treatments were performed on the printed inserts. The resin was first exposed to an oxygen (O_2) plasma followed by an immersion in a SBB solution, after which the resin was coated with a 2 μm thick Parylene C film (Fig. 4.a).

The results indicate that the emission intensity of Bio-Med Clear resin at 388 nm was strongly reduced with SBB and with O_2 plasma prior to SBB treatment, when compared to untreated Bio-Med Clear resin. However, saturation was observed at this wavelength for the untreated condition. The reduction in autofluorescence at this wavelength (at least divided by 3) cannot therefore be quantified. (Fig. 4.b). This decrease in autofluorescence was confirmed with a reduction of 91% and 97% (with O_2 plasma) at 469 nm (Fig. 4.c) and a reduction of 85% and 90% (with O_2 plasma) at 567 nm (Fig. 4.d). These results were confirmed with the calculation of the areas under the curves, validating the significant efficiency of the SBB treatment (Fig. S3).

After 48 h, cell viability was 70% for HPAC-eGFP and 78% for PS-1-TD Tomato for SBB treated inserts. However, this was increased following the Parylene C coating, which ensured high cell viability in both cell lines, reaching 90% for HPAC-eGFP and 97% for PS-1-TD Tomato (Fig. 4.e and f, Table S4). Importantly, Bio-Med Clear treated with both SBB and Parylene C resulted in both reduced autofluorescence and maintenance of cell morphology when compared to the control (Fig. 4.g). In conclusion, the best strategy to reduce autofluorescence and cytotoxicity of the Bio-Med Clear components is a treatment process with O_2 plasma, followed by SBB treatment and final Parylene C coating.

Development of custom 3D printing components to produce a parallelised microfluidic chip

After validating the use of TR300 resin for PDMS moulds and treated Bio-Med Clear resin for incorporated 3D components, a microfluidic chip and its corresponding closing system were designed (Fig. 5.a and b). To maximise its efficiency and usability, the design featured eight independent and rectangular channels (Fig. 5.c, f and g). The chip can be divided into two distinct parts: a 3D printed cap (Fig. 5.d) and a PDMS bottom (Fig. 5.e). This structure allows easy access to the biological sample at any time-point of the culture period. To ensure that the microfluidic chip is leak-proof, the PDMS bottom was bonded to a 1.00 mm thick glass substrate after O_2 plasma, and each channel has a surrounding sealing border. The inlets and outlets have an inner diameter of 2.00 mm and are spaced by 17.98 mm centre to centre while all channels are 8.99 mm apart (Fig. 5.f and g).

Once closed, the culture chamber (yellow), where the biological components are loaded, is created. Its dimensions are 15.01 mm long, 3.00 mm wide, and 0.60 mm high (Fig. 5.h and i). To ensure compatibility with modern robotics and microscopes, each culture chamber was designed to fit a 96-well plate SBS format (Society for Biomolecular Screening) (Fig.

5.j). A custom plate adaptor was also developed (Fig. S4) to facilitate the placement of the device under a microscope.

Flat surface is required for our system to avoid leakage when parts are assembled. Moulds were modified and 3D-printed rings were designed to hold a Polyvinyl Chloride (PVC) sheet over the mould during the reticulation process (Fig. 6.a). This enabled us to obtain the desired flat surface for the bottom part.

The height of the culture chamber is the crucial dimension in the closed microfluidic chip, as it influences the physical constraints imposed on the biological model. The height depends on the 3D printed cap height, the PDMS bottom part height, both influenced by the characterised precision of the fabrication protocols, and the precision of the closing system (Fig. 6.b). If necessary, it can be adjusted based on the biological application by increasing the depth at which the 3D-printed cap penetrates in the PDMS bottom part. Similar to the characterisation of the dimensional precision of the printers and resins, the error in height was measured on the cap and bottom of the device and compared to the error measured for the assembled culture chamber height. The results indicate that the cap and PDMS bottom part fabrication error was on average -5.98 ± 2.4 and -42.63 ± 15.8 μm , respectively (Fig. 6.c and Fig. 6.d), which fits with the previously measured fabrication errors (Fig. 2). From these measurements, the expected culture chamber height h_{expected} (μm) of four pairs of caps and PDMS parts was calculated with the formula:

$$h_{\text{expected}} = 600 + \text{error}_{\text{PDMS}} - \text{error}_{\text{cap}} \quad (1)$$

where $\text{error}_{\text{PDMS}}$ and $\text{error}_{\text{cap}}$ are the mean errors measured on the bottom and top parts, respectively.

The results indicate that on average, the expected culture chamber height was 638.6 ± 15.3 μm . Although this differed from the measured culture chamber height of 606.2 ± 14.6 μm (Fig. 6.e), it validates the design and precision of the closing system components.

3D printing process and microfluidic setup to address different biological culture applications

After designing, producing and dimensionally characterising the parallelised microfluidic chip, we assessed the ability of 3D printing to produce components of the microfluidic circuit. The dimensional characterisations revealed that it is necessary to print hollow parts horizontally to obtain the required diameter. This approach aimed not only to produce custom-made components for the microfluidic circuit, but also to utilise the modularity offered by 3D printing to adapt the geometry to different biological applications.

A 16-in-1 microfluidic connector (Fig. 7.a), a tubing connector (Fig. 7.b) and an 8-tank holder (Fig. 7.c) for custom-made glass tanks were designed. All the elements composing the microfluidic system are presented (Fig. 7.d), with the connector fitted on the cap allowing the connection between the microfluidic channels and the circuit (Fig. S5).

3D printing shows a high modularity and allows us to easily customise the geometry of the microfluidic chip for specific applications. We therefore produced: (i) a 2D cell culture

geometry with modifiable height (Fig. 7.e), (ii) a parasite culture geometry to host whole living organisms (Fig. 7.f) and (iii) a 3D co-culture geometry based on a 96-well plate dimensions (Fig. 7.g). Three different biological models were cultivated using these geometries: (i) a 2D co-culture of epithelial HPAC-eGFP and fibroblastic PS-1-TD Tomato pancreatic cancer cells with two different culture chamber heights (600 and 2400 μm) to produce different flow velocity profiles (Fig. 8.a), (ii) a *Schistosoma mansoni* adult worms culture (Fig. 8.e) and (iii) a 3D co-culture of PaTa-1818x pancreatic patient-derived tumoroids with fibroblastic PS-1-TD Tomato cells in a macroporous matrix (Fig. 8.h).

The flow velocity v ($\mu\text{m/s}$), assumed to be uniform on the channel's cross-section, is calculated as :

$$v = 1000 \frac{Q}{60S} \quad (2)$$

where Q ($\mu\text{L/min}$) is the flow rate and S (mm^2) is the surface of the cross section of the rectangular channel.

This allowed us to tune the system design, either the channel height or width, to obtain different flow velocities in the culture chamber and to match the physiological flow conditions of the targeted biological application (Table S5).

In the first geometry, a morphological difference could be observed between the two channel heights, with fibroblastic PS-1-TD Tomato cells being more activated (larger membrane extensions) in the 2400 μm culture chamber (Fig. 8.b and c). With the 600 μm height channel (34.63 $\mu\text{m/s}$ averaged flow velocity), nuclei counting showed a higher number of HPAC-eGFP cells at day 7 in coculture when compared to the monoculture (day 0 and 3 available in Fig. S6). With the 2400 μm height channel (8.66 $\mu\text{m/s}$ averaged flow velocity), the number of cells was quite similar in mono- and co-culture, except at day 7 where it was less important in co-culture. Moreover, more cells were observed in both mono- and co-culture in the 2400 μm height channel compared to the 600 μm (Fig. 8.c and d).

For the culture of schistosome parasites, one couple of paired adult worms was cultivated in each channel for eight days inside the microfluidic device and compared to a control well plate. The condition of the worms along the culture was determined every 24 h using a five-level scoring method (from "Dead" 0 to "Normal" 4) (Table S6). The parasites at day 0, 1 h after seeding were given a perfect score of 4/4 as they displayed a healthy phenotype with active movements and correct adhesive capacity (Fig. 8.f). They stayed paired for the whole duration of the recording and stayed attached to the glass surface even when the device was turned upside-down. The same schistosome pair at the end of the experiment (day 8) was given a degraded score of 2/4 as their mobility was strongly reduced and they were detached from the surface, in addition to being unpaired (Fig. 8.f). During the culture, the perfect score of 4/4 could be maintained for at least one day. The worms were then kept in an excellent condition with a score greater than 3.75/4 for at least two more days (Fig. 8.g). As of day 4, a non-negligible degradation of the worms' condition was

observed, after which the score decreased linearly from 2.75/4 to 2.13/4 at day 8 (overall -0.25 score per day). This represented a significant difference with the control.

