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BIOINGEGNERIA**

**“3D biofabrication of an in-vitro compartmentalized model to study the tumor
microenvironment”**

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Abstract

Cancer is one of the leading causes of mortality worldwide, with approximately ten million deaths annually, according to the World Health Organization. The tumor microenvironment (TME) is highly complex, characterized by a heterogeneous cellular composition and dynamic microenvironmental signals.

Traditional methods for reproducing the biological environment in vitro have primarily relied on animal models and two-dimensional (2D) cultures. However, animal models are expensive and often fail to replicate critical aspects of human tissues due to biological differences between species. While some of these limitations can be overcome using 2D in vitro cultures, key features of tumors and of the TME, such as cell-cell and cell-extracellular matrix (ECM) interactions, can't be accurately reproduced in monolayer cultures.

To address these limitations, this thesis introduces the use of 3D bioprinting, a technology that enables the creation of three-dimensional structures capable of better representing the complexity of the tumor microenvironment. Extrusion-based bioprinting involves the precise deposition of bioinks made up of biomaterials and cells; using this technique, a three-dimensional structure was created to reproduce a multilayer compartmentalization, with the aim of studying the biological mechanisms occurring in the TME.

The 3D structure was designed to represent an internal environment corresponding to the solid tumor, surrounded by an external compartment simulating the stromal tissue. Once the structure has been designed, a detailed analysis of the materials to be used was conducted, selecting biomaterials that best supported cell viability while also meeting the printability requirements. Furthermore, different cell concentrations were evaluated to optimize experimental conditions. The bioprinted constructs were then monitored over time to evaluate tumor proliferation and interactions at the interface between compartments.

This work highlights the potential of 3D bioprinting in tissue engineering and cancer research, providing a versatile platform for the development of in vitro tumor models that can accelerate the discovery of new therapeutic approaches and enhance the understanding of complex interactions within the TME.

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1. Introduction

The tumour microenvironment (TME) is an extremely complex ecosystem in which tumour cells coexist and interact with multiple cell types; these dynamic interactions profoundly influence tumour evolution and progression.

The first part of this thesis will provide a detailed overview of the role of the TME and the extracellular matrix (ECM), with a particular focus on cell-matrix interactions. This will be followed by a comparison between 2D and 3D in vitro cancer models, highlighting their main differences and focusing on the advantages of three-dimensional models, which are able to more realistically mimic the tumor microenvironment.

The thesis introduces the application of 3D bioprinting as an interesting technique to generate advanced in vitro tumor models. Tumor models fabricated through 3D bioprinting have been shown to better reproduce the complexity of the TME, due to the possibility of precisely arranging cells of different natures (such as tumour and stromal cells) within 3D printed structures.

The second part of the thesis will focus on the analysis of the *Materials and Methods* used to fabricate a 3D bioprinted structure capable of simulating the TME. The selected biomaterials, incorporated cell types, and the critical importance of both cell-matrix and cell-cell interactions will be discussed in depth.

Finally, the *Results* section will present the main findings obtained during the study, along with the challenges encountered throughout the experimental process and the strategies adopted to overcome both technical and, more importantly, biological issues.

1.1 Extracellular matrix

The extracellular matrix (ECM) is a fundamental component of tissues, playing multiple crucial roles in both structural and functional aspects of cellular biology. It provides essential mechanical support to cells, modulates the microenvironment, and acts as a reservoir for signalling molecules. Through these functions, the ECM regulates a wide range of cellular processes, including adhesion, differentiation, proliferation, migration, apoptosis, and even the progression of diseases. [1]

This complex interplay between the ECM and cells is important in maintaining tissue homeostasis and to respond to physiological changes or injuries. The proper development of tissues and the maintenance of their functionality depend on a carefully controlled exchange of information between cells and between cells and the ECM. [1]

Cells interact with the ECM via specialized membrane receptors, which mediate adhesion and enable them to receive and respond to external signals. These membrane receptors process and amplify these signals, transmitting them into the cell through a series of signaling molecules and coordinated interactions among the cytoskeleton, receptors, and ECM components. This process, known as mechanotransduction, ensures that cells not only perceive but also adapt to the mechanical and biochemical cues in their environment. For example, mechanotransduction plays a critical role in tissue remodeling, wound healing, and even the progression of certain cancers. [2], [47]

Notably, cell adhesion to the ECM is not a static process; rather, it is highly dynamic, allowing cells to continuously remodel their connections and respond to changes in the surrounding matrix.

The ECM (*Figure 1*) is composed of three main classes of biomolecules, each contributing to its diverse functions:

- **Structural proteins** such as collagens, which provide strength and help organize the ECM, and elastins, which impart resilience and elasticity to tissues.
- **Glycosaminoglycans (GAGs)**, which are large, highly charged polysaccharides. In the ECM, GAGs are typically covalently bound to core proteins, forming proteoglycans that embed structural proteins within a hydrated gel, creating a supportive and flexible matrix.
- **Adhesive glycoproteins**, including fibronectin and laminins, which anchor cells to the ECM and guide cell migration, settlement, and differentiation in specific tissue locations.

While the basic organization of ECM components is conserved, the ECM can be classified into two main types based on its location and composition.

The *stromal matrix* forms a three-dimensional, porous network that surrounds cells, especially within connective tissues. This type of ECM is composed of fibrillar collagens, elastic fibers, and GAGs/proteoglycans, creating highly complex composites that support tissue structure and function.

In contrast, the *basement membrane* is more compact, and forms specialized layer immediately adjacent to cells. This structural organization not only provides mechanical support but also plays a crucial role in processes such as cell polarization and tissue compartmentalization. [1]

The major components of the ECM will be now described in detail.

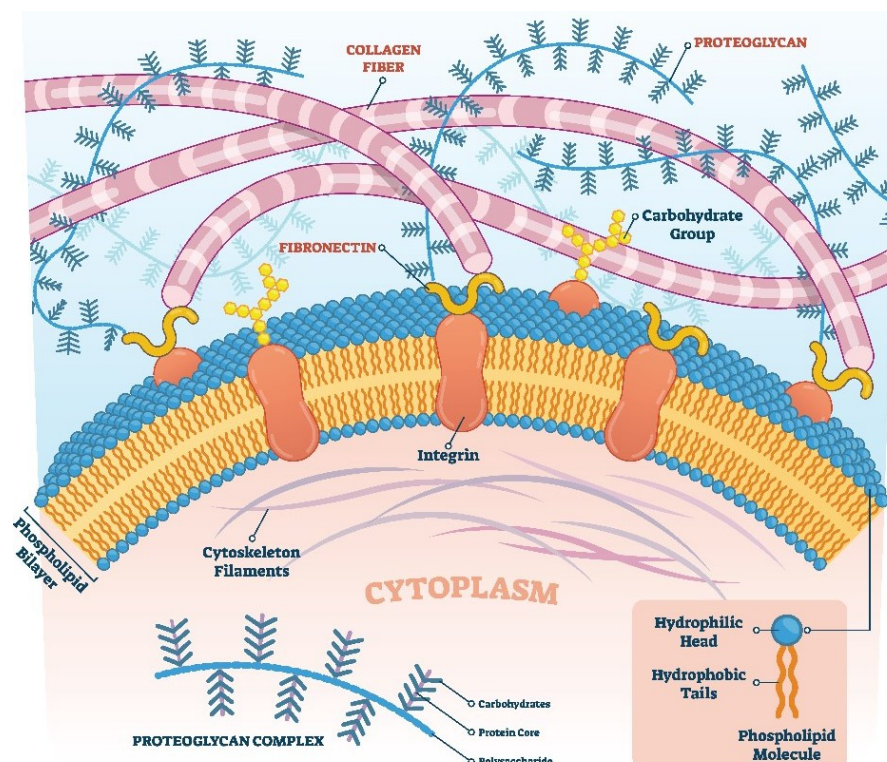


Figure 1: Extracellular environment.

1.1.1 Structural proteins

The structural proteins of the extracellular matrix are essential components that provide strength, elasticity, and support to body tissues. Among them, the most significant are collagen, which accounts for approximately 25% of the total proteins in the human body and is critical for mechanical resistance, and elastin, which provides elasticity to tissues. These proteins are key elements of the ECM, playing a crucial role in offering mechanical support, organizing the three-dimensional structure of tissues, and facilitating cell adhesion. Additionally, they interact with other ECM components, such as proteoglycans and glycosaminoglycans, to regulate tissue hydration and functionality.

Collagen

Collagen is the most abundant structural protein in animals. In humans, it constitutes one-third of the total protein content and accounts for 75% of the skin's dry weight. Within the ECM, collagen provides the structural framework for tissues, giving them the tensile strength required to resist physical forces such as stretching and pulling. This contrasts with glycosaminoglycans (GAGs), which primarily resist compressive forces. [3]

A hallmark of collagen is its unique triple-helical structure, as shown in *Figure 2*. Each collagen molecule consists of three polypeptide chains called α chains, which coil tightly around one another to form a supercoiled triple helix. This structure is both long and rigid, with glycine, the smallest amino acid, occupying the crowded interior of the helix. This arrangement allows for tight packing and contributes to the molecule's stability. The α chains are composed of repeating Gly-X-Y triplets, where glycine appears every third amino acid, while X is often proline and Y is commonly hydroxyproline, a modified version of proline synthesized during collagen production. [3],[4]

Multiple triple helices form collagen microfibrils (10-300nm in diameter) which then associate together to make up fibrils. [5]

The structural organization of collagen plays a vital role in its function. Within the triple helix, interstrand hydrogen bonds form a "ladder," further stabilizing the molecule. The staggered arrangement of the three chains enhances the strength and flexibility of the helix. These properties make collagen an essential scaffold within the ECM, providing thermal stability, mechanical strength, and the ability to interact with other biomolecules. These interactions are key to maintaining the integrity of tissues and supporting cell adhesion, migration, and differentiation. [1]

Collagen serves as a framework for cellular organization and contributes to the ECM architecture. The molecular variety and precision of collagen enable it to do a wide range of biological roles, from forming porous interstitial networks to creating compact, supportive structures such as the basement membrane. These structures play a central role in regulating cellular behaviour and tissue functionality. [3],[4]

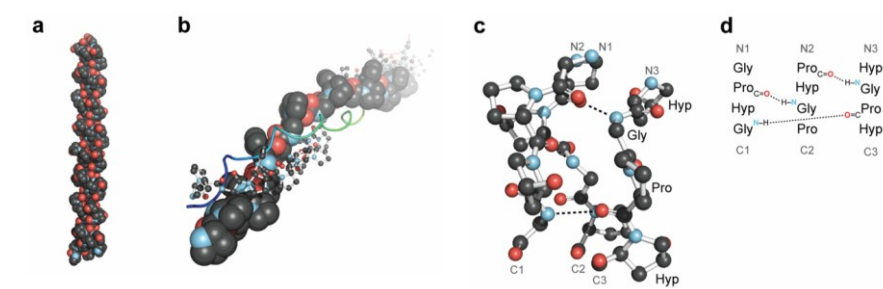


Figure 2: Overview of the collagen triple helix. [3]

In *Figure 3-A*, the first high-resolution crystal structure of a collagen triple helix is shown, formed by (ProHypGly)₄–(ProHypAla)–(ProHypGly)₅. In *Figure 3-B*, a view down the axis of a (Pro-Pro-Gly)₁₀ triple helix is presented, with the three strands depicted using space-filling, ball-and-stick, and ribbon representations. *Figure 3-C* displays a ball-and-stick model of a segment of the collagen triple helix, highlighting the interstrand hydrogen bond ladder. Finally, in *Figure 3-D*, the staggered arrangement of the three strands in the segment from panel (C) is illustrated. [3]

Different types of collagens can be found in many places throughout the body: “Type I” collagen is the type commonly found in skin, bone, and tendons. “Type II” is common in cartilage. “Type III” collagen is prevalent in the vasculature structures (i.e., blood vessel walls), and can also be found in small amounts in skin. “Type IV” collagen is unique in that it is largely non-helical and does not form fibrils. This is found in the basement membrane that separates epithelial and mesodermal tissues.

“Type I” is by far the most common, being the principal collagen of skin, bone and tendons. Over 90% of the collagen in the human body, is type I collagen. [5]

Elastin

Elastic fibers are crucial for the functionality of many tissues, as they provide elasticity to the extracellular matrix (ECM). The main component of these fibers is elastin, a hydrophobic protein that is secreted into the extracellular space, where it aggregates to form elastic fibers. Elastin molecules establish a large number of cross-links between each other, contributing to the overall elasticity of tissues (*Figure 3*). However, elastic fibers are not composed solely of elastin. In fact, the central core of elastin is surrounded by a sheath of microfibrils. [4]

The elastin core constitutes about 90% of the elastic fibers, and the microfibrils that surround it have a diameter of approximately 10 nm. Microfibrils are composed of several distinct glycoproteins, including fibrillin, which binds to elastin and is essential for the integrity of elastic fibers. The microfibrils themselves are elastic and, in some cases, they can persist even in the absence of elastin. [1]

Microfibrils appear before elastin in developing tissues and seem to play an important role in the aggregation and deposition of elastin. They provide scaffolding to guide elastin deposition, and the arrays of microfibrils form a skeleton onto which elastin molecules are deposited. [4]

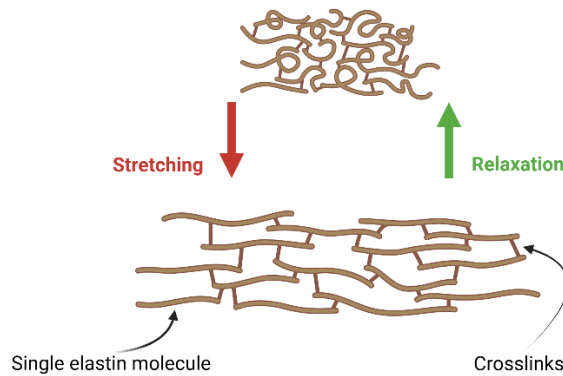


Figure 3: Representative image of the elastin molecule [created with Biorender]

1.1.2 Glycosaminoglycans

Glycosaminoglycans (GAGs) are unbranched polysaccharide chains composed of repeating disaccharide units. One of the two sugars in the repeating disaccharide is always an amino sugar (N-acetylglucosamine or N-acetylgalactosamine), which is often sulphated. The other sugar is typically a uronic acid (glucuronic acid or iduronic acid). Due to the presence of sulphate or carboxyl groups on most of their sugar residues, GAGs are highly negatively charged (polyanionic). [1]

Based on the sugar residues, the type of linkage between them, and the number and position of sulphate groups, glycosaminoglycans (GAGs) are classified into several main groups: hyaluronan, chondroitin sulphate and dermatan sulphate, heparan sulphate, and keratan sulphate.

Polysaccharide chains are too stiff to fold into compact globular structures, and they are strongly hydrophilic. Thus, GAGs tend to adopt highly extended conformations that occupy a huge volume relative to their mass and form gels even at very low concentrations, due to the high density of their negative charges they attract a cloud of cations, such as Na^+ , that are osmotically active, causing large amounts of water to flow into the ECM.