The third model aimed at developing a chip to coculture patient-derived pancreatic tumoroids with pancreatic activated stellate cells within a macroporous matrix. To this aim, the geometry of the culture chamber was transformed from a rectangular shape to a circular configuration (**Fig. 8.i and j**). This modification allowed the seeding of tumoroids and cells in the matrix, their subsequent transfer to the microfluidic device, and cultivation over a 7-day period with direct perfusion across the matrix. Calcein-AM staining was performed to measure viability of cells in the device. After a 7-day culture, a limited number of cells in both PaTa-1818x and PS-1 monocultures could be observed (**Fig. 8.k**) whereas when cocultured, a higher cell density was observed (**Fig. 8.k**).

Discussion

The development of alternative biological models is now driven by the development of microfluidic systems. They aim at mimicking the physicochemical microenvironment of cells but also of more complex and larger organisms. Our aim was to design an easily modifiable and versatile microfluidic device to reliably host various biological models and study their behaviours. As the fabrication of microfluidic systems inherently demands accuracy and reproducibility, along with the use of biocompatible materials, 3D printing has been explored as an alternative fabrication technique to overcome the shortcomings of more traditional methods.²⁷

In this context, we first assessed the precision of SLA- and LCD-based printers using a tailored CP featuring different geometrical patterns and dimensional ranges. Subsequently, we extended this characterisation to four commercially available resins, to choose the best fit for PDMS moulds (TR300) and constitutive parts (Bio-Med Clear) of the developed system. A SBB surface treatment was used to minimise the constitutive parts' autofluorescence,⁴⁵ which facilitated fluorescence-based microscopy imaging. We then assessed the cytotoxicity of the three selected resins. Given the significant residual cytotoxicity observed with the Bio-Med Clear resin, a complementary treatment with Parylene C was evaluated and validated to improve its cytocompatibility, thereby validating its integration in the microfluidic chip. These adaptations leveraged the full potential of 3D printing by fabricating intricate components of the parallelised microfluidic chip and its accompanying circuit. In the end, we obtained a versatile device with three distinct culture chamber geometries that allow studies of different biological questions related to cancer or infectiology, cancer cell behaviour, cell-cell and cell-matrix interactions.

The LCD-based printer has proved easier to use thanks to the overall control over printing parameters provided by the software, contrary to the SLA printer. We successfully adapted the Precisa 780 resin, originally intended for the SLA printer, and used it in the LCD printer to subsequently characterise the printing errors of both printers. However, as the resin is manufactured for a printer with a much more powerful laser

than the LCD's UV lamp, printing times have increased drastically, from 7 h on SLA to 42 h on LCD, making the use of this resin too restrictive. Our characterisation demonstrated that the cheaper LCD-based printer was more accurate in regard with pattern dimension error regardless of printing orientation, which is in agreement with other studies.²⁸

In addition, we evaluated the impact of part orientation during printing. Vertical printing generally resulted in more accurate patterns, which again is in accordance with the literature,²⁸ while horizontal printing allowed to minimise the surface roughness. This enabled us to choose the printing orientation depending on the targeted application. It is generally accepted that the dimensions of microsystems hosting living cells or organisms are usually of the order of 1 to 1000 μm .²⁹ Below 250 μm , our results showed that printing height accuracy is approximate, whereas assembling larger components allowed to form a channel height of 600 μm successfully with low variability. We have also highlighted that the combined choice of printer, resin and printing orientation has major impacts on the undesired curvature of the printed elements, which can lead to dramatically altered shapes and dimensions. By applying our tailored characterisation protocol, we selected the TR300 resin for the fabrication of PDMS mould as it demonstrated superior precision and is temperature resistant which is important for PDMS moulding.

Our work showed that Bio-Med Clear resin was the optimal option for ensuring viability with both cell lines. Although the threshold for cytocompatibility is usually set at 80% for biomedical applications,²⁶ we considered this viability still insufficient for long-term incorporation into the chip and its circuit for culture purposes. Several options were explored to increase the resin's cytocompatibility such as modifying the post-printing washing and curing.³⁰ Rather than drifting from the manufacturer's recommendations, we coated our Bio-Med Clear components with Parylene C. Indeed, Parylene C has previously been shown to improve viability in both short and long-term cell cultures by forming an insulating protective polymer layer that prevents toxicity phenomena induced by the material.³¹ This enabled us to enhance the viability of Bio-Med Clear components to over 90% for cell culture (and viable parasite culture, data not shown), approaching that of traditional 2D *in vitro* culture systems and thus validating our process.³²

We also had to improve the quality of fluorescent imaging as we noticed strong autofluorescence when performing fluorescence-based microscopy with Bio-Med Clear components, questioning its use in a microfluidic chip. We therefore decided to treat the resin with SBB, which had been described to reduce the autofluorescence of polymers either by absorbing emitted photons, or by changing its refractive index.³³ This allowed us to strongly reduce the emission intensity by at least 70% at the wavelengths of interest for these applications, which is consistent with a previously established SBB coating protocol.^{34,45} The images obtained thereafter were more contrasted and allowed precise nuclei counting.

Parallelised microfluidic systems on varying scales (channel number) are a hot topic since they enable a larger number of

experiments or conditions to be carried out simultaneously on more complex models, limiting bias of sequential experiments.²⁷ Our established fabrication protocol enabled the design of a parallelised chip geometry compatible with SBS standard (96 well plate) incorporating a dedicated closing system. Contrarily to commercially available parallelised microfluidic systems, we have shown that we could easily adapt the connection between the circuit and our device, demonstrating the capability of 3D printing to control all aspects of a microfluidic platform compared to other fabrication methods (SU-8 lithography, aluminium milling). This was facilitated by the fabrication of complex 3D-printed components, such as the 16-in-1 connector. To ensure long-term culture, a closed circuit was used featuring an adequate volume of culture medium alongside a peristaltic pump to facilitate the dynamic perfusion within the system. This allowed for the recirculation of the medium, saving a considerable volume (3.5 mL in a closed loop vs. 37.5 mL for an open circuit with 3.74 $\mu\text{L}/\text{min}$ flow rate) and allowing continuous actuation of metabolites.

Our final goal being to design a modular microfluidic device usable for different biological applications in different biological fields, we wanted to have the possibility to easily adapt the geometry of the device by using 3D printing. For this end, we studied the impact of different flow velocities on 2D cultures, previously performed on endothelial cells.³⁵ Our study on epithelial cells showed that their proliferative and adhesive properties were enhanced in the presence of stellate cells or when the flow velocity decreased. By applying different flow velocities, we mimicked the heterogeneous interstitial flows present in solid tumours.^{36,37} Thus, the versatility of our system allows the use of a wide range of flow velocities, and their application to various cell types. In particular, we showed that this system is particularly suited to study and understand the interactions between tumour and fibroblastic cells, with the example of human pancreatic epithelial cancer cells and pancreatic stellate cells.

Another advantage of our system is the possibility to integrate a synthetic macroporous matrix that is not degraded when subjected to a direct flow,³⁸ as it is an important factor in tumour response to treatments and tumour drug resistance.³⁹ This enabled us to implement dynamic culture conditions to our 3D co-culture model of human pancreatic tumoroids and pancreatic stellate cells. In these conditions, tumoroids were more resistant to the applied flow when co-cultured compared to monoculture. This preliminary result opens the way to studies aiming at evaluating the behaviour of tumoroids when subjected to a flow. This device could thus allow more valuable and relevant studies aimed at investigating cell-cell and cell-matrix interactions in a co-culture system subjected to an interstitial flow and measure tumour chemoresistance and aggressiveness when submitted to chemotherapeutic drugs.

Regarding the needs in microbiology and infectious diseases research, the microfluidic device geometry was also adapted to culture *Schistosoma mansoni* parasitic adult worms for several days. Worms have much larger dimensions than tumoroids and are able to move into the system against or with the flow. To

make its integration possible, the culture chamber was delimited by an anti-escape filter in the channel. With that adaptation, the device can be used to study parasite biology and behaviour in dynamic culture conditions, for instance egg production, couple pairing and viability, and worm potential interactions with cells from the host. The study of this parasite in a dynamic *in vitro* environment mimicking a blood vein is highly original. The few works that have been published address the larvae stage.⁴⁰ Our microsystem makes it possible to screen the action of antiparasitic molecules, as has already been done in other microfluidic systems for the parasites *Schistosoma mansoni*⁴¹ or *C. Elegans*.⁴²

As our system is made up of two separable parts, we intend in future works to open it for biological analyses and perform protein or RNA extraction, that are difficult to do when the system is closed. For example, it has been shown that atomic force microscopy analyses can be carried out using an openable device, which is impossible when the system is closed.^{43,44}

Conclusion

We have developed a new versatile 3D microfluidic device, using LCD-based 3D printing with TR300 resin for PDMS moulding and Bio-Med Clear resin for constitutive parts. We treated Bio-Med Clear parts to decrease its cytotoxicity and autofluorescence, and then validated the use of the device for different biological applications. This device is also adapted to modern robotics and microscopes with new and tailored-made culture chamber geometries. Overall, this versatile microfluidic platform can be used to study various complex biological models and establish dynamic culture conditions, allowing to address a variety of use and applications in many biological fields, such as cancer or infectiology.