Although the amount of GAGs in connective tissue is less than 10% of that of fibrous proteins, their ability to form porous, hydrated gels means that they occupy a large volume of the ECM of connective tissue. GAGs allow the diffusion of water-soluble molecules and cell migration and determine the mechanical support function of various organs, as well as resistance to compressive forces. [4]

Hyaluronan is not a typical GAG because it contains non-sulphated sugars, all its disaccharide units are identical, it generally has high molecular weights, and it is not linked covalently to any core protein. HA is thought to have a role in resisting compressive forces in tissues and joints. [1]

1.1.3 Adhesive glycoproteins

Glycoproteins are proteins that contain oligosaccharide chains (glycans) covalently attached to polypeptide sidechains: the covalent addition of oligosaccharides to proteins is one of the major biosynthetic functions of the endoplasmic reticulum (ER). Approximately half of the membrane-bound proteins and the soluble secreted extracellular proteins that are processed in the ER are glycosylated. [1]

Fibronectin

Fibronectin (FN) is the first of the ECM's adhesive proteins to have been characterised. These proteins are responsible for both organising the ECM and allowing cells to connect with it. Fibronectin binds to several other components of the ECM, including collagens, especially collagen type III, and proteoglycans via specific domains, and to cells via integrin receptors. [1]

Fibronectin is also implicated in the transformation and progression as well as metastasising of tumours. Tumour cells often show quantitative and qualitative alterations in fibronectin synthesis: the presence of specific fibronectin isoforms in tumour lesions can be used as a diagnostic marker. [4]

Laminin

Laminins (LM) are the most abundant glycoproteins in the ECM. They are a large family of multi-domain glycoproteins, each consisting of three long polypeptide chains (α , β and γ) held together by disulfide bonds and arranged in an asymmetric structure. Through their binding sites for other proteins, laminin molecules play a central part in organizing basal laminae and anchoring them to cells. [1]

The first laminin discovered was isolated from the ECM of Engelbreth-Holm-Swarm sarcoma (EHS) and initially appeared to consist of two chains, held together by disulphide bridges. Subsequent studies have shown great variability in this protein, due to the presence of three genetically distinct but homologous chains: the α , β and γ chains. It was seen that the EHS tumour-derived laminin is only one member of a large family of proteins consisting of the different combinations of these chains, of which there are multiple variants. [4]

1.2 Tumor microenvironment

The tumor microenvironment is a complex ecosystem surrounding a tumor, composed of cancer cells, stromal tissue (including blood vessels, immune cells, fibroblasts and signaling molecules) and the extracellular matrix. Mutual interaction between cancer cells and the different components of the tumor microenvironment supports its growth and invasion in healthy tissues which correlates with tumor resistance to current treatments and poor prognosis. [6]

Stromal cells recruited to the tumor microenvironment are different and include vascular endothelial cells, pericytes, adipocytes, fibroblasts, and bone-marrow-derived mesenchymal stromal cells. [7] The TME is highly dynamic, as tumors continuously influence their surroundings by releasing extracellular signals, promoting angiogenesis, and inducing peripheral immune tolerance. At the same time, immune cells within the microenvironment can significantly impact the growth and evolution of cancer cells. [6]

These recruited stromal cells contribute to critical processes such as tumor angiogenesis, cell proliferation, invasion, and metastasis, as well as mediate mechanisms of therapeutic resistance. Their diversity further highlights the complex interplay between cancer cells and their supportive stroma. [7]

Tumor cells stimulate significant molecular, cellular, and physical changes within their host tissues to support tumor growth and progression. An emerging tumor microenvironment is a complex and continuously evolving entity: early in tumor growth, a dynamic and reciprocal relationship develops between cancer cells and components of the tumor. Tumors become infiltrated with diverse adaptive and innate immune cells that can perform both pro- and anti- tumorigenic functions. [8]

1.2.1 Components of the tumor microenvironment: cells and ECM

Cancer cells recruit supporting cells from the surrounding endogenous tissue stroma to promote critical steps in tumor formation, making these stromal cells an essential component of the tumor microenvironment (*Figure 4*). The composition of stromal cells can vary significantly between tumor types and includes vascular endothelial cells, fibroblasts, adipocytes, and stellate cells.

Once integrated into the tumor microenvironment, stromal cells secrete a wide range of factors that influence key processes such as angiogenesis, cellular proliferation, invasion, and metastasis. This dynamic interaction between stromal and cancer cells is pivotal in driving tumor progression.

Among stromal components, cancer-associated fibroblasts (CAFs) represent a major population and play a crucial role in mediating crosstalk between cancer cells and the surrounding microenvironment. Although CAFs often originate from tissue-resident fibroblasts, they can be different in origin.

Within the tumor microenvironment, CAFs are responsible for producing the majority of extracellular components, such as growth factors, cytokines, and elements of the extracellular matrix. Through these functions, CAFs actively shape the structural and biochemical landscape of the tumor, thereby influencing its growth, invasiveness, and response to therapy. [8]

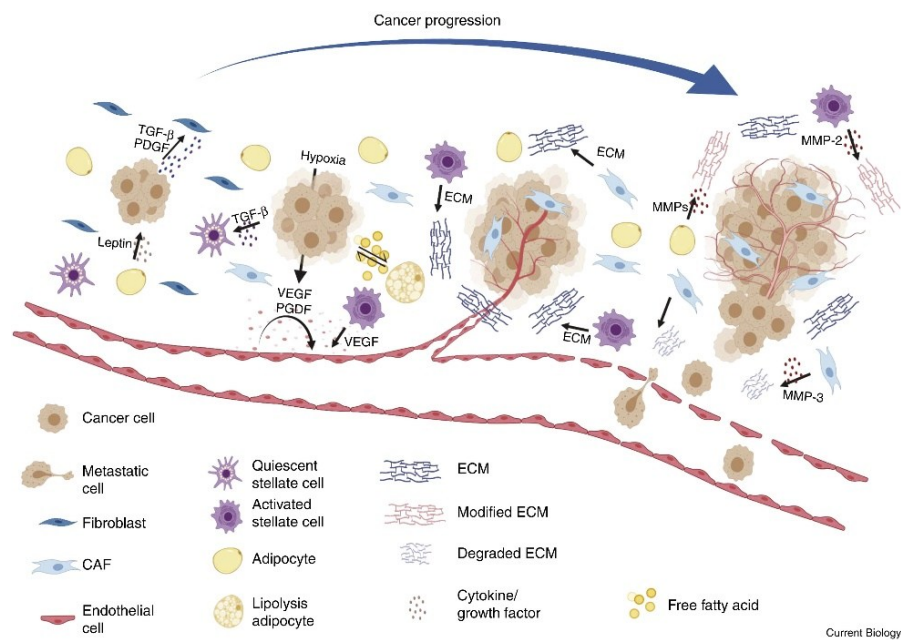


Figure 4: Image illustrating cancer progression and the role of cells in the mechanism. It highlights how the composition of stromal cells can vary significantly among tumor types and includes vascular endothelial cells, fibroblasts, adipocytes, and stellate cells. [8]

The ECM is an important molecular component of the TME. Not only provide a physical scaffold for cells, but it also plays a key role in promoting tumor cell dissemination (*Figure 4*). Tumors contain large extracellular matrix deposits that constitute up to 60% of tumor mass. Many cells within the TME secrete components of the extracellular matrix, although cancer-associated fibroblasts are the predominant source. [8]

Indeed, by secreting ECM components and ECM-modifying enzymes, cancer cells modify their environment. But even more, CAFs, driven by cancer cells, produce and deposit substantial amounts of ECM components, thus altering the ECM of the tumor stroma qualitatively and quantitatively. Caused by these biochemical alterations, the ECM also changes in its biophysical parameters, such as topography, stiffness or rigidity, and tension.

The topography of the ECM components and the tension on the ECM are biophysical parameters, which reinforce CAF differentiation and tumor progression. Both the swelling potential of proteoglycans and the high contractility of CAFs are responsible for the mechanical tension within the tumor stroma ECM. [9]

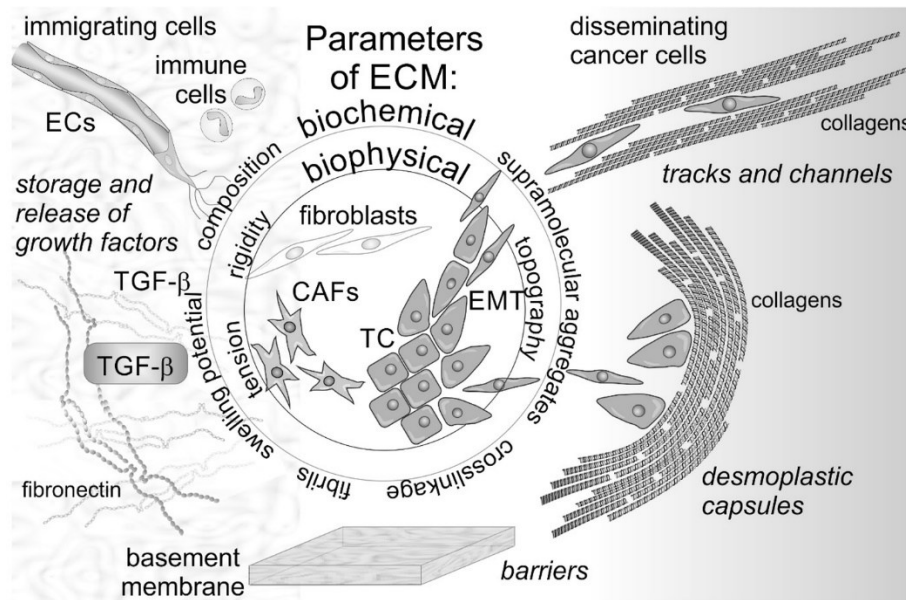


Figure 5: Potential roles of the ECM in tumor progression and metastasis [9]

1.2.2 Collagen's role in TME remodelling

When engineering a 3D model, the choice of biomaterial used is of utmost importance to provide the cells with an environment resembling the native TME.

Among the most used natural polymers is collagen. Collagen I is the most abundant structural protein in the human body and has bioactive sites that support cell attachment and proliferation as well as ECM production. It allows for construction of biologically relevant tumor models and thus is one of the most widely used polymers to engineer the TME.

Moreover, collagen hydrogels help study how fibers alignment affects cancer cell migration by restricting protrusions and inducing cell alignment along the fibers by forcing directional movement.

Simultaneously, cell-mediated remodelling of this important component can be investigated. Tumor cells and tumor-associated stromal cells, indeed, remodel the ECM. As an example, the alignment of the collagen fibers radially can form fibrous “highways” through which tumor cells can migrate. [17]

Recent studies have highlighted several abnormalities that occur during cancer progression, including degradation, remodelling, fragmentation, and fibers alignment. Research confirms that collagens are

fundamental not only in the early stages of *precancerous lesions* but also in *tumor development*. A deeper understanding of their involvement at different stages of cancer progression has led to significant advancements in both diagnosis and prognosis.

As an example, stromal alterations are the reference precursors to predict the progression of carcinoma: the occurrence of fibrosis and the base membrane abnormality are important cues with significant clinical meaning.

In the development of cancer progression, a collagen transition from a passive barrier resisting cancer cells to an accomplice in promoting progression can be observed *in vivo*.

In the initial stages of carcinogenesis, the basement membrane serves as a protective barrier that separates tumor cells from healthy tissue. However, as the tumor progresses, this barrier becomes compromised, primarily due to the degradation of collagen IV, which can be triggered directly by tumor cells or indirectly through tumor-induced enzymatic activity. In more advanced stages, the mechanical stress exerted by the growing tumor alters the organization of collagen fibers, causing them to realign toward the periphery of the tumor. This spatial arrangement provides a "highway" to cancer cell invasion directly.

The analysis of collagen fibers is therefore significant in predicting cancer. In this framework, Despotovic et al. acquired SEM images of the perimalignant tissue (*Figure 6*). They found that the altered organization of collagen fibers was observed at 10 cm and 20 cm away from the malignant tumor. The alignment of collagen fibers is step increased as proximity to the tumor. [18]

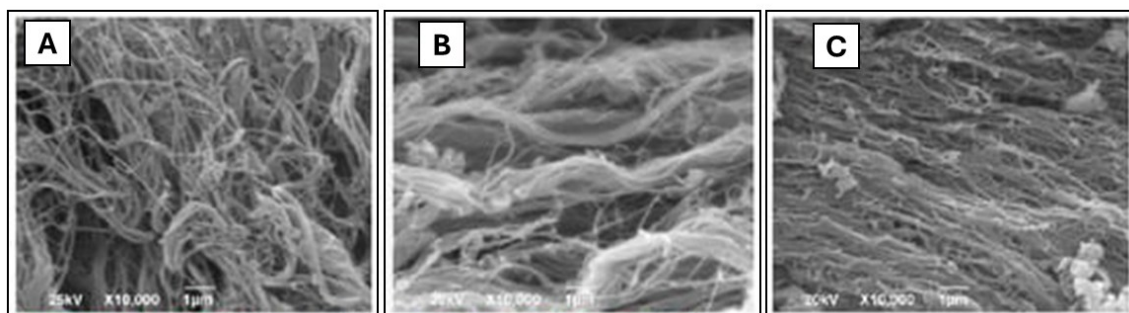


Figure 6: SEM images of collagen fibers in three different conditions: (A) within healthy tissue, (B) at a distance of 10 cm from the malignant tumor, and (C) at a distance of 20 cm from the malignant tumor. [18]

As shown in *Figure 6-A*, the collagen fibers in healthy tissue are thin and form a dense network. In contrast, *Figure 6-B* illustrates that the collagen fibers located 20 cm away from the tumor are thicker and aligned. Finally, *Figure 6-C* reveals that the collagen fibers at 10 cm from the tumor are highly aligned. Based on these observations, it can be inferred that as the tumor is approached, the collagen fibers become increasingly dense and tend to align.

In *cancer progression*, remodelled collagens play also a crucial role in facilitating angiogenesis. Due to the rapid growth of cancer cells, oxygen and nutrients often become insufficient, leading to the induction of angiogenesis (*Figure 7*). The collagen network serves as a scaffold for endothelial cells migrating during angiogenesis, and even distant collagen fibers are influenced by tumor cells to orient and guide this process. [18]

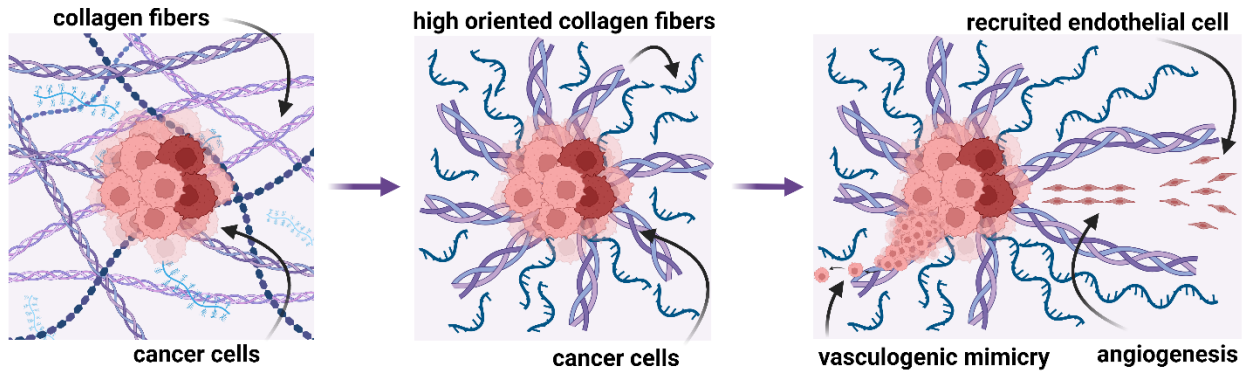


Figure 7: The process of angiogenesis induced by collagen fibers [created with Biorender]

Beyond angiogenesis, collagen also contribute significantly to cancer cell invasion. Collagen fibers guide cancer cell migration, especially when aligned perpendicularly to the tumor, facilitating basement membrane invasion, as shown in *Figure 8-D*. [18]

Remodeling of the mammary TME structure

Breast cancer is one of the most common cancer types. In 2018, the estimated number of new breast cancer cases was 270,000 in the United States alone, and 2.1 million across the world. In addition, breast cancer has the potential to metastasize to secondary tissues.

Normal epithelium is composed of epithelial (luminal) and myoepithelial (basal) cells. These cells are connected to the basement membrane, a thin layer of ECM separating the epithelium from the surrounding stromal tissues. This organization is important for a functional mammary gland.

Cell-cell adhesion is reduced in tumor cells, leading to their dissociation from the epithelium and from each other, rapid proliferation, and formation of ductal carcinoma in situ (DCIS) that is a solid tumor masses in the lumens of epithelial tissues (*Figure 8*). Due to uncontrolled cell division, these cells are heterogenic, and this characteristic is further fortified by the differential oxygen and nutrient supply to cells at different sites in the tumor. Cells in the core of the solid tumors receive less oxygen and thus are necrotic, cells in the middle layer are senescent, and those in the outer layer are proliferating. [49]

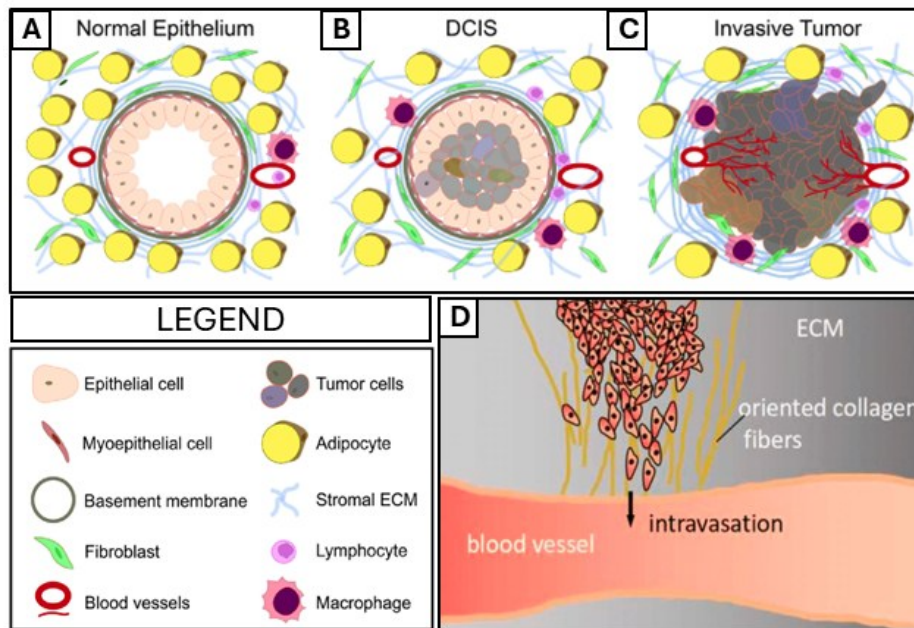


Figure 8: Interactions between cells and ECM lead to alteration of normal epithelium towards the tumor. (A) Normal epithelium, (B) ductal carcinoma in situ (DCIS), and (C) invasive tumor. [49] (D) Collagens promote cancer invasion: schematic representation of oriented collagen fibers providing a "highway" to guide cancer cells in realizing the intravasation process. [18]

Tumor cells and stromal cells secrete soluble factors (growth factors and cytokines) affecting both the other cells and the ECM, which eventually leads to the disruption of normal epithelial organization. The tumor microenvironment plays a crucial role in tumor progression and metastasis, it is a complex environment, with dynamic cell-cell and cell-ECM interactions contributing to cancer initiation. This complex interaction between the cells and the ECM eventually leads to more invasive tumor cells that can break the connective tissue and metastasize.