Materials and methods

Design and printing process

All the components were conceptualised using Autodesk Fusion 360 Computer-Aided Design (CAD) software (version 2.0.20256 x86_64). The 3D files were then exported in STereoLithography (STL) format and prepared for printing in the CHITUBOX Basic software (version 2.1). Specifically, the parts to be printed were virtually positioned on the printing plate and a raft was placed as a base to facilitate detachment. Furthermore, pillars were placed as a support to ensure the print of all the parts' details. The printing parameters were then chosen depending on the resin used (**Table S7**) and a '.fictor' and '.nauta' and '.ctb' file was created and transferred to the printer, based either on SLA (DWS, Thiene, Italy, XFAB 2000) or LCD (Phrozen, Hsinchu, Taiwan, Sonic Mini 8K) technology.

Once printing was complete, the parts were lifted off the printing plate and separated from the construction pillars. The post-printing process depended on the resin used; details are given in a **Table S8**. Briefly, the parts were cleaned with isopropyl alcohol (IPA) (VWR, West Chester, Pennsylvania, United States, 20922.368) in a propeller bath (Formlabs,

Somerville, Massachusetts, United States, Form Wash) or ultrasound cleaner (Vevor, France, 020), followed by drying with compressed air. The parts were then left to dry for about 30 min, before being put in a UV oven (Formlabs, Form Cure) to reticulate the resin. To ensure the manufacturer's properties of each resin, including biocompatibility, one single vat was associated to each resin, and IPA used for cleaning was not mixed between resins. Both temperature and hygrometry were recorded regularly in the printing room over a 3-month period using a digital thermometer and hygrometer (Atome 3D, Toulouse, France, AFA1040). The obtained temperature was pretty stable over time with an average of 20.82 °C as the laboratory is heat-regulated, with only local variations mainly due to day/night cycle and current weather (Fig. S7). The hygrometry increased over time due to the seasonal weather changes, with an average of 44.97%.

System fabrication process

The bottom closing plate and wedge were made by machining aluminium using a Computer Numerical Control (CNC) milling machine (Datron, Annecy, France, Datron Neo). The aluminium plate (RS, 188-321) was placed in the milling machine and held in place with a vacuum plate. The desired patterns were then produced according to the previously created Fusion 360 design files, using micro-end mills (Datron, 0068020E). The upper closing plate was 3D printed in Bio-Med Clear resin. The lower part of the microfluidic chip was fabricated in polymerised polydimethylsiloxane by mixing 10:1 (w/w) ratio Sylgard 184 Silicone Elastomer with the corresponding curing agent. The solution was then mixed and degassed for 30 min. The solution was then poured into the master moulds, which were closed using a set of 3D printed rings that held a 200 µm thick Polyvinyl Chloride (PVC) sheet (Fellowes brands, Itasca, Illinois, United States, CRC53761), ensuring a smooth surface after demoulding. The mould was then degassed for an additional 20 min, then placed in an oven (Virtus GmbH, Hamm, Germany, 5695TD) at 70°C for 3 h as glass temperature of PVC is 73.4°C. Once cured, the PDMS was demoulded using a scalpel and tweezers, and washed for 3 min in IPA using an ultrasonic cleaner. To complete the process, the PDMS chip was bonded to a glass substrate after exposure to an oxygen (O₂) plasma (Harrick Plasma, Ithaca, New-York, United States, PDC-002-CE) for 60 s and subsequently placed on a hot plate at 120°C for 2 min.

Characterisation platform design

A Characterisation Platform (CP) was designed and produced both in horizontal and vertical orientations to assess the dimensional precision of the printer. Protrusions and cavities were designed to assess the printing precision both in the positive and negative directions, ranging from 50 to 5000 µm in height for 2 mm constant width (for 10 mm base length). Additionally, cylindrical and punch-shaped features were incorporated to assess the integrity of pillars and holes, as well as the precision in diameter dimensions, ranging from 50 to 5000 µm in diameter for 2 mm constant height. Angled and inverted-angled structures were employed to evaluate the

accuracy of slope reproduction during printing in both printing orientations, ranging from 100° to 160° and from 20° to 80°, respectively. The details of the size ranges for each pattern type are given in Table S9. The platform also included a designated flat surface area without any specific pattern for conducting average surface roughness measurements. The platforms were printed in triplicates, for each printer and resin (DWS, Precisa 780; Phrozen, TR300 Ultra-High-Temp; Phrozen, Aqua-Clear; Liqcreate, Bio-Med Clear).

Characterisation of printing precision using a digital microscope

The dimensions of the produced CPs were measured using a digital microscope (Keyence, Osaka, Japan, VHX-7000) also allowing 3D profiles of the imaged object. Protrusions, cavities and angles were imaged as 3D acquisitions, using a 100X magnification, with complete annular, complete coaxial, and lower annular illumination. The cylinders and punches were imaged as 2D acquisitions using a 50X magnification with complete annular illumination. Inverted angles were measured in the same manner as cylinders and punches, but with the characterization platform wedged vertically under the objective. For all the acquisitions, the whole range of a pattern type was imaged all at once by assembling images along a 40 mm linear track. Following the measurements, raw data was then exported in Comma Separated Values (CSV) files to be analysed (Fig. S8). From the raw pattern measurements, the absolute error compared to the design was calculated for all pattern sizes (Fig. S9). For the cavities, the measurements corresponding to the 5000 µm height were not considered because for most of the replicates the cavities were breached due to insufficient platform width. The measurements of the protrusions and cavities heights were considered characteristic of the printers and resins precision in both Z-axis (height) and XY-axis (surface pattern), for horizontal and vertical printing orientations, respectively. Protrusions were considered positive patterns as they were additive to the platform bulk, and cavities negative as they were subtractive. That defines the four-dimensional indicators used for the precision comparisons (Table S10). Following the characterisation of the dimensions of the produced CPs, the microfluidic devices were assembled and closed using the aluminium plates. The culture chambers were then imaged as 3D acquisitions using a 100X magnification with complete annular illumination to extract their resulting height.

Curvature characterisation

CPs curvature was analysed using the VHX-7000 microscope using a 50X magnification with complete annular illumination for each replicate. The height profile measurement was performed along a line following the whole edge using the microscope integrated software, and data were directly exported in CSV files. Data were processed automatically using a custom python script that extracts the height profiles and calculates for each one the area between the profile and a straight line connecting its ends by cumulating the point-to-point difference. This area is considered representative of the curvature of the platform (Fig. S10), thus allowing to prevent

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the impact of the imperfect horizontality of the CPs under the microscope objective during the measurement.

Profilometer surface roughness evaluation

Surface roughness of CPs was evaluated using a stylus profiler (KLA Instruments, Milpitas, California, United States, Alpha-Step® D-600 Stylus) with a tip diameter of 2 µm. The measurements were realised with a stylus force of 15 mg, over a length of 1 mm and at a speed of 0.05 mm/s. For each platform, six measurements (three horizontal and three vertical measurements) were carried out (Fig. S11). All the profiles were then determined and analysed using a custom-made Python script to extract the average roughness R_a based on the definition by The American Society of Mechanical Engineers.⁴⁵ The surface roughness was also imaged using the digital microscope (Fig. S2).

Sudan Black B and Parylene C device treatment

0.3% (w/v) Sudan Black B (SBB) (Sigma-Aldrich, St. Louis, Missouri, United States, 199664-25G) solution was prepared as described.⁴⁶ The 3D-printed objects were exposed to O₂ plasma for 60 s and immersed in the SBB solution overnight at 4°C. After staining, the objects were rinsed in 1X Phosphate-Buffered Saline (PBS) (Gibco, Grand Island, New York, United States, 14190-144) three times, then sterilised by UV exposure for 30 min. Thin film Parylene C coating was done by depositing a 2 µm thick Parylene C (Specialty Coating Systems, Indianapolis, Indiana, United States 28804-46-8) film using SCS Labcoter 2 Parylene C deposition system (Specialty Coating Systems, PDS 2010). Briefly, 4g of DPX-C was placed in a vaporizer under vacuum. The process began at a base chamber pressure of 7 mTorr, and the furnace was heated to a temperature of 690°C, after which the vaporizer was ramped up to 175°C to sublime the dimer which is then deposited onto the component.

Resin fluorescence assessment

The emission spectrum of Bio-Med Clear resin was analysed using pellets of the size of a 24-well plate (15 mm diameter and 3 mm height). These pellets were printed, treated or not with SBB and placed in conventional 24-well culture plates (Falcon, 353047). The spectrum was obtained by exciting the resin at 388, 469 and 567 nm, and the emission intensity was read every 2 nm from 408 to 800 nm, 489 to 799 nm and 587 to 799 nm, respectively (Perkin Elmer, Waltham, Massachusetts, United States, EnSpire Multimode Plate Reader).