Microarchitecture, i.e. fiber structure, is an important mediator of cancer cell invasion and migration. For example, the presence of dense collagen structures in the stroma promotes tumor invasiveness in vivo, since the confinement of tumor cells in small pores triggers ECM degradation. Tumor cells and tumor-associated stromal cells remodel the ECM such that radially aligned collagen fibers are formed. These aligned fibers act as highways through which tumor cells can travel. ECM alignment in vitro was shown to result in an elongated cell morphology and a higher migration speed, with the most aligned cells migrating faster. In a study, alignment of collagen was reported to enhance migration of the invasive breast cancer cells more than the non-invasive cells, suggesting that a selective environment for the migration of the invasive cancer cells is created. [49]

In fact, these results are supported by a recent study (Conklin et al., 2018), in which collagen alignment in breast stromal tissues of 227 women patients with DCIS was evaluated. The study showed that patients with poor prognosis had higher amounts of collagen fibers that were perpendicular to the ducts. [50]

1.3 In vitro cancer models

Recreating the tumor microenvironment, encompassing tumor–stromal interactions, cell–cell adhesion, and cellular signalling, is fundamental to advance cancer-related research.

Traditional two-dimensional (2D) cell cultures and animal models have been effective in specific areas, particularly in explaining the behaviour of cancerous cells and exploring hypotheses about underlying mechanisms (*Figure 9*). However, a precisely defined three-dimensional (3D) in vitro cancer model that accurately mimics tumor structures observed in vivo while facilitating cell–cell and cell–matrix interactions investigation has gained strong interest for a wide variety of diagnostic and therapeutic applications. [10]

Monolayer culture of cancer cells in two-dimensional (2D) environment represents the most straightforward approach for conducting in vitro cancer studies. However, 2D in vitro models typically offer an overly simplified representation of the complex conditions found in vivo and fail to address numerous physiological questions.

The tumor microenvironment is defined by dynamic, bidirectional interactions between a wide range of cellular and noncellular components. Thus, reconstruction of the complex microenvironment assumes great significance in modern cancer biology, which can be attempted in a three-dimensional (3D) model. [12]

Supported by advanced engineering techniques, the development of a novel 3D in vitro cancer model system provides an opportunity to explore critical cancer mechanisms and develop innovative clinical therapies. For these reasons, in vitro cancer models are in high demand as they can accurately and efficiently replicate the microenvironment where cancer cells reside and where tumors grow.

An ideal 3D in vitro cancer model should be capable of reliably replicating the invasive behaviour of tumors and accurately simulating the interactions between tumor and stromal cells. Additionally, from an ethical perspective, an in vitro model helps reduce the use of animal models in many research laboratories. [10]

When compared to those in 2D models, gene expression, cell proliferation, cell migration or invasion, cell morphology and heterogeneity in 3D in vitro models are closer to in vivo. All these make 3D in vitro models a very valuable tool for cancer research.

Since 3D models closely recapitulate the tumor in vivo, these models can be used in drug screening and selection. Tumor cells in 3D models are usually more resistant against drugs and more invasive compared to those in 2D models.

The 3D models can be useful in dissecting the interactions of the tumor cells with immune and stromal cells and ECM, and in solving the signal transduction pathways involved in tumor initiation and progression. The 3D in vitro models can also be designed to study angiogenesis and drug efficacy. [17]

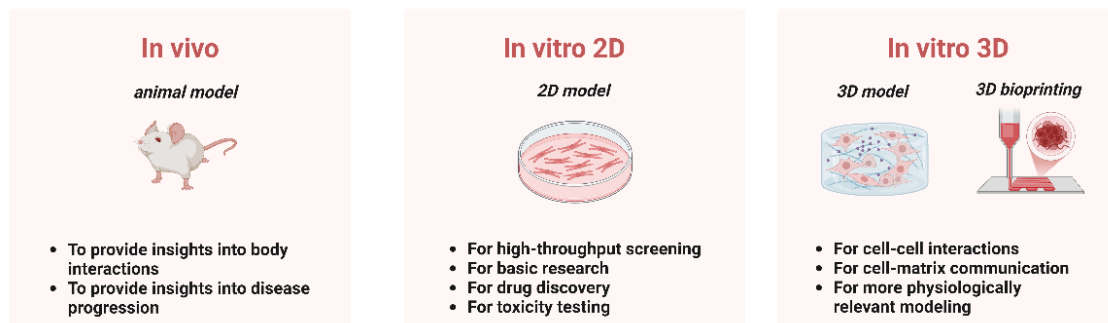


Figure 9: Advancing cancer research models: from in vivo to 2D and 3D in vitro approaches [created with Biorender]

1.3.1 Advantages of 3D bioprinted cancer models

In the last few years, 3D bioprinting has been used for the progression of improved cancer research cellular models. With the use of 3D bioprinting, researchers may simulate the disease in a lab setting using models created from cancer cells. The ability to automate the fabrication of these 3D bioprinted models while maintaining high repeatability and reliability matches up well with scalable production. [11]

Three-dimensional printing is an additive manufacturing technique, in which computer-aided 3D geometries are produced through layer-by-layer deposition of the materials often referred to as bioinks; in this way, complex 3D structures can be created with living cells. [17]

The long-term objective of 3D bioprinting is the fabrication of highly functional three-dimensional tissue-engineered constructs. In this way, crucial cell–cell and cell–ECM interactions would be stimulated, and the complex ECM structures would be mimicked.

3D bioprinting has a lot of potential for automating the fabrication of extremely complex 3D tissue constructs in a scalable and repeatable manner. Some of the bioprinting methods used to create 3D cancer models include:

- Extrusion-based bioprinting,
- Laser- assisted bioprinting
- Stereolithography,
- Inkjet-based bioprinting

Each of these technologies contributes to advancements in cancer research by providing more accurate and functional tumor models for studying disease mechanisms and testing potential therapies. Beyond this, there are the potential to address a variety of medical research problems and find applications in areas such as functional organ replacement, regenerative medicine, and drug delivery. [11]

Some studies have shown that 3D-bioprinted cancer constructs more accurately replicate physiological cancer tissues, particularly in terms of the tumor microenvironment and cellular behaviour, while preserving the characteristic spatial distribution of cells. For instance, Wang et al. demonstrated that 3D-bioprinted lung cancer models better mimic in vivo tumor growth and interactions compared to traditional 2D cultures, providing a more physiologically relevant platform for drug testing and disease modelling. Similarly, Maloney et al. developed a 3D-bioprinted glioblastoma model that effectively recreates the tumor microenvironment, enabling improved drug screening and a deeper understanding of glioblastoma progression. [11]

In addition to these benefits, bioprinting offers numerous advantages over traditional biofabrication methods, including the ability to create complex structures with viable cells, accurate reproducibility, cost-effectiveness, scalability, high throughput and efficiency.

Furthermore, bioprinting systems allow for using multiple cartridges that can be filled with different bioinks, and thus it is possible to spatially and temporally control the location of cells and other components to fabricate physiologically relevant constructs.

Beyond general cancer modelling, 3D bioprinting has been particularly impactful in breast cancer research. For instance, Reid et al. developed reproducible and reliable arrays of human mammary organoids within 3D collagen matrices using bioprinting. Their study demonstrated the superiority of bioprinted models over manually embedded matrix models in terms of efficiency and morphological consistency. [17]

3D bioprinting is revolutionizing in vitro cancer research by providing highly functional, physiologically relevant tumor models. The ability to fabricate complex 3D constructs with precise control over cellular and biochemical components enhances the study of tumor development, drug responses, and therapeutic strategies.

1.4 3D bioprinting

Three-dimensional printing is a term referring to additive manufacturing, as first described by Charles Hull in 1986. He reported the fabrication of complex 3D structures through stereolithography, a technique that relies on the photopolymerization of materials that are sequentially deposited as thin layers. Since then, many 3D printing modalities have been developed, including bioprinting.

3D printing has attracted particular attention in the biomedical field, given the complexity surrounding the personalized nature of tissues and the wide variety of human diseases. For this reason, the development of the technology has been linked to the idea of creating and printing in 3D personalized devices, including orthopedic devices and customized implants for the patient, such as surgical guides or in the field of regenerative medicine. This is possible thanks to the modelling of tissues through image reconstruction following computed tomography (CT) and magnetic resonance imaging (MRI).

An example of application, in this context, dates to 2013, an infant with tracheobronchomalacia (TBM) received a 3D-printed bioresorbable external airway splint and healed.

Bioprinting is a bottom-up manufacturing technique that involves depositing materials, so consists of computer-aided transfer processes for patterning and assembling structures through the combination of biomaterials, growth factors, and cells. Common bioprinting techniques used to fabricate biomaterials have included inkjet-based, extrusion-based, and laser-assisted bioprinting methods. [19]

Three-dimensional bioprinting has emerged as a promising approach for engineering functional tissues and organs by precisely positioning biological materials, living cells, and biochemical components layer-by-layer. Compared with non-biological printing, 3D bioprinting involves additional complexities, including selecting biomaterials that satisfy both printability and functionality requirements.

Most materials used in 3D bioprinting come from natural or synthetic biomaterials, each having its advantages and disadvantages. The limited availability of biological structures for the rehabilitation of lost or damaged tissues and organs has been the biggest motivation driving the field of 3D bioprinting.

One of the goals of 3D bioprinting is to develop and provide an accessible and affordable alternative to tissue implants, organ donors, animal testing procedures, and cancer research by printing cancer structures in vitro, so that tumor progression can be easily and reproducibly studied using this new technology. [13]

1.4.1 Bioprinting techniques

As mentioned earlier, bioprinting involves the controlled deposition of biomaterials in a predefined spatial arrangement, utilizing a methodology similar to conventional 3D printing. Over recent years, a variety of bioprinting techniques have been developed, with the most prominent approaches including stereolithography, inkjet-based, laser-assisted, and extrusion-based bioprinting. [15]

Figure 10 shows a workflow of the 3D bioprinting process, with reference to the "pre-bioprinting" phase, where cells and biomaterials are selected. Once the bioink is prepared, it is transferred into a cartridge and loaded into the printer. The second phase, "processing," describes the various printing techniques used, that will be described in detail below. Finally, in the "post-bioprinting" phase, the process that follows printing is detailed, including crosslinking, if necessary, and the incubation of the printed structure.

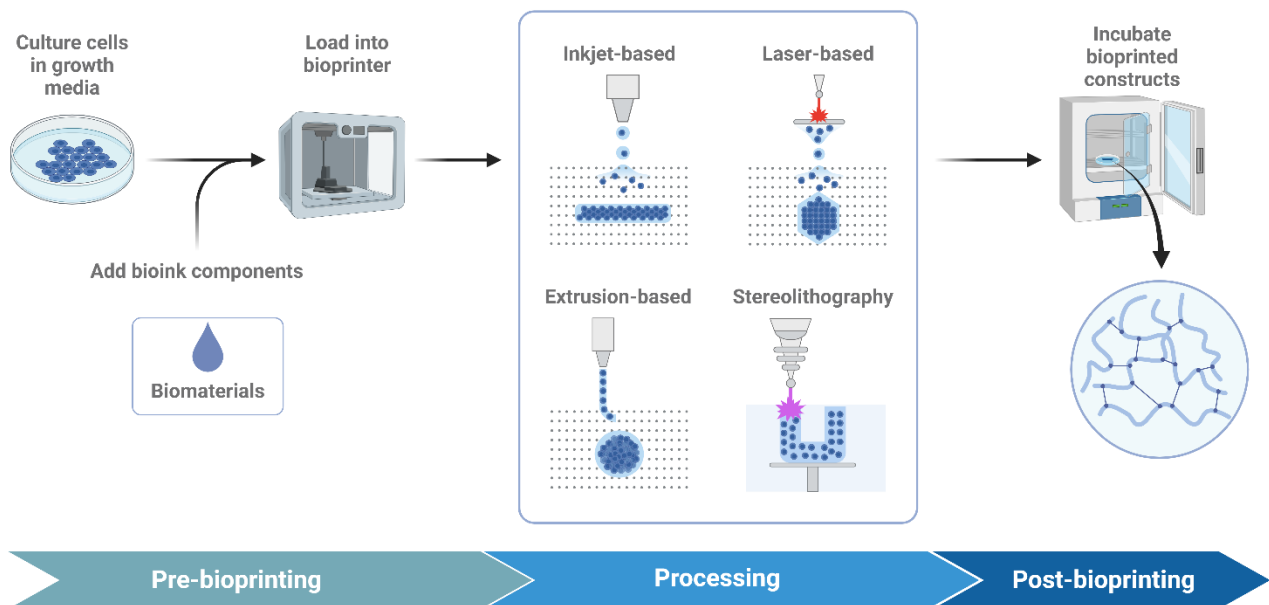


Figure 10: 3D bioprinting workflow [created with Biorender]

Stereolithography

Stereolithography is based on the crosslinking of a photosensitive material, using ultraviolet rays as a light source, ensuring, thanks to their high energy, an extremely rapid process, which can take place in two different ways.

In the laser-based method, the curing of each layer is obtained point-by-point through a scanner system (Figure 11); meanwhile, the digital light projection relies on a digital micromirror device to project and then crosslink an entire cross-section of the three-dimensional structure. [15]

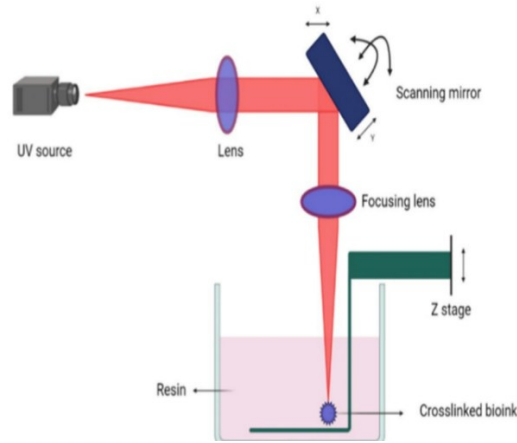


Figure 11: Stereolithography with scanner mirrors that crosslink each layer point-by-point. [14]

Stereolithography has been diffused in tissue engineering for its high speed, especially using the digital light projection method, and for its high resolution of up to 25 μm . Moreover, being a nozzle-free technique, no shear stress is exerted on cells, and thus cell viability is maintained high (>85%) after the printing process.

This technique does not require particularly stringent rheological properties, and a wider range of viscosities can be used compared to other bioprinting types: the biomaterial should be sufficiently viscous to avoid cell settling during crosslinking.

Furthermore, stereolithography offers the possibility of fabricating constructs such as hollow vessels and branching structures, which would otherwise be difficult to realize with traditional biofabrication methods. In this context, Miri et al. [16] incorporated a microfluidic device into a stereolithographic bioprinter to switch between different bioinks and produced different complex biological constructs, including a tumor angiogenesis model, composed of a GelMA matrix laden with breast cancer cells and tortuous channels filled with vascular endothelial cells. [15]

Table 1 presents some advantages and disadvantages of stereolithography.

ADVANTAGES	DISADVANTAGES
• Solid freeform and nozzle-free system • Highest biofabrication accuracy • Quick and cheap • Compatibility with many materials • Light-sensitive hydrogels can be bioprinted layer-by-layer	• Applicable to photopolymers only • Newer processes may be better • Lack of biocompatible and biodegradable polymers • Harmful effects from residual toxic photo-curing reagents • Possibility of harm to DNA and human skin by UV

Table 1: Advantages and disadvantages of stereolithography [14]

Inkjet-Based Bioprinting

Inkjet-based bioprinting dispenses bioink in a drop-on-demand manner onto a substrate, mainly using thermal or acoustic forces (*Figure 12*).

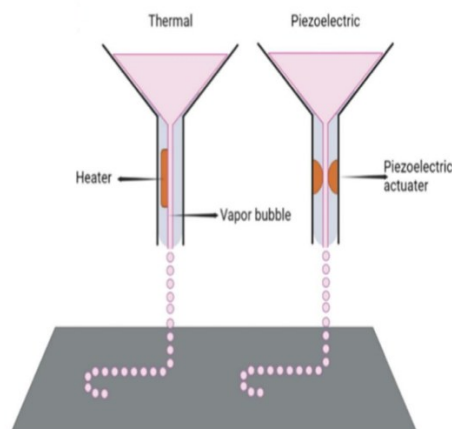


Figure 12: Inkjet-based bioprinting produces droplets through thermal or piezoelectric actuators. [14]

In thermal inkjet bioprinting, bioink is heated, and the vapor generated forces it out of the nozzle. The heating temperature is usually 200–300 °C; however, the overall bioink temperature rises to a maximum of 10 °C above room temperature due to the very short heating time. According to several studies, cell viability is about 70–95%, relying on the cell phenotype and printing parameters. The typical droplet diameter is in the range of 30–80 µm.

In the case of acoustic forces-based bioprinters, a piezoelectric actuator produces sound waves that break the bioink, leading to the drop extrusion. During piezoelectric bioprinting, the droplets are larger than those generated by the thermal method, ranging from 50 to 100 µm. The creation of the droplets by the acoustic method must take place in a frequency range not exceeding 15–25 kHz to prevent damage to the cell membrane. However, with the right printing parameters, the survival rate of cells is higher than 90%.

Inkjet bioprinting can produce up to 10,000 droplets per second, which is a highly desirable quantity in high-throughput screening applications, such as drug testing and cancer screening.

Some typical drawbacks of inkjet bioprinting are the mandatory use of low cell densities and low viscosity bioinks. Further disadvantages of this technique include the nonuniformity of droplet size and the limitation in bioprinting multiple cell phenotypes at the same time. [15]

Table 2 presents some advantages and disadvantages of Inkjet-Based Bioprinting.

ADVANTAGES	DISADVANTAGES
• Low cost • Wide availability • High resolution and high bioprinting speed • Electronic control of drop size and ejection rate • Can introduce concentration gradients in 3D constructs	• Poor vertical structure clogging characteristics • Thermal and mechanical stress on cells • Limited bioprintable materials – liquid only (high viscosity bioinks cannot be used) • Weak mechanical integrity of the construct

Table 2: Advantages and disadvantages of Inkjet-Based Bioprinting [14]

Laser-Based Bioprinting

Laser-assisted bioprinting is a nozzle-free, noncontact method where a laser beam is pulsed on a three-layer ribbon: laser-transparent glass, laser-absorbing metal, such as titanium or gold, and bioink. The laser beam energy is absorbed by the ribbon, which rapidly generates a local bubble on the opposite side which, in turn, ejects a desired amount of bioink on a receiving substrate (*Figure 13*).

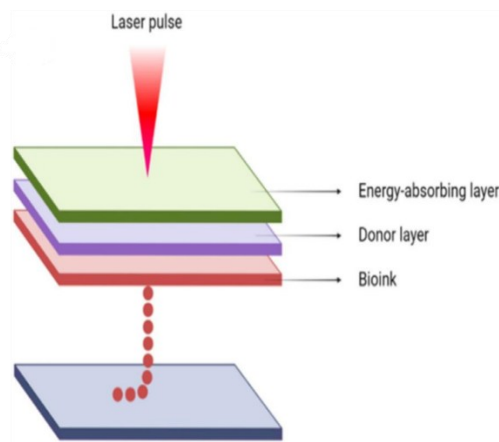


Figure 13: Laser-based bioprinting employs a laser beam to create droplets starting from a bioink ribbon. [14]

This technique is applicable for high cell densities (10^7 – 10^8 cells/mL). The resolution of the laser-assisted bioprinting can reach 10–100 μm and relies on several factors, such as the thickness of the absorbing layer and the bioink layer coated onto the ribbon, the viscosity and surface tension of the bioink, wettability of the substrate, laser energy, as well as the air gap between the ribbon and the receiving stage.

The viscosity range of the bioink is in the range of 1–8000 $\text{mPa}\cdot\text{s}$, while lasers with wavelengths from 193 to 1064 nm are employed. If the laser energy is too low or the bioink viscosity is too high, the jet cannot be developed completely, which may result in no material transfer; therefore, these two parameters are fundamental for the success of the printing.

Being a nozzle-free approach, laser-assisted bioprinting does not impart mechanical shear stress on cells, which exhibit a high viability (>95%) after the printing process. Bioprinters with a high-frequency pulse laser can obtain high-throughput printing, as high as 5000 droplets per second.

The drawbacks of this method are related to the high cost of the printer and to the absorbing layer: the break due to the intense energy absorbed can generate fragments that can contaminate the bioink and damage the living cells. [15]

Table 3 presents some advantages and disadvantages of Laser-Based Bioprinting.

ADVANTAGES	DISADVANTAGES
• Nozzle-less setup – no clogging • Noncontact process • Cells are bioprinted with high activity and microscale resolution • Compatible with a broad range of viscous constructs • High control of ink droplet • Precise delivery • Cells viability is very high (>90%)	• Time consuming ribbon layer preparation • Costly • Difficult to position cells • Require a metal film and thus is subject to metallic particle contamination • High resolution requires fast gel dynamics for high shape fidelity which causes a relatively low total flow rate

Table 3: Advantages and disadvantages of Laser-Based Bioprinting [14]

Extrusion-Based Bioprinting

Extrusion-based bioprinting represents the most popular method for the biofabrication of tissues. Although droplets and microbeads can be produced, bioink usually is extruded in the form of a continuous cylindrical filament through a nozzle or a needle and deposited layer-by-layer onto a print bed. The extrusion bioprinters exploit either pneumatic or mechanical dispensing systems. In some bioprinters, the print head can move in all three spatial dimensions; in others, it can move in the x- and y-directions, while the print bed can move in the z-direction (*Figure 14*).

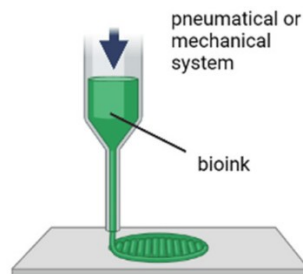


Figure 14: Extrusion-based bioprinting realizes continuous bioink filaments [15]

In general, lower-viscosity materials provide a more suitable microenvironment to support cell growth, whereas higher-viscosity materials often guarantee more desirable mechanical properties and

thus better structural support for the bioprinted models. Moreover, the temperature can be set and controlled during the printing process, which is particularly useful for thermosensitive hydrogels.

Cell viability, after the printing process, achieve a survival rate of 40–85%, depending on the extrusion pressure and on the gauge of the nozzle used, but could be even above 90% if the printing parameters are optimized. Proper cell viability, which is fundamental for functional tissue, can be obtained by reducing the pressure and using a large nozzle, though it decreases the resolution and the print speed.

However, the widespread employment of extrusion bioprinting is primarily due to the possibility of using high viscosity bioink and the capability to realize large-scale 3D models. Other benefits include the versatility of this technology, allowing the simultaneous printing of different cells and different biomaterials, and its affordability. [15]

Table 4 presents some advantages and disadvantages of Extrusion-Based Bioprinting.

ADVANTAGES	DISADVANTAGES
• Reduced amount of shear stress • Wide range of drive forces • Deposition of an array of biological materials with different rheological properties is possible • Most cell support hydrogels with viscosities ranging $30\text{--}6 \times 10^7 \text{ mPa}\cdot\text{s}$, are compatible with this system • Can deposit cell in very high densities	• High dispensing pressure and shear stresses inflicted on cells in viscous fluids • Low viability of cells deposited by extrusion • Loss of bioprinting resolution and speed

Table 4: Advantages and disadvantages of Extrusion-Based Bioprinting [14]

1.4.2 Bioink for 3D bioprinting

The principle of bioprinting can be defined as the placement of cells within biomaterials into spatially defined structures using automated 3D bioprinter technologies. Recently, various bioprinting processes have emerged, as described in the previous paragraph, along with various process-compatible bioink materials. [20]

The term bioink was first used in the context of organ printing in 2003 and was introduced together with the term biopaper. Initially, the concept was to provide, or even print, a biopaper (hydrogel) and then insert living cells as the 'bioink' by bioprinting. Thus, the term bioink originally referred to the cellular component that was positioned in three-dimensional on or within hydrogels.

A distinction is made between printable materials, and, by definition, two categories of materials are identified for bioprinting:

- Bioink
- Biomaterial ink

Bioink is a formulation that must contain cells, which are an essential component of the printed material. Consequently, a formulation without cells cannot be considered a bioink.

On the other hand, biomaterial ink is an aqueous formulation based on polymers or hydrogel precursors that can be used for printing but, by definition, becomes a bioink only when cells are added to its composition. [21]

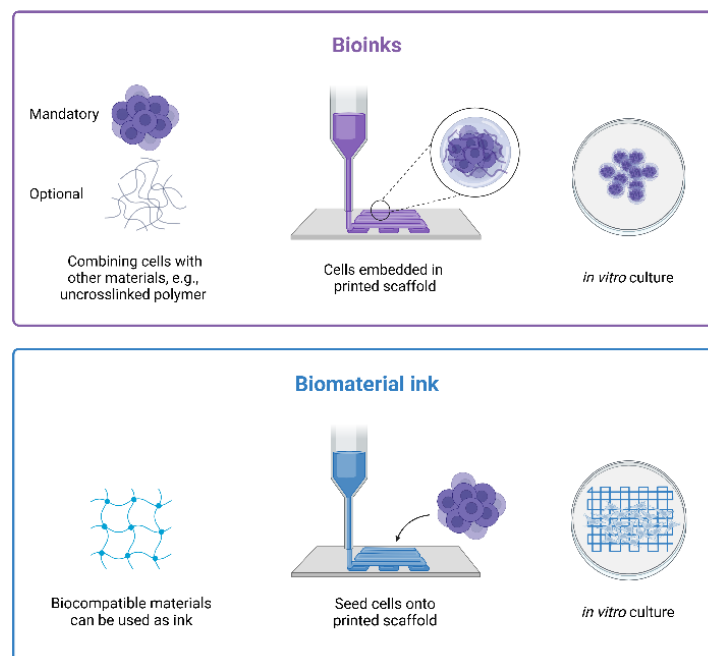


Figure 15: Graphical difference between a bioink and a biomaterial ink used in bioprinting. [modified with Biorender]

In Figure 15 it is possible to analyse the distinction between a bioink (top section), where cells are a mandatory component of the printing formulation in the form of single cells, or also in combination with materials; and a biomaterial ink (bottom section), where a biomaterial is used for printing and cell-contact occurs post-fabrication. [21]

It is important to consider and optimize the properties of a bioink according to the target tissue. These properties include cell source, ink rheology, gelation process, biocompatibility, bioprintability.

As defined above, a hydrogel is considered bioink when living material is added to it, which is why cells are a mandatory component of the bioink. In this regard, the *selected cells* should maintain their

function, mimic their physiological state, proliferate adequately and survive during the biofabrication process. [23]

With the further development of the field of bioprinting, the understanding of material *rheological properties* used in the printing process increased. [21] The pressure the bioink experiences as it is forced through the nozzle can change its properties. It is critical for a perfect bioink to maintain this pressure by acting like a shear-thinning fluid. In addition, the bioink should maintain its structural integrity after being extruded from the nozzle by exhibiting a viscoelastic response. These properties fall under the umbrella of bioink rheology, which determines the success of bioprinting bioinks into well-structured 3D objects.

Another important property to evaluate is the *gelation process*, which occurs when the gel form of the bioink solidifies after extrusion; gelation can impact both the viability and resolution of the bioprint and should therefore be rapid, biomimetic and non-toxic to cells. The gelation process can also affect the compatibility with a particular bioprinting system.

Depending on the composition and material properties of the bioink, several options are available for the gelation process, these include ionic, thermal, photocrosslinking and enzymatic processes. For example, while thermal gelation offers great biocompatibility, the temperatures required for the process can reach potentially dangerous levels; some examples of biomaterials that undergo thermal gelation are Matrigel and collagen. Similarly, photocrosslinking allows the bioink to be crosslinked, but can be dangerous to cell viability; an example of a photocrosslinked biomaterial is gelatin methacrylate, which is photocrosslinked after printing to adjust its mechanical properties. As no one gelation process can give the best results in all areas of interest, it is recommended that the gelation process is chosen according to the objectives of the experiments and the availability of the material.

Advancement in research has deepened and evolved the understanding of *biocompatibility* from the requirement of low cytotoxic effects to positive biological interactions. On the one hand bioinks derived from natural materials offer a favourable environment because they can mimic the natural cellular environment, but they have weak mechanical properties. On the other hand, synthetically developed bioinks may not be as biocompatible but they offer superior mechanical properties. [23]

Synthetic polymers can be chemically modified with not crosslinkable functional groups. [20] Therefore, depending upon the desired function, the target tissue, and the purpose of the experiment, the choice of bioink material can vary between natural and synthetic sources. [23]

From a structural point of view, *bioprintability* constitutes a fundamental requirement, as bioprinted models must be able to be printed in order to reproduce the complex microarchitecture of native tissues in vitro in sufficient resolution. [22]

Based on the characteristics described above, the bioink should be able to maintain its structure after bioprinting and throughout the in vitro observation period, preventing deformations or collapses that could compromise its functionality and reliability. Therefore, its properties must be optimized before, during, and after bioprinting to achieve the desired construct. [23]

1.4.3 3D Bioprinting procedure workflow

The entire process of 3D bioprinting can be summarized into three principal steps: “pre-bioprinting”, “bioprinting”, and “post-bioprinting”, as shown in *Figure 16*.

Pre-bioprinting

As the first step in this pre-processing phase, the target tissue is designed using computer-aided design (CAD) techniques. To achieve this, it is crucial to understand the geometry and structure of the biomimetic scaffold. This is typically accomplished through medical imaging techniques such as computed tomography and magnetic resonance imaging; these imaging techniques allow a plan of the desired final structure to be developed. However, in the case described in this thesis, medical imaging is not required, as the structure to be fabricated is less complex than a tissue or organ. Instead, the 3D structure is designed using modelling software, based on its dimensions and the desired shape.

Digital models can be generated using 3D modelling software or, and these models are then utilised in 3D bioprinting techniques to design the 3D construct. To represent structures within a 3D cartesian coordinate system, CAD-developed models are converted into STL (Standard Tessellation Language) files. This format contains only surface geometry data, providing essential information about the structure’s dimensions, shape, and defined thickness, ensuring accurate 3D reconstruction. A specialized program known as a "slicer" then processes the STL file, converting it into a machine-readable format (.gcode file) that the 3D printer can interpret. The slicing software further refines the data by incorporating machine-specific parameters, optimizing the printing process for precision and efficiency. [24]

Once the printing file has been created, it is necessary to prepare the bioink. As described in the previous section, the selection of the bioink must be based on the type of biological application. After preparation, the bioink is transferred into a cartridge and subsequently inserted into the printhead.

Bioprinting

During the printing phase, various conditions must be evaluated, starting with the type of printing being performed. Many printers are equipped with a display that allows users to select different settings essential for the process. Therefore, using the printer's display, it is necessary to load the previously prepared (. gcode) file and configure the printing parameters, including the temperature of the printheads and the print bed, both of which vary depending on the biomaterial used and the presence or absence of cells, as well as the possible activation of photocrosslinking.

Post-bioprinting

Depending on the bioink and the 3D printing technique used, post-processing procedures will be followed to treat the printed structure; generally, the obtained structure is stored in an incubator at 37°C to ensure cell viability, and then the structure is observed for many days.