Resin cytotoxicity assessment using resazurin assay

To assess the cytotoxicity of the resins, wells based on the size of a 96-well plate (Falcon, 353072) made from 3D printing resins were created. 3D-printed inserts of each resin (Aqua-Clear, TR300 and Bio-Med Clear) were produced to fit into a 48-well plate (Falcon, 353078) to replicate a 96-well plate well's dimensions. The dimensions of the insert were as follows: 9.70- and 10.00-mm bottom and top outer diameters; 6.35 and 6.85 mm top and bottom inner diameters, respectively. The inserts were bonded to a 48-well plate using PDMS. HPAC-eGFP (17×10^3) and PS-1-TD Tomato (8×10^3) cells were seeded and

cultured for 48 h. Follow-up imaging was carried out at 24 and 48 h. After 48 h, a resazurin viability assay was performed and a double absorbance reading at 595 and 570 nm was taken after 16 h. The 570 nm reading was subtracted from the 595 nm reading and the values were compared with the control, which consisted of cells cultured with no 3D-printed components. A 10% (v/v) resazurin solution (150 mg/mL resazurin (Sigma-Aldrich, #R7017), 25 mg/mL methylene blue (Sigma-Aldrich, #MB-1), 329 mg/mL potassium hexacyanoferrate (III) (Sigma-Aldrich, #P8131) and 422 mg/mL potassium hexacyanoferrate (II) trihydrate (Sigma-Aldrich, #P9387)) was used.

Microfluidic setup

The microfluidic device and circuits were prepared, washed and sterilised by autoclaving prior to each experiment. One circuit consisted of a 16-in-1 connector for connecting the eight pairs of fluidic inputs and outputs of the device, a glass media reservoir (MOD'VERRE, Grasse, France, custom-made), 1.6 mm tubes (Ibidi®, Grärfelfing, Germany, 10842), 0.5 to 1.6 mm adaptors (Ibidi®, 10829), and the peristaltic pump (Ismatec™, Glattbrugg, Switzerland, ISM930A). The circuits were closed without the devices at first and connected to the running peristaltic pump (Ismatec™, IPC-N8 ISM936) to fill the tubing with medium. Then, the device was carefully closed and constrained using the screwed closing plates. After cell, tumoroid or parasite seeding, the reservoirs were filled with 3.5 mL of the respective culture medium. The circuits were then connected to the device and the peristaltic pump (0.5 mm tubing, Ibidi®, 10840) for flow actuation.

Human pancreatic cancer cell lines

All cell types were cultured at 37°C in a 5% CO₂ humid atmosphere incubator. The HPAC pancreatic cancer cell line (ATCC® CRL-2119™) was cultured in DMEM medium (Dulbecco's Modified Eagle Medium, Gibco, 41965-039), supplemented with 10% (v/v) Foetal Bovine Serum (FBS) (Gibco, 10270-106), 2 mM Glutamine (Gibco, 25030-024), and penicillin (100 IU/mL) – streptomycin (100 µg/mL) (Gibco, 15140-122). Human pancreatic stellate cells (PS-1) (UK Human TissueBank (Ethics approval; Trent MREC, 05/MRE04/82)) were cultured as previously described (Froeling et al. 2009). The HPAC and PS-1 cell lines were genetically modified to respectively express the green fluorescent protein (HPAC-eGFP) and the TD Tomato protein (PS-1-TD Tomato) (Vect'UB vectorology platform, TBM Core, UMS3427, University of Bordeaux).

Human pancreatic tumoroids

The human pancreatic tumoroids PaTa-1818x were cultured at 37°C in a 5% CO₂ humid atmosphere incubator as previously described.⁴⁷ They were mechanically and enzymatically dissociated every fortnight as previously described.⁴⁸

Schistosoma mansoni material and culture

All animal experimentations were conducted in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS no. 123, revised Appendix A) and were approved by the

local committee for ethics in animal experimentation (authorization no. APAFIS #37320-2022051208133098v3) and by the Pasteur Institute of Lille (agreement no. D59350009). A Puerto Rican strain of *Schistosoma mansoni* was maintained in the laboratory using the intermediate snail host *Biomphalaria glabrata* and the definitive golden hamster host *Mesocricetus auratus*. Adult schistosome worms were obtained by hepatic portal perfusion of hamsters, six weeks after infection by cercariae released from infected snails. Perfused worms were maintained as previously described until further use.⁴¹

2D culture in the microfluidic device

To evaluate the proliferation of HPAC-eGFP and PS-1-TD Tomato cells in 2D under different flow velocity profiles, the height of the cap was modified to have chambers with heights of 600 μm and 2400 μm , respectively. After trypsin detachment, cells (2×10^4 HPAC-eGFP and 2×10^4 PS-1-TD Tomato in monoculture; 4×10^3 HPAC-eGFP and 1.6×10^4 PS-1-TD Tomato in coculture) were seeded after complete closure of the device in a culture medium volume of 100 μL . The chip was incubated for 24 h to allow the cells to attach properly to the glass substrate, after which a 3.74 $\mu\text{L}/\text{min}$ flow rate was delivered with the peristaltic pump. Cells were subsequently cultured for 7 days. Follow up imaging was carried out at days 0, 3 and 7 using an automated fluorescence microscope (ZEISS, Oberkochen, Germany, Cell Discover 7). Nuclei (DAPI staining) were counted using Zeiss Zen Blue 3.5 software and the Image analysis pack.

3D culture in the microfluidic device

To enable the culture of PaTa-1818x tumoroids in a macroporous matrix (HCS Pharma, BIOMIMESYS® custom), the geometry of the culture chamber was modified to a circular 96 well plate format (6 mm diameter – 2 mm high). After trypsin detachment, cells (2×10^4 PaTa-1818x and 4×10^4 PS-1-TD Tomato in monoculture; 2×10^4 PaTa-1818x and 4×10^4 PS-1-TD Tomato in coculture) were seeded in 10 μL of culture media in the lyophilised macroporous matrix and incubated for 24 h for proper attachment.⁴⁹ After 24 h, the matrixes were removed from the 96 well plate with a tweezer and placed in the opened microfluidic device culture chamber. The microfluidic device was then closed and connected to the circuit, after which a 3.74 $\mu\text{L}/\text{min}$ flow was delivered with the peristaltic pump. The cells were subsequently cultured for 7 days. Follow up imaging was carried out at days 0 and 7 using the automated fluorescence microscope (ZEISS, Cell Discover 7).

Parasite culture in the microfluidic device

To enable the culture of schistosome adult worms (up to 10 mm long and 500 μm large), a culture chamber (6 mm long, 1 mm high and 3 mm wide) was added at the centre of the channel. The design of the other parts of the closing system were adapted to the change in height of the device. To prevent worms from escaping the culture chamber, a 70 μm nylon filter was extracted from cell strainers (Falcon, 352350) and shaped precisely to the channel size using a scalpel. Hollow extensions (600 μm high, 1.7 mm long, 3 mm wide, 200 μm thick) were added on the top part design on each extremity of the channel.

A cut filter piece was bound on those extensions' bottom surface using Bio-Med Clear 3D printing resin, that was solidified by UV reticulation for 1h and treated with Parylene C for toxicity prevention, to cover the whole culture chamber. 30 μL of medium was then added in the culture chamber before one adult worm pair was deposited in each channel, using a low-retention pinch to minimize worm damage. The circuit was then closed and 3 mL of medium was filled in the tanks, and the peristaltic pump was set to deliver a 73.9 $\mu\text{L}/\text{min}$ flow rate. The worms were monitored every 24 h for 8 days. A control was realised by culturing one adult worm pair per 12-well plate well with 3 mL of medium over the same culture time.

Evaluation of parasite condition by scoring

The viability of the worms was evaluated using a VHX-7000 microscope with a 45s video of the worm pair at a 50X magnification. The condition of the worms was determined using a phenotypic scoring method, a method classically used for the screening of antischistosomal drugs.⁵⁰ A five-level scale was defined: S4 score as normal healthy condition for worms having active movements, correct adhesive capacity, normal tissues and tegument integrity; S3 score as reduced condition was defined by reduction of movement or abnormal ones and/or the loss of adhesive capacity, or the appearance of anomalous morphological changes; S2 score as degraded condition corresponded to worms with severely reduced activity, displaying only occasional extremities movements; S1 score as minimal condition denoted worms with no external movement, but still displaying an internal activity evaluated by observing gut peristalsis; S0 score as dead condition referred to worms having no movement at all, external or internal, with tissue and tegument degradation. The complete criteria used to determine worm condition during culture is presented in **Table S6**.