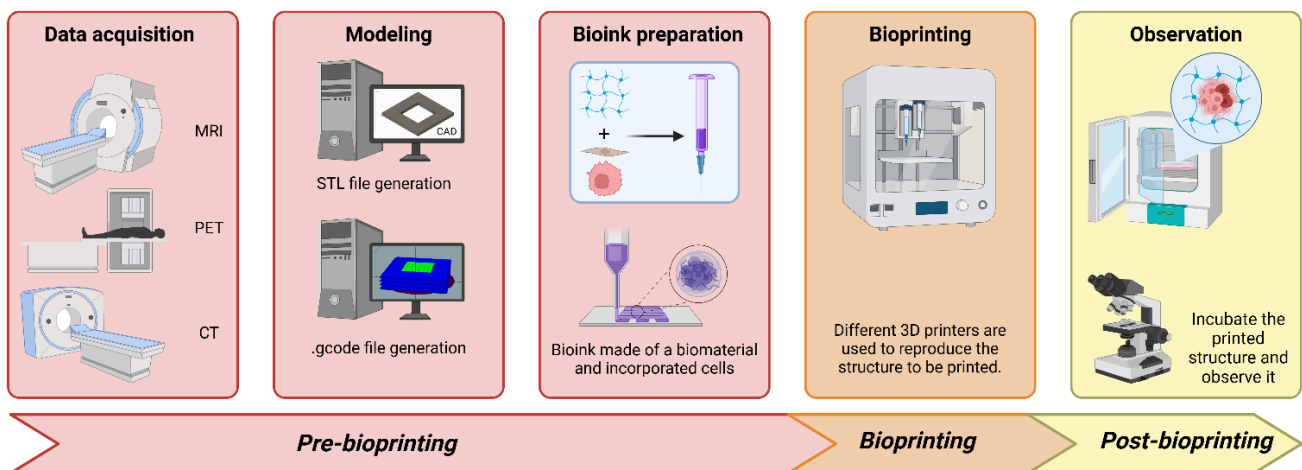


Figure 16: 3D Bioprinting procedure workflow [created with Biorender]

2. Materials and Methods

This section describes the biomaterials used for the fabrication of a bioprinted structure through 3D bioprinting, with a particular focus on the use of the BIOX (Cellink).

An overview of the selected biomaterials will be provided, highlighting their biocompatibility and bioprintability properties. A detailed explanation will follow on the cell lines employed for the development of the bioinks, and the protocols used for cell culture, bioink preparation, and the main biological methods applied.

Finally, an overview of the bioprinting software and hardware used throughout the experimental process of the thesis will be presented.

Acetic Acid Glacial (Merck), Acetone (Sigma-Aldrich), Calcein AM (ThermoFisher), Cell line 4T1-GFP, Cell line MAF tdTomato, Cell line WI38, Deionized water, Dulbecco's Modified Eagle Medium (DMEM F12 1:1) (Sigma-Aldrich), DMEM 1X (Sigma-Aldrich), Ethanol - EtOH 100% (Merck), Fetal Bovine Serum (FBS), GelMA C (Cellink), Glutamine, HBSS +/- Hank's Balanced Salt Solution (Thermo-Fisher), Lifeink® 200 Collagen Bioink (Cellink), Matrigel® (Corning), Milli-Q®, Minimum Essential Medium (MEM) (Sigma-Aldrich), PBS10X (Sigma-Aldrich), Penicillin-Streptomycin, Sodium Hydroxide (NaOH) (Sigma-Aldrich), 3-(Trimethoxysilyl)propyl methacrylate (TMSPM) (Sigma-Aldrich), Trypan Blue (Invitrogen™), Trypsin-EDTA (0.05%) (Sigma-Aldrich), Trypsin-EDTA (0.25%) (Sigma-Aldrich), Ethidium homodimer-1 (ThermoFisher), Type I collagen rat tail (Corning).

2.1 Bioprinting materials

Natural hydrogels are widely regarded as promising scaffold materials due to their biocompatibility and their structural and physical resemblance to natural tissues. However, one of the biggest challenges in this field is their generally poor processability, which makes it difficult to fabricate complex 3D structures.

2.1.1 Gelatin methacryloyl (GelMA)

A material that has recently attracted attention in the field of tissue engineering is gelatin, a water-soluble biodegradable polypeptide derived from partial hydrolyzation of collagen.

Its advantageous characteristics, including biocompatibility, biodegradability, and low toxicity, facilitate enhanced cell adhesion, differentiation, and proliferation. Moreover, gelatin is commonly used to form thermally driven hydrogels and can be enzymatically degraded by body enzymes, without eliciting an immunogenic response. Its cost-effectiveness has led to its application across a wide range of tissues, including bone, skeletal muscle, and neural tissues. Gelatin has been used in various forms, from microparticles that enhance bone regeneration to wound dressings and hydrogels designed for the controlled release of chemotherapeutic agents in cancer treatment. Additionally, the inherent ability of gelatin to absorb water is highly valued in tissue regeneration, as its porosity ensures the diffusion of nutrients and oxygen essential for proper cell growth. [46]

However, despite these benefits, gelatin exhibits certain limitations in tissue engineering applications, such as low mechanical stability, rapid enzymatic degradation, and high solubility under physiological conditions. To overcome these limitations, gelatin can be chemically modified by introducing pendant methacrylamide groups. The resulting material, gelatin methacryloyl (GelMA), is a hydrogel that forms covalent cross-links via radical polymerization when exposed to UV light in the presence of a photoinitiator, as shown in *Figure 17*.

It has been proven that GelMA hydrogels at low concentrations (≤ 5 w/v %) are particularly suitable as cell-laden bioinks due to their ability to support high cell viability and stability. However, they present some problems when used in extrusion-based 3D bioprinting: the low concentration leads to a low viscosity, making the bioink unstable during extrusion, the transition from the liquid to the gelling state is too slow and, once gelled, the hydrogel does not possess the mechanical stability needed to maintain the expected geometry of the support.

On the other hand, while high-concentration GelMA bioinks (> 15 w/v %) offer excellent printability, they come with a trade-off: the reduced cell viability. This creates a fundamental challenge in using

GelMA hydrogels, finding the right balance between physical printability and biological functionality. [25]

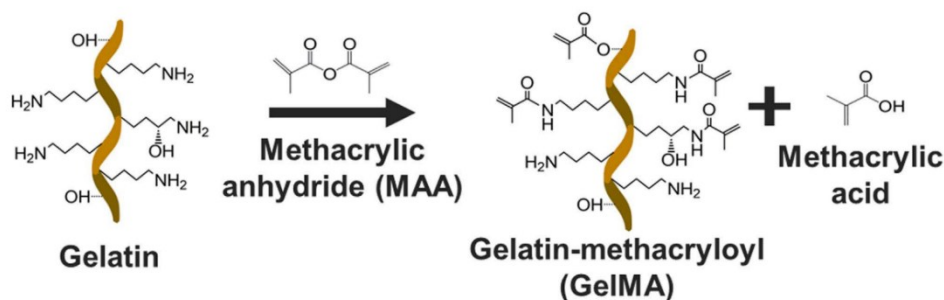


Figure 17: Schematic illustration of gelatin-methacryloyl (GelMA) synthesis. [30]

An alternative approach to improve this issue is the use of viscosity enhancers in combination with low-concentration GelMA bioinks. It has been shown that adding additives, such as nanocellulose, significantly improves printing quality. In this context, GelMA C has been introduced, a bioink that combines GelMA with nanofibrillated cellulose. [26] Cellulose nanofibers (CNFs) are nanoscale cellulose fibrils with typical widths of several tens of nanometers and a wide range of lengths [28]. This additive has attracted particular interest in the field of 3D bioprinting due to its rheological properties, as it exhibits shear-thinning behaviour in aqueous solutions. Numerous research studies have demonstrated that cellulose is a biocompatible and low-toxicity component capable of mimicking the extracellular matrix (ECM). Moreover, it has a highly beneficial impact not only on the printing process but also on cell viability and proliferation. [26]

As demonstrated by Shin et al. (2017), cellulose nanofibers (CNFs) were used to enhance the printability of low-viscosity gelatin derivatives. In this study GELMA C solution containing 5% w/v GelMA and varying concentrations of CNF up to 2.0% w/v were prepared by dissolving the polymers in cell culture medium.

Results showed that the presence of CNFs incorporated in the GelMA solution allowed to significantly increase the bioprintability of the material. The viscosity of the GelMA solution gradually increased as the CNF concentration increased. During the 3D printing process, the printed structure maintained its shape without collapsing. A cytotoxicity analysis was also performed, and the number of live cells gradually increased over the culture period, with most cells remaining viable. The live and dead assay of the cell-loaded GelMA C hydrogels indicated that the hydrogel did not exhibit any significant cytotoxicity. Interestingly, GelMA C showed higher metabolic activity than pure GelMA hydrogels after prolonged incubation, indicating the beneficial effect of CNFs on cell proliferation in the hydrogel system. [27]

Based on these considerations, this thesis explores the use of GelMA C as a bioink for 3D bioprinting, in combination with stromal and tumor cells. GelMA C is jointly patented by UPM-Kymmene Corporation and CELLINK Bioprinting. From the specification sheet, the methacrylation degree is in the range 45-55%, while gelatin concentration is not reported. It is supplied in 3 mL cartridges and pre-mixed with 0.25% w/v LAP photoinitiator. Stored at 4°C, it remains in a solid state under these conditions. The presence of LAP in the bioink formulation allows the material to be photocrosslinked by exposure to UV light at 405 nm, thereby increasing the mechanical stability of the printed structure. A scheme of the photocuring mechanism is represented in *Figure 18*.

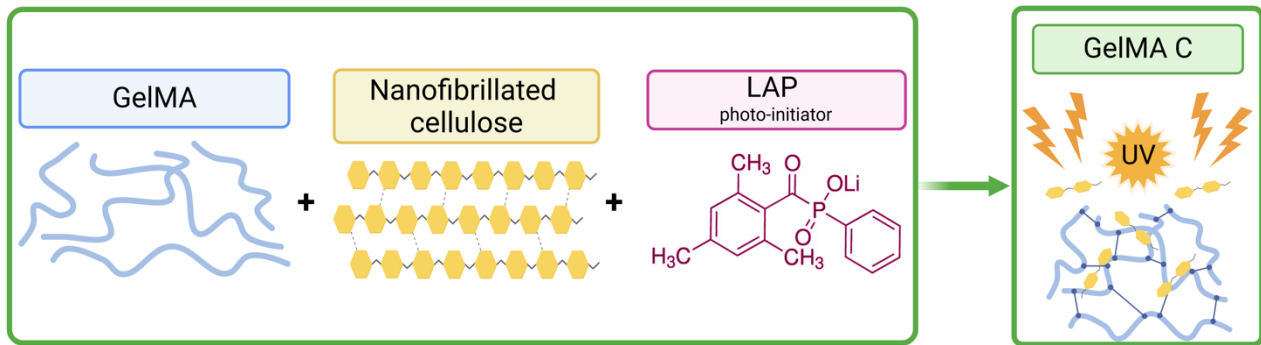


Figure 18: Schematic illustration of GelMA C photocrosslinking synthesis. [created with Biorender]

The presence of nanosized fibrils keeps the bioink translucent, facilitating cell imaging and analysis. This unique formulation ensures smooth printability at ambient conditions and low extrusion pressures, accommodating a wide range of nozzle diameters while preserving the universal cell attachment motifs of GelMA. [29]

The bioink has the following quality criteria (*Table 5*):

PARAMETER	VALUES
Ph	6.5-7.4
Gelma degree of methacrylation	45-55%
Viscosity (measured at 22°C)	≥ 3 kPa·s at 0.01 s ⁻¹ , ≤ 3 Pa·s at 200 s ⁻¹ ,
Base material	GelMA
Stabilization modality	Photocuring

Table 5: GelMA C specifications [29]

2.1.2 Matrigel

Matrigel™, a commercial product, is a gelatinous ECM protein mixture secreted by Engelbreth-Holm-Swarm mouse sarcoma cells (Kleinman and Martin, 2005) [31]. Matrigel is an ECM mixture primarily composed of collagen type IV, laminin, entactin and perlecan. Given its composition, Matrigel resembles the complex basement membrane environments found in many biological soft tissues. [32]

It promotes cellular outgrowth from tissue fragments, vascularization and differentiation of various cell types [31]. Moreover, it facilitates cells self-organization into structures that closely mimic the features of in vivo tissues, making it the most widely used matrix for 3D organoids cultures. Regarding 3D bioprinting applications, Matrigel is commonly used in combination with other bioinks which act as support materials, because its poor mechanical properties and non-thixotropic behaviour makes it difficult to bioprint with common bioprinting strategies. It is used to improve biocompatibility, as many hydrogels lack essential protein components, compromising performance. [32]

One of the major advantages of culturing cells on Matrigel™ is that it promotes complex cellular behaviours that are difficult to observe in other 3D matrices. For example, endothelial cells create capillary sprouts in Matrigel™ (Kleinman and Martin, 2005). Molecules that promote endothelial cell network formation (Paul et al., 2013) are potential candidates for use in tissue regeneration therapies, while molecules that inhibit such formation can be used for anti-cancer therapies. Matrigel™ has been vital in the development of tumor xenograft models in rodents for the discovery of novel cancer treatments (Benton et al., 2011). [31]

Matrigel™ is an expensive material with a composition that is not completely defined and exhibits batch-to-batch variability. Derived from mouse tumors, its clinical applicability in humans is limited. However, thanks to its availability, ease of use, and versatility in culturing various cell types, it remains widely utilized. [32]

The thermal gelation properties of Matrigel™ and collagen type I are similar; however, Matrigel™ gelation is thermally reversible. Crosslinking occurs between 24 °C and 37 °C, and gelation takes about an hour to complete, beginning at temperatures above 5 °C.

In extrusion-based bioprinting, Matrigel™ needs to be bioprinted before becoming fully crosslinked; thus, a cooling chamber or temperature-controlled printheads are essential [31]. To accelerate the crosslinking process after deposition and to maintain the printed shape, a heated printbed or an incubator should be employed.

2.1.3 Rat Tail Collagen

The hierarchical organisation of collagen (*Figure 19*) is a widely investigated feature because it impacts on the properties of both collagenous tissues and engineered collagen structures.

Collagen fibers, which contribute to the mechanical strength of various tissues and organs, are composed of collagen fibrils. These fibrils, with diameters ranging from 100 to 500 nm and lengths of a few millimetres, are formed by the staggered arrangement of five collagen molecules. At a more fundamental level, collagen molecules, also known as tropocollagen, consist of three left-handed polypeptide chains that coil together to form a right-handed triple-helix structure. Each of these polypeptide chains follows a repeating amino acid motif, Gly-X-Y, where glycine (Gly) serves as the core, while X and Y represent other amino acids. [39]

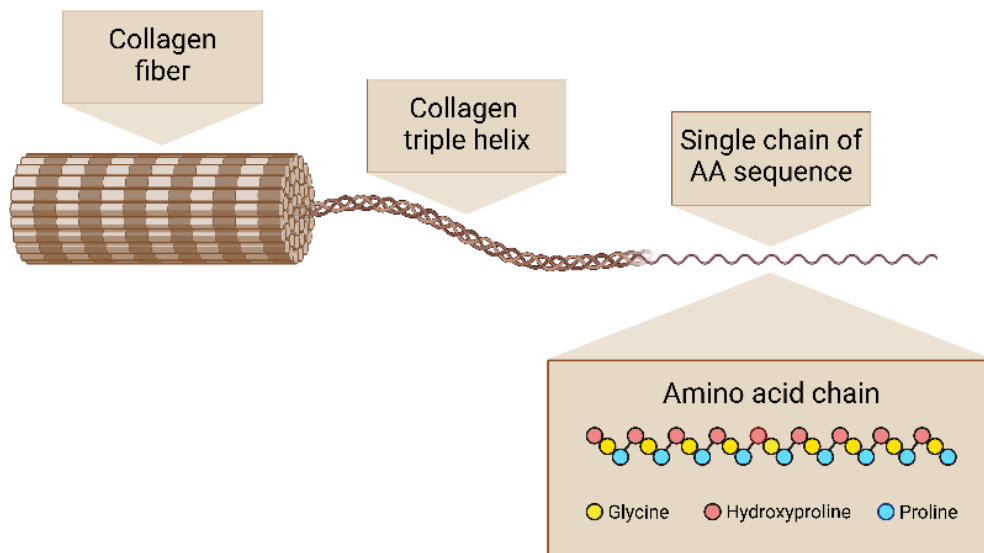


Figure 19: Hierarchical representation of collagen [created with Biorender]

Collagen Type I is the most prevalent type of collagen used in biomedical research, with extensive documentation on its properties and applications. It is a natural biomaterial with excellent biocompatibility and low antigenicity, making it ideal for tissue engineering and regenerative medicine. This type of collagen is predominantly found in the skin, bones, tendons, ligaments, and cornea of animals, where it plays a crucial role in maintaining structural integrity. [33]

In general, collagen is the most abundant extracellular matrix (ECM) component in the body, forming the majority of the interstitial matrix. It accounts for approximately 30% of the total body protein content in humans. Collagen plays key roles in cell adhesion, migration, tissue scaffolding, and repair. Despite its natural abundance, commercially available collagen sources are often expensive and subject to high inter-batch variability, which affects reproducibility in large-scale biomedical applications. [34],[35]

Among the 28 different types of collagens identified, fibrillar collagens (Type I, II, III, V, and XI) represent more than 90% of the total collagen in the human body. These fibrillar collagens form structural fibrils that provide tensile strength and contribute to tissue stability and integrity. Other collagen types, such as Type IV and VII, are crucial for forming basement membrane networks. [34]

The primary sources of collagen for biomedical use come from animal tissues, mainly from skin and tendons. Among these, rat-tail tendon collagen is one of the most widely used due to its well-characterized properties and high purity. Different species produce collagen with unique chemical, physical, and biological characteristics, making source selection crucial for specific applications. Rat-tail tendon is considered one of the original and most reliable sources of Type I collagen extracts. [33]

The composition of collagen varies across tissues. In tendons, collagen constitutes about 30% of the dry mass, of which 60-80% is Type I collagen, while elastin makes up only 2% (*Figure 20*). [34]

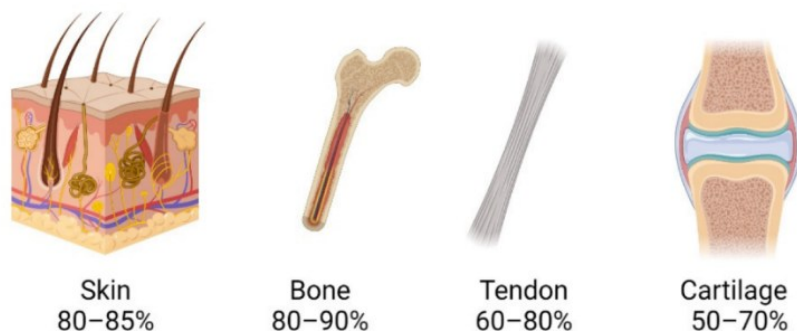


Figure 20: Collagen type I sources and approximate content in each tissue. N.B. Cartilage percentage is based on all collagens in their dry weight, as it is predominantly collagen type II. [34]

In this thesis, Corning® Collagen I, Rat Tail, was used. This collagen is derived from rat tail tendon and has a purity of $\geq 90\%$, as determined in the specification sheet. It has been tested for its ability to promote attachment and spreading of HT-1080 human fibrosarcoma cells. Additionally, it forms a firm gel up to a dilution of 1:10, while further dilution decreases the rigidity of gel. In terms of formulation, Corning® Collagen I is supplied as a liquid solution in 0.02 N acetic acid, with a concentration range of 3-4 mg/mL. It remains stable for at least three months when stored at 2° to 8°C but should not be frozen. For handling, it is effective both as a gel and as a thin coating, with a recommended concentration of 5-10 $\mu\text{g}/\text{cm}^2$ of growth surface, depending on the cell type. [36]

2.1.4 Lifeink 200 Collagen

Lifeink® 200 Type I Collagen Bioink is a high-purity, pH-neutral, and isotonic bioink specifically designed for FRESH 3D bioprinting.

With a collagen concentration of 35 mg/mL, it provides a biomimetic environment that closely mimics the native extracellular matrix. This bioink exhibits shear-thinning properties, ensuring high print resolution and mechanical integrity after deposition. Additionally, its cytocompatibility and ability to undergo cellular remodelling make it an ideal candidate for tissue engineering and regenerative medicine applications. [38]

The unique properties of Lifeink® 200 have enabled significant advancements in bioprinting functional tissues and organ models, as demonstrated in a groundbreaking study by Lee et al. [37], where collagen bioinks were successfully used to print anatomically precise heart components with physiological functionality. More specifically, they present a method to print collagen using FRESH technique to engineer components of the human heart at various scales, from capillaries to the full organ.

This study highlights the potential of collagen-based bioinks in the fabrication of complex, vascularized tissues that closely mimic native structure. [37]

Advanced BioMatrix supplies Lifeink® 200, that is in a sterile, aseptically processed form, and should be stored at 2–8°C. It maintains a shelf life of at least six months, with optimal printing temperatures ranging from 2–8°C to preserve its bioactive properties.

The collagen is purified from bovine sources, ensuring a >99% purity level, and undergoes strict quality control testing for endotoxin levels and sterility. Due to its superior mechanical properties, ability to support cell viability, and biocompatibility with continuous flow extrusion bioprinting, Lifeink® 200 is a cutting-edge biomaterial for advanced biofabrication strategies in tissue engineering. [38]

2.2 Cells culture

To establish and maintain a cell culture, specific cell lines are required. In this thesis 4T1-GFP, MAF-tdTomato, and WI38 cells were used. 4T1-GFP cells are a green fluorescent protein (GFP)-labeled mouse mammary carcinoma cell line (4T1), making them visible under green fluorescence. WI38 cells are human fibroblasts derived from fetal lung tissue. Lastly, mouse adult fibroblasts (MAFs) are primary cells derived from mouse skin; after extraction, MAF were subsequently genetically modified to express tdTomato, a constitutively orange fluorescent protein, making them instantly visible under a fluorescence microscope.

In the experiments of this thesis, fibroblasts were used to simulate the stromal environment, while cancer cells were employed to mimic the tumor microenvironment.

For cell detachment, Gibco™ Trypsin-EDTA (Sigma-Aldrich) is used. The typical use for this product is in removing adherent cells from a culture surface. The concentration of trypsin necessary to detach cells from their substrate is dependent primarily on the cell type and the age of the culture. In detail, WI38 cells require trypsin 0.05%, whereas the other cell lines are treated with trypsin 0.25%.

All cell lines are detached following the standard splitting protocol, which will be described below, and then resuspended in their respective culture medium.

4T1-GFP cells are cultured in DMEM/F-12 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S), and 1% glutamine. WI38 cells are cultured in Modified Eagle Medium (MEM) with 10% FBS, 1% P/S, and 1% glutamine. Lastly, MAF cells are cultured in DMEM 1X supplemented with 20% FBS, 1% P/S, and 1% glutamine.

To ensure fibroblast adhesion (MAF and WI38) to the flask surface, the substrate must be coated with a collagen-based solution: coating with collagen is necessary because fibroblasts do not adhere directly to the flask wall but rather to the collagen. This coating solution consists of 0.02M acetic acid and Corning® rat tail collagen at a final concentration of 0.05 mg/mL. The solution must remain in contact with the surface for at least 40 minutes and be incubated at 37°C with 5% CO₂. After incubation, the coating solution is removed, the flask surface is washed with PBS 1X or culture medium, and the surface is ready for cell culture.

Cell splitting protocol

Before starting a cell split, it is crucial to verify that all necessary equipment and conditions are in place. This includes: a certified biological safety hood to protect both the cells and the operator from potential biological contaminants; a centrifuge, preferably one capable of refrigeration and equipped

with suitable containment holders for cell culture use; a microscope to examine the cell cultures accurately; a humidified incubator set at 37°C with 5% CO₂ in the air to maintain the optimal conditions for cell growth; and a 37°C water bath, filled with water containing bacterial and fungal growth inhibitors, can also be helpful for warming media before use. [40]

To begin with, the flask containing the cells should be observed under the microscope to assess the cell confluency and evaluate the overall health and condition of the cells. Under the hood, the exhausted culture medium is removed from the flask, which may contain dead cells. Depending on the specific cell line, one or more washes are performed with Trypsin-EDTA. This step ensures that almost only healthy cells are carried forward and initiates cell detachment from the flask wall. Subsequently, an appropriate amount of trypsin is added to the flask for cell detachment. The flask is then placed in the incubator for some minutes at 37°C (the precise incubation time is contingent on the cell type). After the incubation period, the cells are checked under the microscope to determine if they have detached from the surface of the flask. If necessary, additional incubation time can be given for complete detachment. At this point the action of trypsin is stopped by adding an appropriate volume of fresh cell culture medium, which should be approximately four times the volume of trypsin used earlier. This step is essential to prevent any further enzymatic activity. The cell suspension is transferred into a Falcon tube and centrifuged at 1500 rpm for 4 minutes. This process allows the cells to form a pellet at the bottom of the tube, separating them from the medium. After centrifugation, the supernatant is carefully removed, and the pellet is gently resuspended in an appropriate volume of culture medium to ensure a uniform suspension of cells.

Based on the observed confluency of the cells, the area covered by the cells is calculated. This is done using the formulas:

$$100 [\%] : \text{flask total area [cm}^2\text{]} = \text{observed confluency [\%]} : x [\text{cm}^2]$$

$$100 [\%] : \text{flask total area [cm}^2\text{]} = \text{desired confluency [\%]} : x [\text{cm}^2]$$

Once the observed confluency area and the desired confluency area are determined, the volume of the cell suspension required to achieve the desired confluency in the new flask is calculated using the following formula:

$$\text{total suspension volume [mL]} : \text{observed confluency area (cm}^2\text{)} = x [\text{mL}] : \text{desired confluency area [cm}^2\text{]}$$

The calculated volume corresponds to the amount of cell suspension to transfer to the new flask to continue cell culture. The described protocol is schematically illustrated in *Figure 21*.

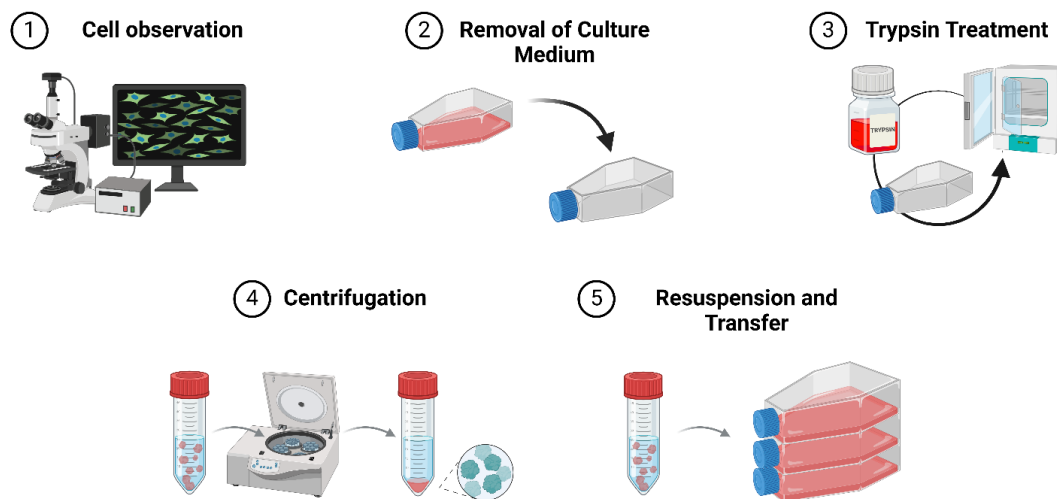


Figure 21: standard splitting protocol [created with Biorender]

2.3 Biological methods

This section outlines the biological methods employed in this thesis, which are fundamental for the development of 3D bioprinted in vitro models. Specifically, it details the preparation of cell suspensions in bioinks, cell counting method, and live/dead staining for viability assessment. These techniques ensure the reliability and reproducibility of the experimental workflow, ultimately contributing to the accuracy of the bioprinting process.

2.3.1 Cells suspension in bioinks

After performing the *cell splitting protocol*, as described in the previous section, the cell suspension can be used for different purposes. For instance, it can either be employed to continue the cell culture or as a component of a bioink.

In the first case, enough cell suspension is used to maintain the cell line, and an appropriate volume of fresh culture medium is added to the flask. In the second case, the cell suspension is processed following two different procedures, depending on the viscosity and type of biomaterial used to obtain the final bioink.

- **Direct mixing:** a specific volume of cell suspension is collected based on the desired cell concentration in the bioink. It is then transferred into a tube and centrifuged. After centrifugation, the supernatant is removed, and the remaining cell pellet is directly resuspended in the selected biomaterial for printing. This method is generally used for low-viscosity biomaterials.

- **Mixing with resuspension:** a volume of cell suspension is collected to obtain the desired final cell concentration. The suspension is transferred into a tube and centrifuged. After centrifugation, the supernatant is removed, and the cell pellet is resuspended in a volume of medium. This cell suspension is subsequently loaded into a proper syringe and mixed with the selected biomaterial prior to printing.

The second method is described in detail in Cellink protocol for using GelMA C. The protocol recommends diluting GelMA C following a 1:10 ratio of the final volume, meaning $\frac{9}{10}$ should be GelMA C, while $\frac{1}{10}$ should be the cell suspension. During the development of this thesis, different dilution ratios were tested. A second trial followed a 3:10 ratio of the final volume, meaning $\frac{7}{10}$ should be GelMA C, while $\frac{3}{10}$ should be the cell suspension. In this case, the cell suspension and biomaterial are mixed using a specific procedure recommended by Cellink.

This protocol involves using two syringes: one containing the defined cell suspension and the other containing the pure biomaterial. The two syringes are connected using a connector, and their contents are mixed thoroughly by repeatedly transferring the solution between them. Once a homogeneous solution is obtained, it is transferred into one of the syringes and then loaded into the printing cartridge (Figure 22) [41].

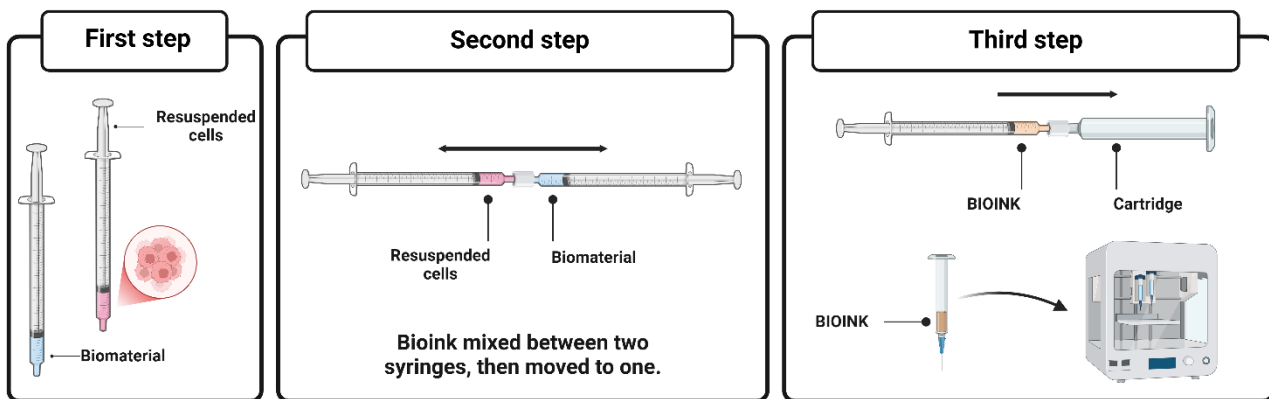


Figure 22: Schematic representation of the “Mixing with resuspension” process, divided into three steps: (first) preparation of syringes with cell suspension and biomaterial, (second) mixing via repeated transfer between syringes, and (third) transfer of the BIOINK into the printing cartridge. [created with Biorender]

2.3.2 Cells counting

Cell counting is a fundamental step in determining the concentration of cells in a suspension. Materials used for this procedure include cell suspension, Trypan Blue, Countess cell counting chamber slides, and Countess™ 3 automated cell counter (Thermo Fisher Scientific).

The protocol begins by mixing 15 μL of Trypan Blue with 15 μL of cell suspension; then 10 μL of the solution is pipetted into sections A and B of a chamber slide. Finally, the slide is inserted into the automated cell counter, which automatically determines the measured cell concentration, expressed in units of cells/ml. To reduce variability of the measurement, the counting procedure is repeated for both sections of the chamber slide. The estimated concentration is then determined by the arithmetic mean of the two measurements.

2.3.3 Live and dead staining

This protocol is designed to identify live and dead cells under a microscope, using specific optical filters in fluorescence microscopy: FITC and RFP. FITC filter is designed to detect green fluorescence, typically emitted by fluorochromes such as Calcein AM, while RFP filter allows the observation of red fluorescence, such as that emitted by Ethidium homodimer-1. [42]

Required reagents for the assay are Calcein AM (ThermoFisher), Ethidium homodimer-1 (ThermoFisher) and HBSS [-/-]. The protocol is used to evaluate the percentage of live and dead cells in the samples to be analysed. Live/Dead staining is a test based on the use of two fluorescent dyes that allow the distinction between live and dead cells. Calcein AM penetrates live cells and is metabolized into a fluorescent green compound, indicating cell viability. Ethidium Homodimer-1, on the other hand, cannot cross the membrane of viable cells but binds to the DNA of dead cells, producing red fluorescence. This test provides a clear image of cell survival under different experimental conditions. Dead cells appear red, while live cells appear green. After identifying the samples to be analysed, they are placed in separate wells of a multiwell plate, and approximately HBSS [-/-] solution added to each well in sufficient volume to ensure complete coverage of the sample. This step is needed to remove salts and lasts for 10 minutes. Meanwhile, the staining solution containing Calcein AM, Ethidium homodimer-1, and HBSS [-/-] is prepared according to the ratios reported in *Table 6* and then covered with aluminium foil since it is photosensitive.

MATERIAL	%V/V	EXCITATION/EMISSION WAVELENGTH	STORAGE CONDITIONS
HBSS [-/-]	99.7506	-	Room temperature
Calcein AM	0.0498	494/517 nm	-20 °C
Ethidium homodimer-1	0.1995	528/617 nm	-20 °C

Table 6: The table provides relevant information on the materials used, including the quantities to be considered for the staining solution.