Statistical analysis

Statistical analyses were carried out using GraphPad Prism 8.0.2 software. Normality and homoscedasticity for the data were checked for each performed statistical test using quantile-quantile and residual plots, respectively. Depending on these results, appropriate parametric or non-parametric tests were chosen. Errors for the different printers and resins depending on the pattern size were analysed using two-way ANOVA followed by Tukey's tests. The overall printer and resin precision, CP curvature, average surface roughness, the area under the curve representing decrease in fluorescence emission, and PS-1-TD Tomato viability was analysed using one-way ANOVA followed by Tukey's tests. Average assembled culture chamber heights were analysed using an unpaired T test. HPAC-eGFP cell viability was analysed using a Brown-Forsythe and Welch one-way ANOVA followed by Games-Howell's tests. The condition of the worms in the microfluidic system was analysed using Repeated Measures two-way ANOVA followed by Bonferroni's tests for parametric and homoscedastic data, and sphericity assumption. A $p < 0.05$ value was considered statistically significant and was denoted with '*' for $p < 0.05$, '**' for $p < 0.01$, '***' for $p < 0.001$ and '#' for

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$p < 0.0001$. Data are represented as mean $\pm$ standard deviation. The number of replicates is described in each figure legend.

Author contributions

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Conflicts of interest

There are no conflicts to declare.

Data availability

The data supporting this article have been included as part of the Supplementary Information.

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Figure legends

Figure 1. Assessment of fabrication precision of obtained parts with SLA and LCD printer technologies using Precisa 780 resin

(a) Design of the characterisation platform. (b) Schematic of the indicators used to characterise the precision. Comparison of the precision of the printers (Phrozen Sonic Mini 8K (LCD), XFAB (SLA)) with (c) 'Z+' protrusions in height, (d) 'Z-' cavities in height, (e) 'XY+' protrusions in length and (f) 'XY-' cavities in length, as a function of the pattern design dimension and averaged on the whole dimension range (n=3). (g and h) Comparison of the incurvation and surface roughness depending on the printing orientation (n=3). Error bars denote

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SD. * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$ and # for $p < 0.0001$.

Figure 2. Assessment of fabrication precision of obtained parts with TR300, Bio-Med Clear, Aqua-Clear and Precisa 780 resins using the LCD Phrozen Sonic Mini 8K printer

Resins precision comparison and averaged on the whole dimension range using a matrix with **(a and b)** 'Z+' protrusions in height, **(c and d)** 'Z-' cavities in height, **(e and f)** 'XY+' protrusions in length and **(g and h)** 'XY-' cavities in length ($n=3$). The elements above the matrix's diagonal represent the difference in absolute error in pattern dimension between the two compared resins. The elements under the matrix diagonal represent the calculated statistical significance of that difference. Comparison of the incurvation **(i)** and average surface roughness **(j)** depending on the printing orientation ($n=3$). Error bars denote SD. * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$ and # for $p < 0.0001$.

Figure 3. Viability of HPAC-eGFP and PS-1-TD Tomato cells in an in vitro 2D culture system including LCD 3D printed parts

(a) Upon completion of the printing process, the insert is positioned within a 48-well plate. The external dimensions of the insert correspond to a 48-well plate well, while the internal dimensions match those of a 96-well plate well. Consequently, the integration of the component into the 48-well plate results in a 96-well 3D-printed resin well, maintaining uniform inner dimensions. **(b)** Images of the plate's wells obtained with the inserts. **(c)** HPAC-eGFP and **(e)** PS-1-TD Tomato cells at 24 and 48 h in wells made with Bio-Med Clear, Aqua-Clear and TR300 inserts. Histogram of resazurin assay (resin/control ratio) at 48 h for **(d)** HPAC-eGFP and **(f)** PS-1-TD Tomato cells ($n=3$). Scale bar = 500 μm ($n=3$). Error bars denote SD. * for $p < 0.05$ and # for $p < 0.0001$.

Figure 4. Study of the cytotoxicity and autofluorescence of LCD 3D printed parts through Sudan Black B and Parylene C coating post-processing

(a) The desired part is printed, cleaned and cured according to the resin. The part undergoes an oxygen plasma treatment for 60s to create free radicals. It is then directly immersed in a SBB solution overnight at 4°C to reduce autofluorescence. The part is then coated with Parylene C for 3 h using a parylene coating system to decrease cytotoxicity. Emission spectrum of Bio-Med Clear resin treated with SBB with and without O_2 plasma, excited at **(b)** 388 nm, **(c)** 469 nm, **(d)** 567 nm, with the area under curve represented ($n=3$). Histogram of resazurin viability test (resin/control absorbance ratio) at 48 h in the presence of untreated Bio-Med Clear resin or treated with SBB with or without Parylene C coating for **(e)** HPAC-eGFP and **(f)** PS-1-TD Tomato cells ($n=3$). **(g)** Fluorescence of HPAC-eGFP and PS-1-TD Tomato cells at 48 h in wells made with untreated Bio-Med Clear or with different treatments (SBB, Parylene C). Scale bar = 500 μm ($n=3$). Error bars denote SD. * for $p < 0.05$ and ** for $p < 0.01$.

Figure 5. Design and assembly of the multi-component parallelised microfluidic chip

(a) Exploded view of the system showing the 7 parts composing the microfluidic chip: 1: M3x10.8 and M3x17.8 closure screws 2: 3D printed upper closure plate 3: 3D printed resin cap 4: PDMS bottom made from 3D printed mould 5: glass substrate 6: aluminium wedge for closure precision 7: aluminium lower closure plate. **(b)** Illustration of the closed chip. The microfluidic chip consists of eight individual culture chambers **(c)** formed by inserting the cap part **(d)** in the bottom part **(e)**. The cap part is 3D printed and the bottom part is in PDMS moulded in 3D printed mould. The culture chamber has two fluidic ports for connection to the fluidic network. **(f)** Technical diagram of the cap part, top and side views. **(g)** Technical diagram of the bottom part, top and side views. **(h and i)** The height of the culture chamber is defined by the depth of the cap. **(j)** The design of the microfluidic system ensures that the chambers are perfectly aligned with the wells of a 96-well plate making it compatible with automation systems (robotics and microscopy). All units are in mm.

Figure 6. Fabrication process of the microfluidic chip and characterisation of its components' key dimensions

(a) The PDMS mould is printed, cleaned and cured. It is then filled with an uncured PDMS and sealed with a 200 μm thick polyester sheet and 3D printed rings. It is then cured for 3h at 70°C, after which the PDMS is demoulded to obtain the bottom part of the microfluidic chip. **(b)** Schematic of the cap (amber), PDMS bottom section (transparent) and culture chamber (yellow) with their dimensions. Analysis of the fabrication error of the PDMS bottom part ($n=4$) **(c)** and of the 3D printed cap ($n=4$) **(d)**. The error is calculated with respect to the dimensions specified in the CAD software file. **(e)** Comparison of the expected culture chamber height with the measured culture chamber height once the device is sealed using closing plates ($n=4$). The expected height of the culture chambers is calculated using the measured fabrication errors shown in graph (c and d). The yellow line represents the theoretical height of 600 μm . Error bars denote SD. * for $p < 0.05$.

Figure 7. Development of complex microfluidic circuit components and customisable culture chambers using 3D printing

The circuit that connects the eight culture chambers is made up of 3D printed parts, including **(a)** the 16-in-1 connector that connects to the adaptors on the cap (inlet and outlet), **(b)** the tube connectors for connecting the tubes to the tank outlets, **(c)** the 8-tank holder. **(d)** Images of the complete microfluidic system with the (a), (b) and (c) printed parts, combined with tubings, connectors and peristaltic pump. Modularity permitted by 3D printing was evaluated by developing three geometries of the culture chamber: **(e)** a classic channel with variable height to vary the speed of the flow on a 2D culture **(f)** a culture chamber designed to culture a pair *Schistosoma mansoni* adult worm parasites, delimited by a 70 μm nylon anti-escape filter with the flow passing over it, and **(g)** a culture chamber in the format of a 96-well plate well to accommodate a macroporous

matrix and a flow passing through to cultivate different cell types (epithelial and fibroblastic) and tumoroids.

Figure 8. Application of the microfluidic chamber geometries to 2D, 3D human cells co-culture and worm culture

The first geometry **(a)** is a simple channel whose height has been adjusted at 600 and 2400 μm to modulate the flow velocity. HPAC-eGFP cells were grown in either monoculture or cocultured with PS-1-TD tomato for 7 days. **(b)** full channel imaging, **(c)** channel zoom and **(d)** nuclei counting (DAPI staining) ($n=1$). The second geometry **(e)** allows a pair of motile *Schistosoma mansoni* adult worm parasites to be enclosed while feeding on the perfused culture medium. The pairs were cultured for 8 days and their condition assessed every day. **(f)** images of the parasites on day 0 and day 8 and **(g)** assessment of their health status ($/4$) during culture ($n=1$). The third geometry **(h)** is the size of a 96-well plate well and allows the integration of a macroporous culture matrix **(i and j)** to co-culture pancreatic tumoroids (PaTa-1818x) with PS-1-TD Tomato cells. **(k)** After 7 days of monoculture and co-culture, calcein and fibroblasts (TD Tomato) were observed. Scalebar = 1mm. Error bars denote SD.

Figure 1. T. Meynard* et al. 2025

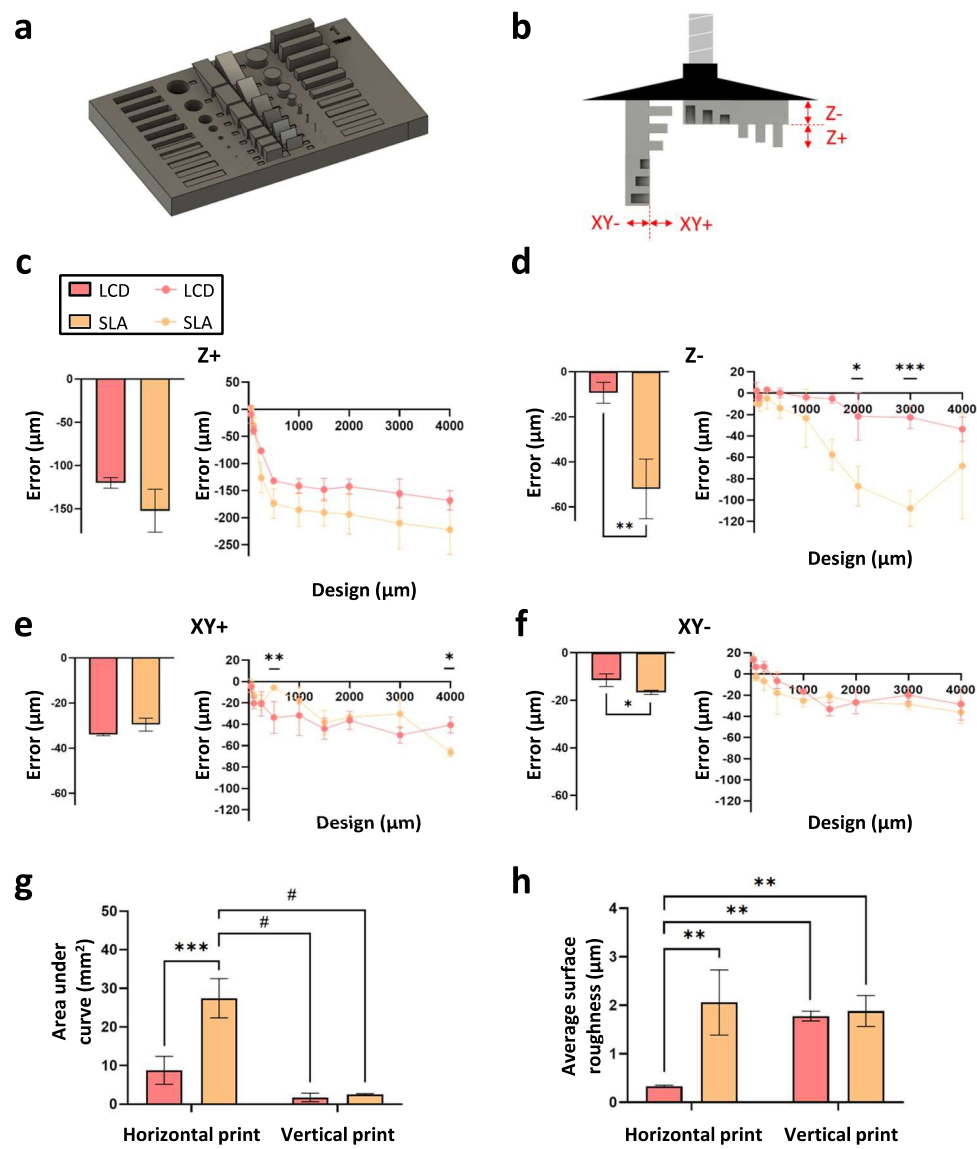


Figure 2. T. Meynard* et al. 2025

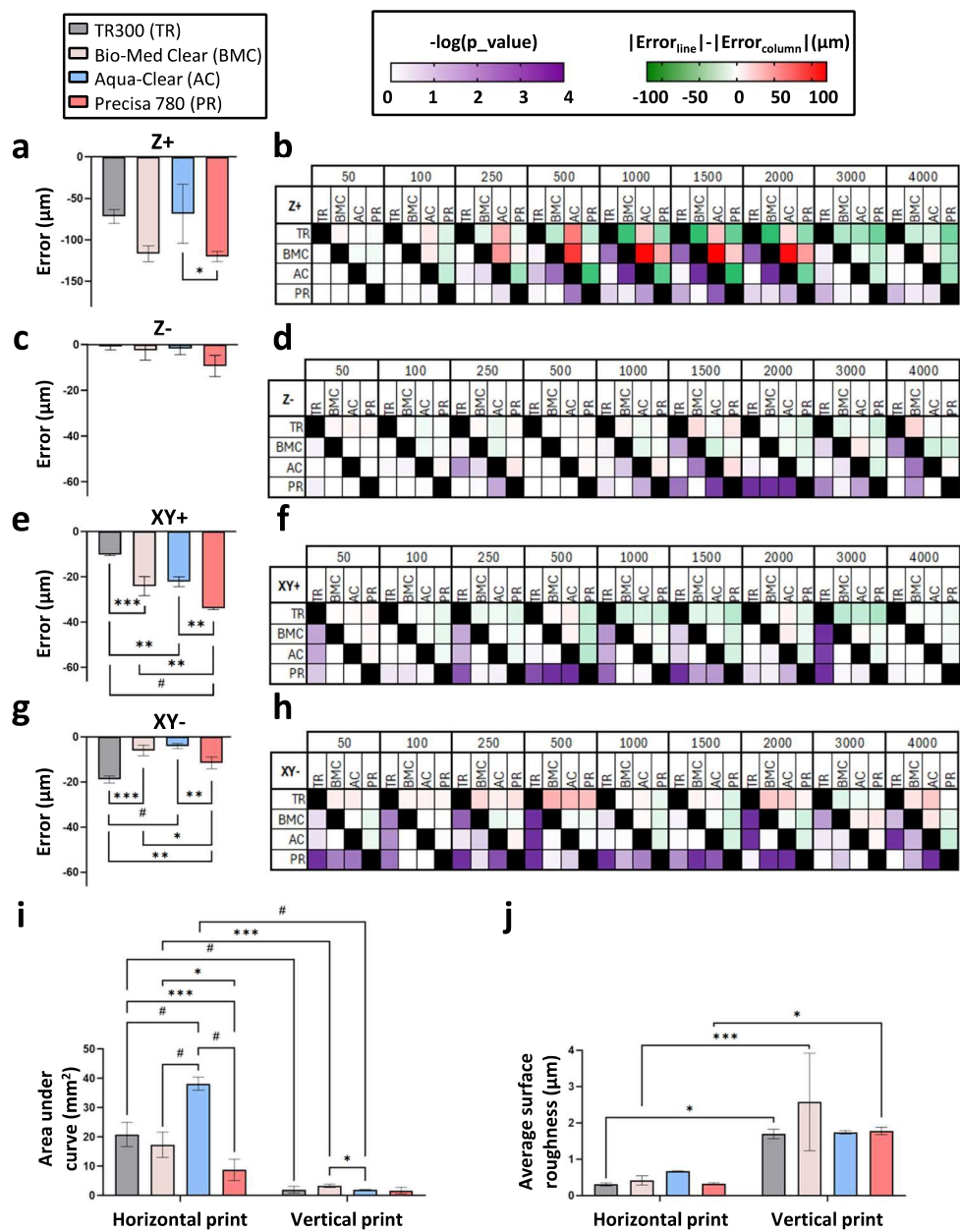


Figure 3. T. Meynard* et al. 2025