A dedicated box is also used as incubation chamber for the live and dead staining. The box is thoroughly disinfected with 100% EtOH and lined with MilliQ-soaked paper, which facilitates the adhesion of sterile parafilm over its entire surface. After sample incubation with HBSS, using a micropipette, approximately 200 μ L of the staining solution is dispensed onto the parafilm surface. The sample is then removed from the multiwell plate, lightly dried, rotated 180°, and placed on top of the freshly dispensed droplet. This procedure is performed for each sample, using separate droplets. The samples are then left into the incubation chamber for 40 minutes.

After this period, the sample must be removed from the droplet, rotated again by 180° and placed in a well of the multiwell plate, previously filled with HBSS [-/-].

At this point, the samples can be observed under the microscope using the different optical filters. Fluorescence lasts for about 4 hours; after this time, cells will no longer be visible. Finally, once images are acquired, the live and dead cells can be counted, either manually or using an automated counting software.

The entire procedure for the live and dead assay is represented schematically in *Figure 23*.

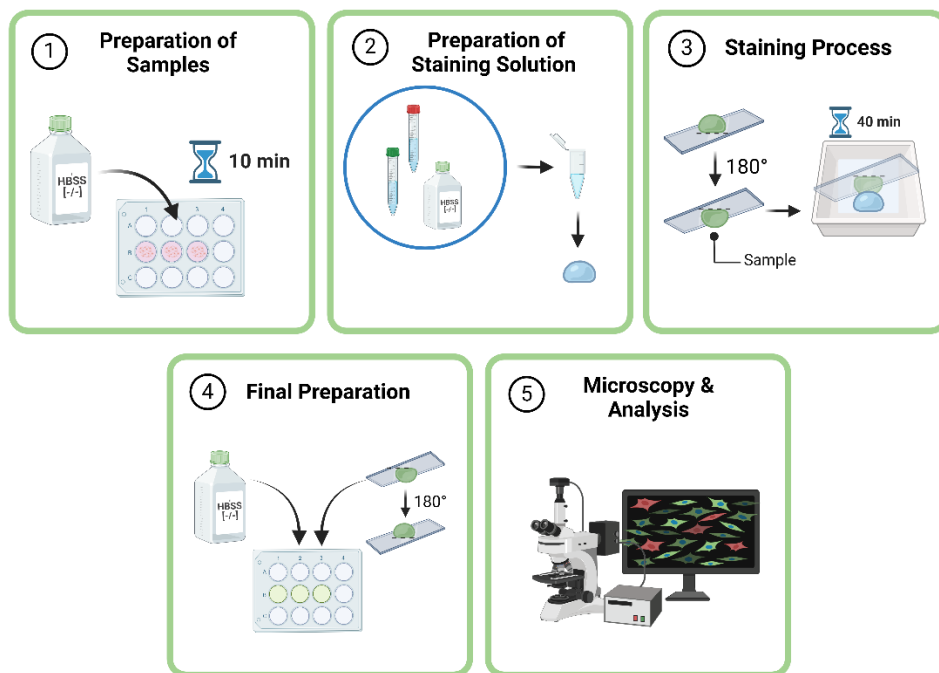


Figure 23: Live and dead staining process [created with Biorender]

2.4 Bioprinting software and hardware

This paragraph will provide a detailed analysis of the software used in this research study for designing the structures, as well as the hardware components, with a particular focus on the 3D bioprinter (BIO X™) and its technical specifications.

2.4.1 Bioprinting hardware

The BIO X™ by Cellink is an advanced 3D bioprinter designed for applications in cell biology, tissue engineering, and biomedical research. With its innovative technical features, it provides a complete and highly flexible system (*Figure 24*).

Equipped with a touchscreen interface, BIO X™ guides users through every step of the printing process, from model preparation to printing parameters adjustment. The printbed supports various surfaces, including multi-well plates, Petri dishes, and glass slides, and is temperature-controlled with a range between 4°C and 65°C.

The printer features a pneumatic extrusion system with adjustable pressure: the internal pump operates within a range of 0-200 kPa, while the external air supply allows pressures between 0 and 700 kPa.

There are three printhead slots, supporting temperature-controlled printheads, with a temperature range from 4°C to 250°C. This precise temperature control is essential for working with temperature-sensitive materials such as collagen, Matrigel and GelMA C, expanding the possibilities of working with a wide range of biomaterials and cell types.

Additionally, BIO X™ includes a chamber with the possibility of UV-light sterilization and supports multiple built-in photocuring modules, which can be selected directly from the display. For the experiments of this thesis, the 405 nm photocuring source was used, as its intensity is considered less harmful to cell viability than shorter wavelength UV modules (such as the 365 nm module). To have a quantitative control over the UV dose, the UV power intensity was detected using a dedicated gauge. Specifically, at a distance of 5 cm from the light source, the measured power intensity emitted from a UV light source at 405 nm was 43.6 mW/cm².

To ensure optimal accuracy, the system offers both automatic and manual calibration modes. Finally, the printer is compatible with various file formats. [43]



Figure 24: BIO X 3D Bioprinter

2.4.2 Bioprinting software

In the 3D bioprinting process, the choice and use of the appropriate software play a key role in ensuring the correct design and fabrication of the construct of interest.

In this context, there are numerous software packages that are used depending on the specific sector and application. In the case of this thesis study, the main software used are *Autodesk Fusion 360*, *PrusaSlicer* and *Repetier-Host* (Figure 25).

Autodesk Fusion 360

Autodesk Fusion 360 is an advanced CAD (Computer-Aided Design) software that enables parametric and direct modelling of three-dimensional objects. Thanks to its intuitive interface and numerous features, this software allows users to design complex 3D models and optimize their geometries before the printing phase.

In the context of bioprinting, Fusion 360 is used to create customized constructs, ensuring dimensional accuracy and adaptability to the specific requirements of the printing process. In the specific application of this thesis, a structure with defined dimensions was first created in 2D by setting its length and width. Subsequently, the extrusion function was applied, allowing the 2D construct to gain volumetric structure by adding the third dimension, height, resulting in a fully developed 3D model.

PrusaSlicer

PrusaSlicer is an open-source software developed by Prusa Research, designed to convert 3D models (contained in a “.stl” file) into “G-code” instructions executable by a bioprinter. This slicer plays a crucial role in optimizing the printing trajectories by adjusting parameters according to the specific properties of the material used and the complexity of the construct. The slicing process requires configuring specific settings that instruct the software on how to divide the 3D model into printable 2D layers and guide the bioprinter in building the construct.

To use this software, it is first necessary to configure it by selecting the specific options corresponding to the bioprinter in use (BIOX™ bioprinter in this case), by defining key parameters such as the firmware type, the shape of the print bed, and its dimensions.

After configuring these settings, the next step involves uploading the STL file, previously designed using *Autodesk Fusion 360*. At this point, the print settings can be adjusted in the *Print Settings* section, where various parameters, including layers and perimeters, infill, skirt and brim, speed, and other advanced options, can be fine-tuned to meet the specific requirements of the construct.

Then, the model can be previewed in the *Plater* section to ensure that the layout is correct and meets the intended design. Once everything is set, the slicing process can be executed, and the output file can be exported in G-code format, making it ready for printing.

Repetier-Host

Repetier-Host is a 3D printer control software that enables management of the printing process. In bioprinting, Repetier-Host is used to write and optimize the G-code, ensuring precise material deposition and process accuracy, during construct fabrication.

This software allows further optimization of the print file by importing the G-code previously exported from *PrusaSlicer* and manually modifying it line by line within the *Print Preview* section. This step is crucial for fine-tuning of the printer's movements according to the desired structure, as it enables customization of key parameters, such as the extrusion speed along the X and Y axes, which can be set differently from the speed along the Z-axis for more precise motion control.

Another particularly useful feature is *Travel Visualization*, which allows users to preview the exact path the printer will follow during printing. This tool is essential for verifying the printing trajectory and preventing errors that could damage the construct or lead to material waste. Once the optimization phase is complete, the file can be re-exported in G-code format and sent to the printer to start the 3D printing process.

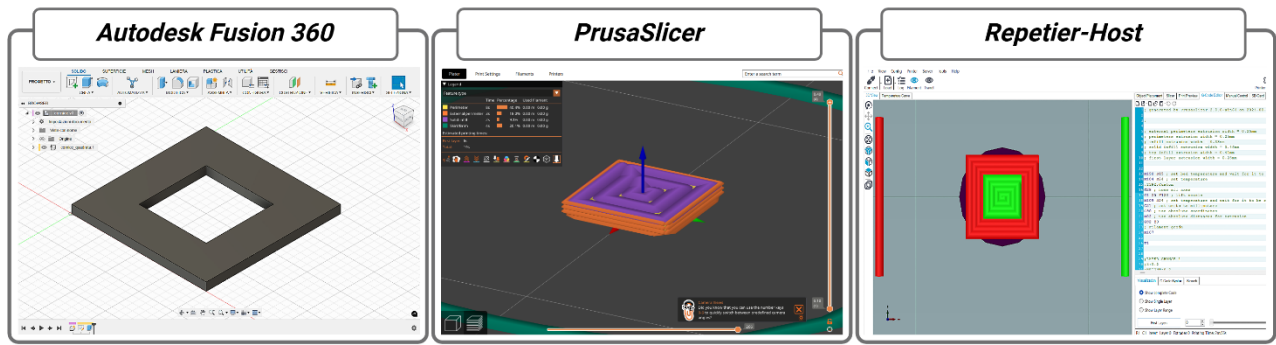


Figure 25: Software workflow for use in the bioprinting process [created with Biorender]

2.4.2.1 Code optimization process

For the in vitro reconstruction of a tumour microenvironment through a 3D bioprinted model, which was the scope of this thesis, the optimization of the printing code played a crucial role in ensuring the quality of the final construct. The implemented strategies focused on various key aspects, from structural design to fine-tuning printing parameters and solving emerging issues.

Structural design

The main goal is to replicate a compartmentalized tumor microenvironment using a multi-layered 3D structure. The design includes a central zone printed with a bioink simulating the tumor microenvironment and an outer zone made with a bioink mimicking the stromal microenvironment (Figure 26).

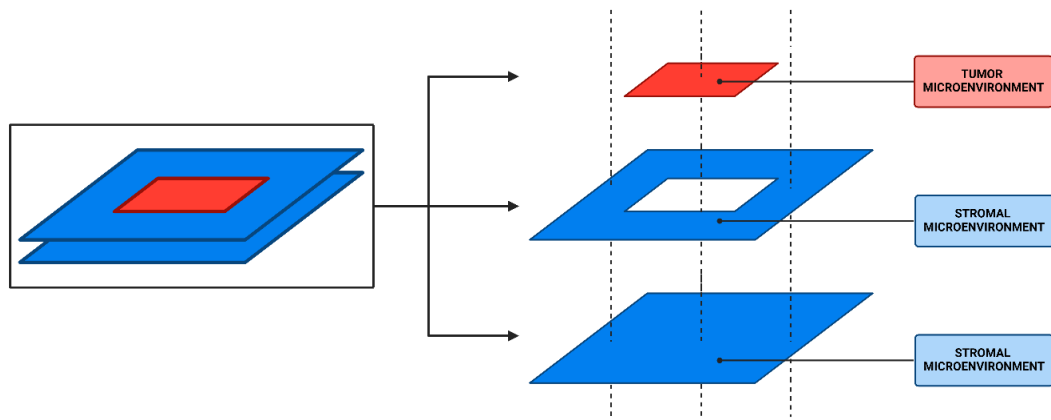


Figure 26: Graphic representation of the 3D structure with its layers, followed by a detailed view of each layer along with its characteristics. [created with Biorender]

To ensure precise bioinks deposition, two temperature-controlled printheads were used, allowing the materials to be maintained at optimal temperatures according to their composition and properties.

The printing process was structured as follows:

- The first layer was a full square base printed with stromal bioink.
- The second layer combined both bioinks: the stromal bioink formed a square frame with an empty centre, which was then filled with the tumor bioink (*Figure 27*).

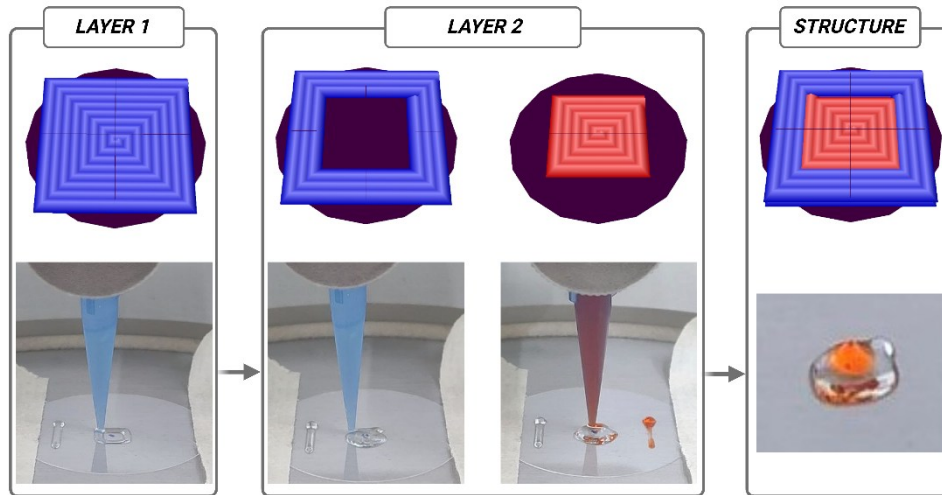


Figure 27: Workflow of the 3D structure printing process, featuring a schematic preview of the structure followed by an exemplary image of the printing in progress.

Fine-tuning of printing parameters

Various filling methods were tested to ensure uniform deposition. The most effective approach was the "outside-in" filling method, where printing began from an outer vertex of the square and progressively moved toward the centre, ensuring a homogeneous and orderly structure.

Different construct sizes were evaluated to balance printing quality and material efficiency. After testing formats that were either too large (20×20 mm), leading to excessive bioink waste, or too small (2.5×2.5 mm), making detailed construct analysis difficult, an intermediate size of 4×4 mm was chosen, optimizing both material usage and post-printing analysis.

Furthermore, the nozzle diameter directly affects the filament thickness and, consequently, the construct's height. To ensure accurate printing, the code was adjusted by modifying this parameter in *PrusaSlicer*.

The resolution of emergent issues

One of the main challenges was the correct implementation of the photocrosslinking step during the printing process, which is essential for materials such as GelMA C to ensure their mechanical

stability and structural integrity once printed. However, the application of the UV phase interfered with the correct execution of printer commands, causing undesired movements that risk the damaging of freshly printed layers. This problem was solved by adding a non-extruding movement immediately after the UV phase, preventing errors in subsequent movements.

During printing, blob formation inside the nozzle was observed due to the lack of thermal regulation once the bioink exited the printhead, this compromised extrusion pressure. To solve this problem two strategies were implemented: the first is adding lateral lines before printing the target layer to eliminate excess material in a non-critical area; the second is a manual pause for extrusion tests, allowing the removal of blobs before resuming printing in the target area with the correct pressure.

These optimizations significantly improved printing precision and construct quality, ensuring a reliable replication of the desired structure.

2.5 Bioink preparation

As anticipated, the printing process consists of three main phases: pre-bioprinting, bioprinting, and post-bioprinting. The pre-bioprinting phase includes a crucial step, i.e., the preparation of the bioink, which varies depending on the biomaterial used.

This chapter provides a detailed description of the bioink preparation procedures using GelMA C, Matrigel, Rat Tail collagen, and LIFEINK 200 collagen (described in paragraph 2.1).

In the experiments conducted for this thesis, these materials were often combined to achieve the desired compartmentalized final structure. A thorough analysis of the materials and their methods of use was carried out to better understand their interaction, behaviour over time, biological response when in contact with cells, and overall printability.

2.5.1 GelMA C Bioink

The GelMA C used in this experiment is patented by Cellink. It is stored at 4°C, where it appears as a viscous liquid, while its viscosity decreases at higher temperature.

For bioink preparation, the GelMA C cartridge must be heated to 37°C until it becomes a smooth, viscous liquid. Heating can be performed in a printhead or incubator and typically requires 30-60 minutes if taken directly from the fridge, where it is usually stored.

To ensure bioink homogeneity after heating, connect the cartridge to a syringe using a Luer lock connector, remove the end cap and gently pull the syringe plunger to transfer the necessary amount

of bioink. To eliminate any air bubbles from the dead volume inside the syringe, separate the syringe from the cartridge while keeping the Luer lock attached. Hold the syringe with the tip facing upwards, gently tap it to move air bubbles to the tip and carefully extrude the air.