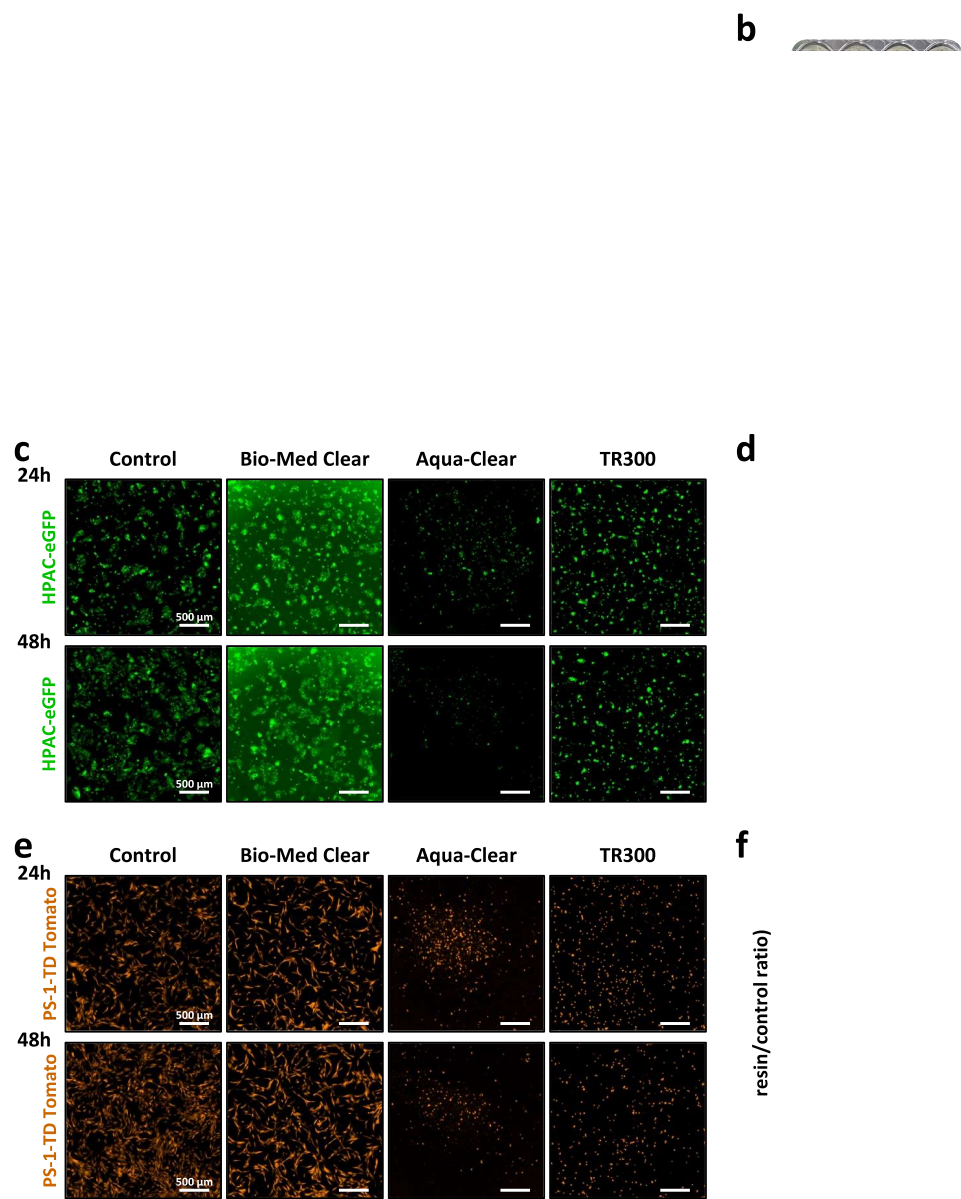


Figure 4. T. Meynard* et al. 2025

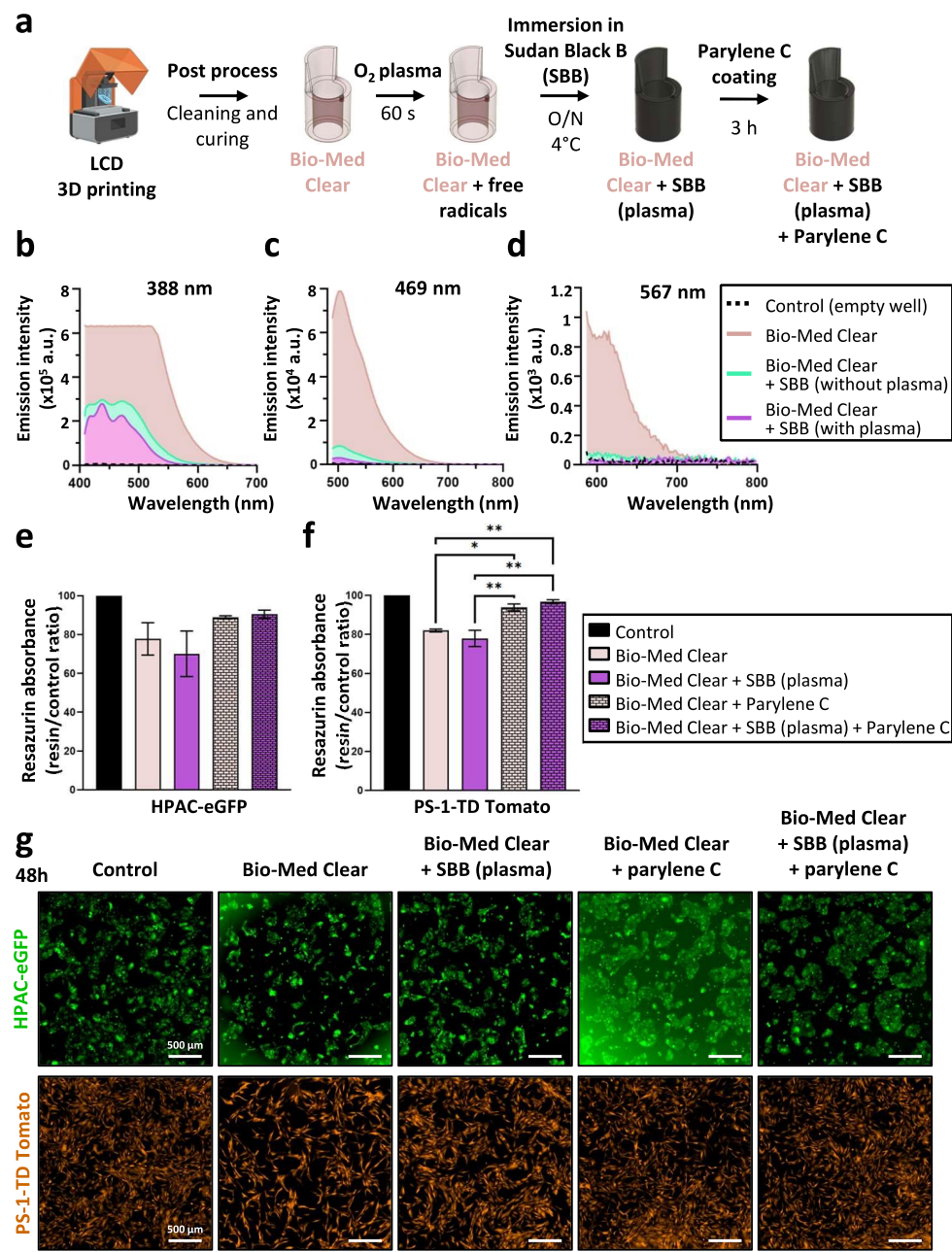


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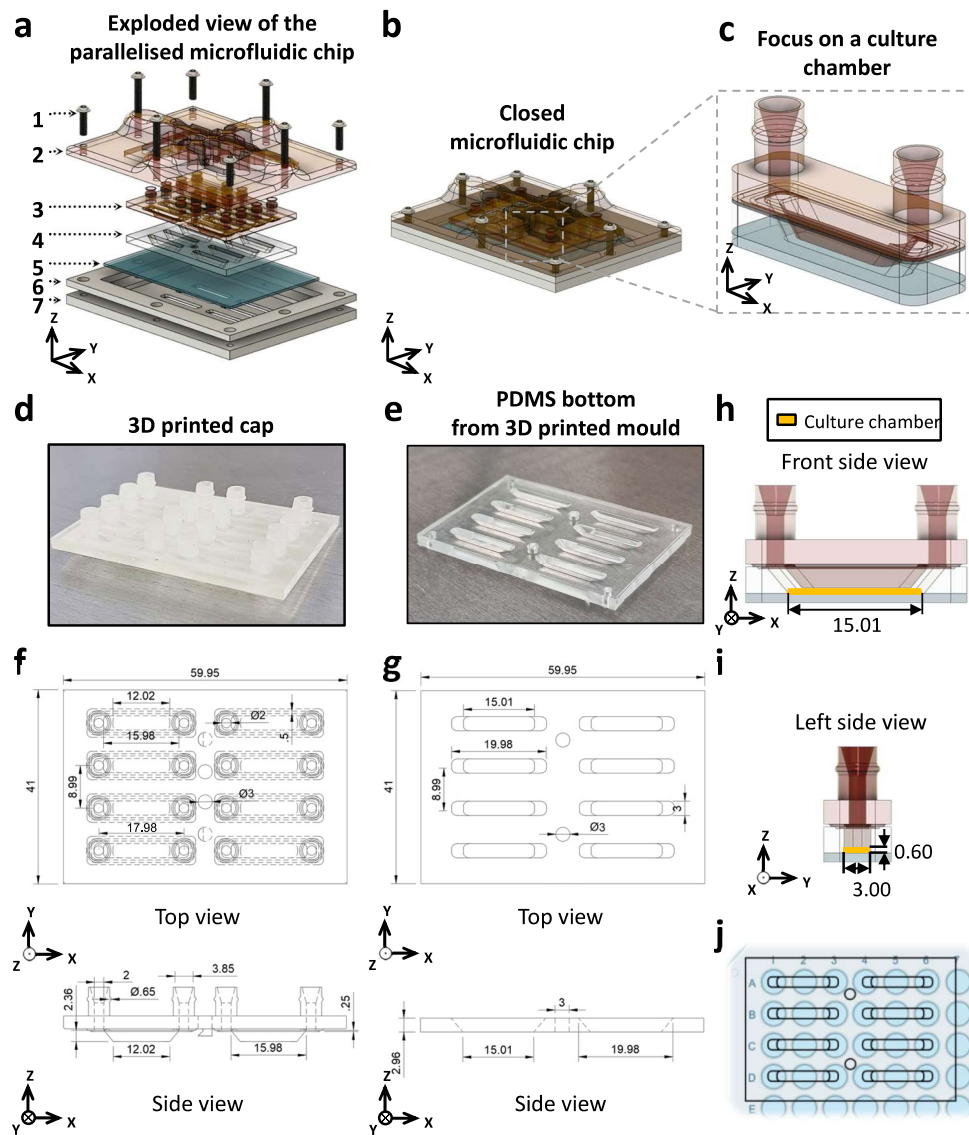


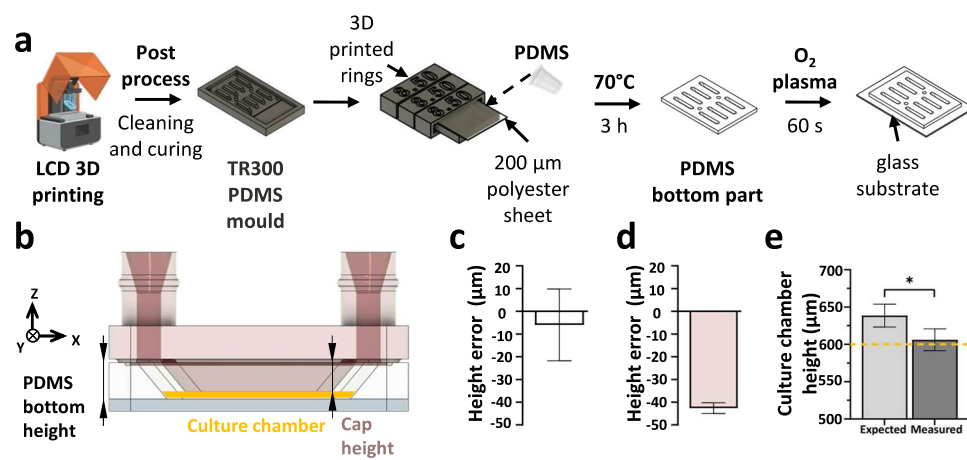
Figure 6. T. Meynard* et al. 2025

Figure 7. T. Meynard* et al. 2025

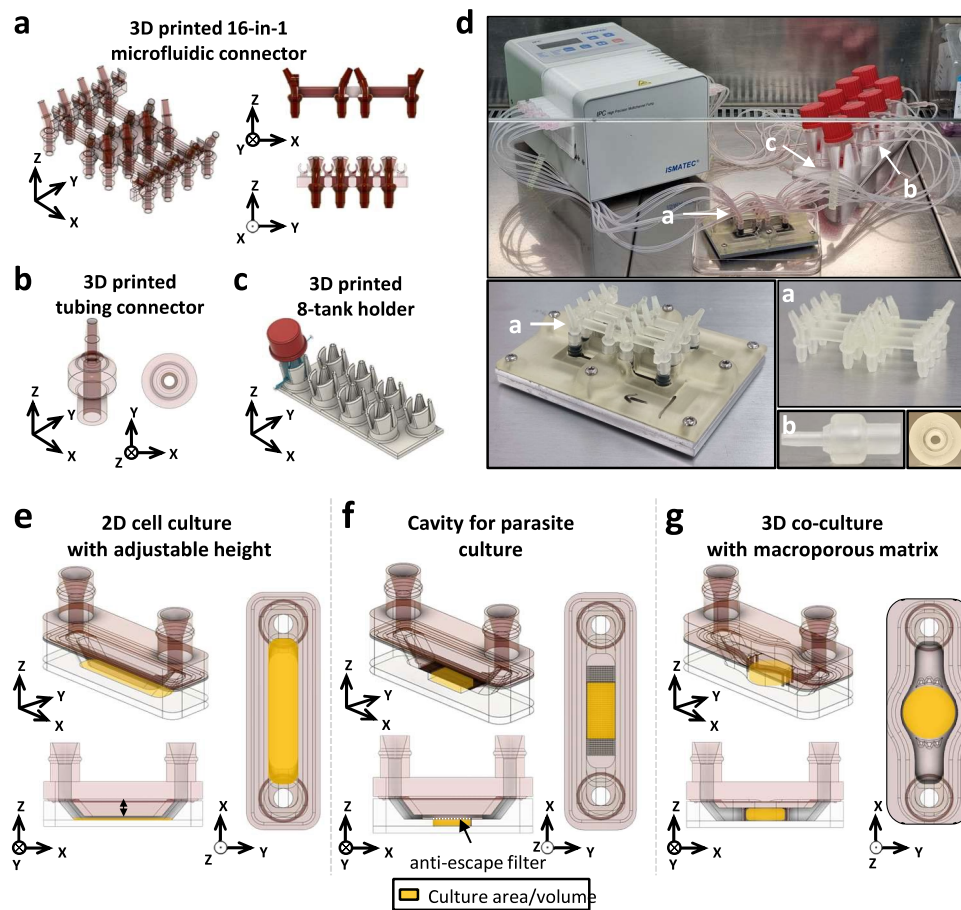
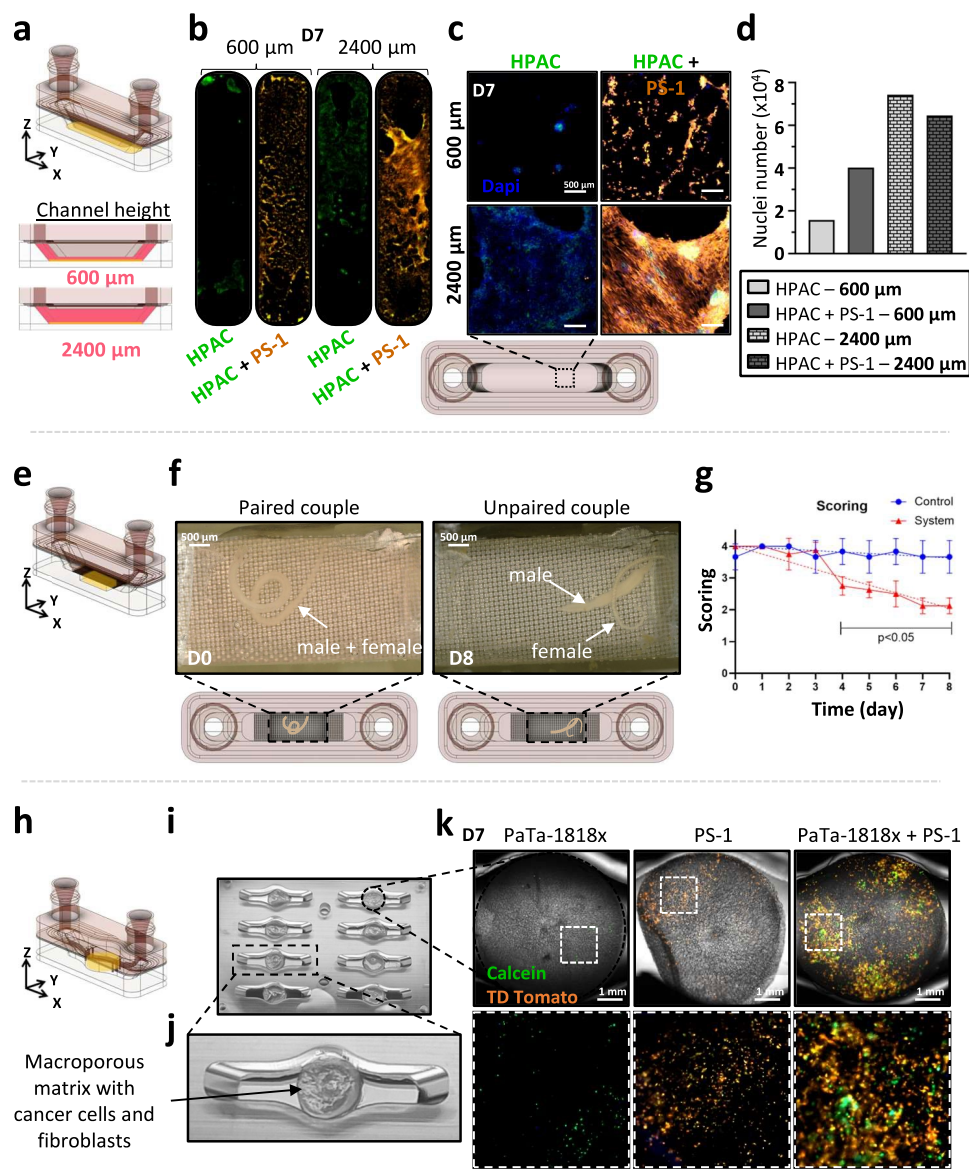


Figure 8. T. Meynard* et al. 2025



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