At this point, transfer the entire volume of bioink into an Eppendorf. If not using the entire 3 mL volume of the cartridge, store the remaining bioink under optimal conditions. If air bubbles are present in the bioink inside the Eppendorf, perform a quick centrifugation for 30 seconds at 600 x g to remove them. Meanwhile, store the Eppendorf in the incubator and prepare the cell suspension, following the protocol described in the previous section.

For the preparation of GelMA C-based bioink, one of the following dilution protocols were followed, depending on the specific experiment.

Preparation protocol for bioink based on GelMA C at 90% concentration

According to Cellink's printing protocol for GelMA C, the material should be diluted at a 1:10 ratio relative to the total bioink volume. The following ratios should be followed:

$$\text{Cell suspension volume} = \frac{1}{10} \text{ of the final bioink volume}$$

$$\text{GelMA C volume} = \frac{9}{10} \text{ of the final bioink volume}$$

Preparation protocol for bioink based on GelMA C at 70% concentration

By modifying the standard protocol, in some experiments of this thesis GelMA C was diluted at a 3:10 ratio using the following proportions:

$$\text{Cell suspension volume} = \frac{3}{10} \text{ of the final bioink volume}$$

$$\text{GelMA C volume} = \frac{7}{10} \text{ of the final bioink volume}$$

Once cell concentration has been determined, the necessary volume of cell suspension is centrifuged, and the supernatant is removed. A proper volume of fresh culture medium, based on the chosen protocol, is then added to the cell pellet.

Next, the cell suspension is transferred into a syringe. To prevent bubble formation the syringe plunger is removed, and the entire cell suspension is aspirated using a micropipette before slowly

injecting it into the syringe. The plunger is then reinserted and gently pushed towards the end of the syringe.

At this stage, take the Eppendorf containing the GelMA C stock from the incubator and transfer the required volume of material (according to the chosen protocol) into a second syringe.

Once both syringes are ready, they are connected using a Luer lock adapter for mixing. Once the contents of the two syringes are perfectly mixed and a homogeneous bioink is obtained, the entire mixture is transferred into one of the syringes. Using a Luer lock adapter, the syringe containing the bioink is then connected to the cartridge, which is equipped with a piston.

Since GelMA C rapidly crosslinks through photocuring, it is crucial that crosslinking occurs after printing and not inside the cartridge. For this reason, UV-shielding cartridges, which are transparent but designed for UV- and light-sensitive materials, should be used. Additionally, the nozzle must also be UV-shielding to prevent premature crosslinking.

Before printing, place the cartridge in a temperature-controlled printhead of the BIO X, preheated to 24°C, and wait for at least 15 minutes before printing.

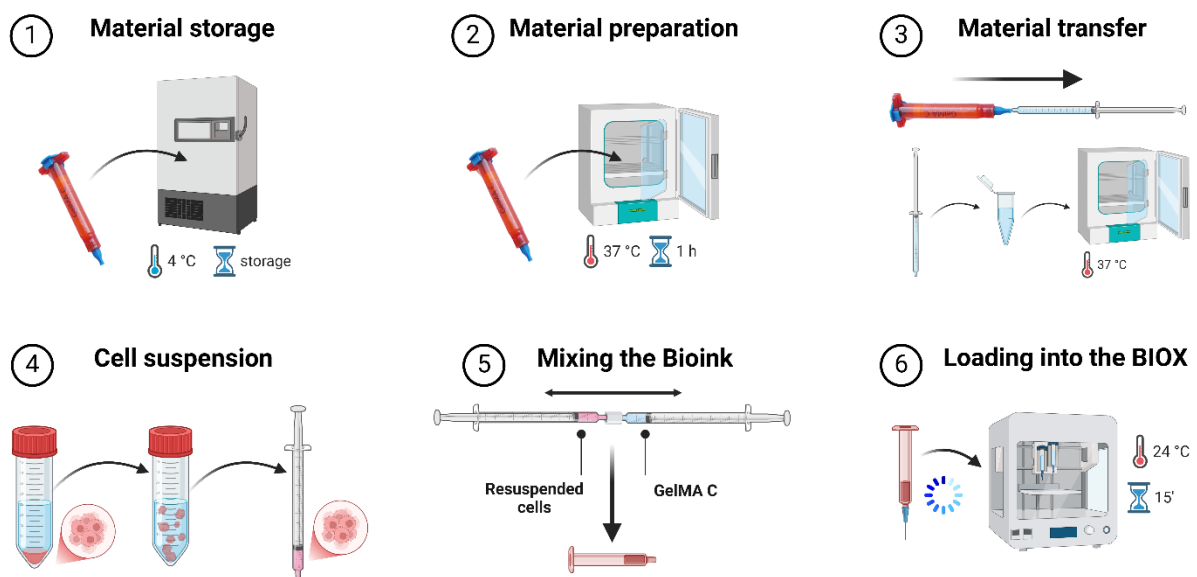


Figure 28: Workflow of GelMA C Bioink Preparation with Cell Suspension [created with Biorender]

During the printing process, photocrosslinking is activated to enable the bioink to crosslink after deposition. Bioink containing LAP can be crosslinked using either the 405 nm or 365 nm LED module. When working with cells, it is recommended to use the 405 nm module instead of 365 nm to minimize potential damage. [44]

Photocrosslinking parameters used in this thesis are reported in *Table 7*:

WAVELENGTH	EXPOSURE TIME	DISTANCE FROM UV LIGHT
405 [nm]	20 [seconds]	5 [cm]

Table 7: Photocrosslinking parameters

2.5.1.1 Glass cover functionalization

For printing GelMA C-based constructs, it was necessary to functionalize the glass covers to create an adhesive substrate ensuring good adhesion of the printed constructs.

Glass cover functionalization is a procedure used for the scope of this thesis to introduce methacrylate groups onto the glass surface, thereby facilitating the adhesion of biomaterials such as GelMA C.

Reagents employed in this procedure are TMSPM, acetic acid, and 100% ethanol. First, the glass slides are picked up with tweezers and treated with a flame: by quickly passing them in front of the flame, an opaque film will form on their surface. This heat treatment oxidizes the glass, introducing hydroxyl groups (-OH) and making the substrate more reactive.

The solution is prepared using the proportions reported in *Table 8*:

REAGENT	%V/V
100% ethanol	93.1372
Acetic Acid	4.9019
TMSPM	1.9607

Table 8: Concentrations used for preparing the glass cover functionalization solution.

TMSPM contains reactive methacrylate groups, which can react with the hydroxyl groups present on the glass surface. Acetic acid acts as a catalyst, accelerating the condensation reaction, while ethanol serves as a solvent.

At this point, the glass slides are placed inside a glass Petri dish and covered with the prepared solution. Pouring the solution onto the flame-treated glass slides allows the methacrylate groups to react with the hydroxyl groups, forming covalent bonds and creating a functionalized surface. After a 15-minute waiting period, the cover slips are washed with acetone (to remove any unreacted reagents) and then placed on an absorbent surface to ensure drying, before being covered with aluminium foil.

2.5.2 Matrigel Bioink

In the experiments developed in this thesis, Matrigel® (Corning) was used (details are described in paragraph 2.1.2). [45]

To prepare a Matrigel-based bioink, the "*Direct Mixing*" protocol described in section 2.3.1 was followed. Under a biological hood, the cell suspension was prepared at the desired concentration and then transferred into an Eppendorf tube and centrifuged to obtain a pellet.

Matrigel is handled while maintaining constant contact with ice and under sterile conditions. It is essential to use a box with ice to store Matrigel during the preparation process under the hood. Using a micropipette, the required amount of Matrigel was collected and transferred into the Eppendorf tube containing the cell pellet. Subsequently, the Matrigel-based bioink was aspirated into a syringe that had been pre-cooled in the refrigerator. At this stage, the bioink is transferred into the cartridge and quickly loaded into the BIOX temperature-controlled printhead, set at 5°C for printing.

After printing, the construct is incubated at 37°C for about one hour where the Matrigel begins to gel. Finally, the construct is immersed in cell culture medium and kept in the incubator for the desired observation period.

2.5.3 Rat tail Collagen Bioink

In the experiments developed in this thesis, Collagen I Rat Tail (Corning®) was used. This collagen is supplied in a liquid formulation in 0.02 N acetic acid, with a concentration ranging from 3-4 mg/mL (batch-specific). In this state, the pH is acidic, but to make it suitable for cells, it must be brought to a physiological pH, so neutralization is required.

Collagen I is stored in a refrigerator at a temperature between 2-8°C. Similar to Matrigel, it must be handled in contact with ice, under a biological hood and in sterile conditions, to prevent it from fibrillating before printing. [36]

To neutralize collagen, the following reagents are used: PBS 10X, Collagen I Rat Tail (Corning®), NaOH 1M, MilliQ.

- PBS 10X: its volume corresponds to 1/10 of the final bioink volume and ensures a physiological salt concentration, as cells require a saline environment to survive.
- Collagen I Rat Tail (Corning®): collagen volume is calculated using the formula:

$$C_{in} \times V_{in} = C_{fin} \times V_{fin}$$

The initial concentration C_{in} corresponds to the one provided by Corning, while the final concentration C_{fin} depends on the experimental goal. The initial volume V_{in} corresponds to the volume of collagen to be collected, while the final volume V_{fin} is the desired bioink volume.

- NaOH 1M: added to fine-tune the pH to approximately 7. The exact volume is not predefined and must be determined experimentally. Once the proper volume is defined, it is used for subsequent experiments with the same material.
- MilliQ: its volume is calculated by subtraction:

$$V_{MilliQ} = V_{bioink} - (V_{NaOH} + V_{collagen} + V_{PBS\ 10X})$$

Collagen neutralization must be performed under a biological hood and in contact with ice. All reagents must be kept at a low temperature to prevent premature collagen fibrillation.

The neutralization procedure starts by taking a sterile Eppendorf and adding the reagents in the following order: PBS 10X, Collagen I Rat Tail (Corning®), NaOH 1M, and finally MilliQ. After adding each reagent, the solution must be mixed thoroughly to ensure complete homogenization, and the final pH should be checked.

At the end of collagen neutralization, the Eppendorf containing neutralized collagen is stored at 4°C for about one hour. After this time, neutralized collagen is ready for bioink preparation. First, the desired cell suspension is transferred into a syringe, connected to a Luer Lock, and kept on ice, then a second syringe is prepared, loaded with the neutralized collagen ink. The mixing between the two syringes is performed according to the *Mixing with resuspension* protocol (section 2.3.1) and the bioink is transferred into a cartridge with a piston and loaded into the temperature-controlled printhead at 5°C for printing.

After printing, the construct is incubated at 37°C for about one hour to allow collagen fibrillation. Finally, the construct is immersed in cell culture medium and kept in the incubator for the desired observation period.

2.5.4 Lifeink 200 Collagen Bioink

Lifeink® 200 Collagen Bioink, patented by Cellink, is a commercial high-concentration type I collagen-based bioink with a 35 mg/ml concentration. Thanks to this high concentration, it can be easily printed in air.

This bioink is stored in a refrigerator at a temperature range of 2-8°C and is supplied in a 5 ml syringe. It has a neutral pH, ranging from 6.9 to 7.6, so collagen neutralization is not required.

To prepare the bioink, Lifeink® 200 Collagen and the desired cell suspension are required. Since collagen must be handled at low temperatures to prevent fibrillation before printing, an ice box is prepared and kept under a biological hood to maintain sterile conditions.

First, the desired cell suspension is transferred into a syringe, connected to a Luer Lock, and kept on ice; then a second syringe is loaded with the Lifeink® 200 Collagen.

The mixing between the two syringes is performed according to the *Mixing with resuspension* protocol and the bioink is transferred into a cartridge with a piston and loaded into the temperature-controlled printhead at 5°C for printing.

After printing, the construct is incubated at 37°C for about one hour to allow collagen fibrillation. Finally, the construct is immersed in cell culture medium and kept in the incubator for the desired observation period.

2.6 Experimental procedures

For the fabrication of a compartmentalized structure using 3D bioprinting, two distinct bioinks must be prepared: one to simulate the stromal microenvironment and the other for the tumor compartment. These bioinks must be loaded into the printheads of the bioprinter and deposited in the correct sequence to create the previously designed structure.

During this thesis, various material combinations were tested to recreate this compartmentalized microenvironment, including Matrigel, GelMA C, type I collagen, and others. The processing of these materials mainly differed in their preparation methods, e.g., in terms of processing temperatures.

The following sections will describe the bioink preparation procedures, outlining the pre-bioprinting phases.

2.6.1 GelMA C and Matrigel: pre-bioprinting procedures

In this experiment a Matrigel-based bioink was used to simulate the tumor environment, incorporating tumor cells, and formed the central region of the structure. At the same time, a GelMA C-based bioink was employed to simulate the stromal environment, containing fibroblasts, and deposited in the outer

region of the structure. GelMA C and Matrigel are two materials with distinct characteristics, requiring specific processing steps. To ensure optimal results, they must be prepared separately.

GelMA C bioink was prepared as described in section 2.3.1, under the hood at room temperature, and then loaded into a UV-shielding cartridge. This cartridge was subsequently placed in the temperature-controlled printhead of the BIOX printer, set at 24°C, and left to rest for at least 15 minutes before printing.

Meanwhile, the Matrigel™-based bioink was prepared under the hood and kept continuously in contact with ice to maintain its temperature stability. After mixing Matrigel™ with the cell pellet, the bioink was carefully transferred into a syringe, and then quickly loaded into the printing cartridge, pre-cooled at 5°C.

After 15 minutes of resting for the GelMA C-based bioink, the bioprinting process can begin.

2.6.2 GelMA C: pre-bioprinting procedures

Another type of experiment for this thesis involved the use of a GelMA C-based bioink to model the tumor environment, incorporating tumor cells, and another GelMA C-based bioink to simulate the stromal environment, including fibroblasts.

Using GelMA C for both the inner and outer regions of the structure simplifies the preparation process, as the same material follows a single processing procedure. However, to ensure optimal results and minimize errors, it is still recommended to prepare the bioinks separately.

First, the bioink for the tumor microenvironment was prepared as described in section 2.3.1. Under the hood, the cell suspension was mixed with GelMA C and subsequently loaded into a UV-shielding cartridge. This cartridge was then placed in the temperature-controlled printhead of the BIOX, set at 24°C and left to rest for 15 minutes. Meanwhile, the second GelMA C-based bioink, for the stromal microenvironment, was prepared following the same protocol as above but embedding a different cell suspension. Similarly, this second bioink was transferred into a UV-shielding cartridge, placed into a separate temperature-controlled printhead, also set at 24°C, and left to rest for 15 minutes prior to initiating the bioprinting process.

Influence of GelMA C concentration on fibroblast viability and matrix interaction

To better understand the biological mechanism underlying the interaction between GelMA C and fibroblasts, three experimental trials were conducted using different concentrations of GelMA C.

The objective of this study was to evaluate how different formulations of the biomaterial influenced cell viability and fibroblast behaviour over time.

The experimental conditions tested were:

1. Bioink with 90% GelMA C in cell suspension,
2. Bioink with 70% GelMA C in cell suspension,
3. Bioink with 70% GelMA C in cell suspension and Type I collagen rat tail.

In the first case, the bioink consists of 9/10 of the final volume of GelMA C and 1/10 of the final volume of culture medium with cells. In the second case, the bioink consists of 7/10 of the final volume of GelMA C and 3/10 of the final volume of culture medium with cells. In the third case, the bioink consists of 7/10 of the final volume of GelMA C, 3/10 of the final volume of culture medium with cells, with the addition of Type I collagen.

For each experimental condition tested, bioink droplets were deposited onto glass coverslips previously functionalized, as described in section 2.5.1.1. After deposition, the droplets were UV-cured following the photocrosslinking protocol reported in *Table 7*.

Cell behaviour was observed for 7 days using an optical microscope to analyse fibroblast interaction with the GelMA C matrix at different concentrations. A Live/Dead assay was performed on the samples of the three formulations on days 1, 4 and 7 from which meaningful considerations were drawn on which of the three experimental conditions promoted higher cell survival within the matrix.

2.6.3 GelMA C and Lifeink 200 Collagen: pre-bioprinting procedures

A third type of tumor microenvironment model was developed in this thesis. It involves a GelMA C-based bioink to simulate the tumor environment, printed it in the central region of the structure and a Lifeink 200 Collagen-based bioink to model the stromal compartment, containing fibroblasts and deposited in the outer region of the structure.

GelMA C and Lifeink 200 collagen are materials with different characteristics, requiring distinct preparation protocols and printing temperatures to achieve optimal results.

GelMA C bioink was prepared following the protocol described in section 2.5.1. After preparation, the bioink was transferred into a UV-shielding cartridge equipped with a piston, which was then loaded into the BIOX temperature-controlled printhead set at 24°C.

Meanwhile, Lifeink 200 collagen bioink required a significantly lower preparation temperature; thus, it was handled under the hood, maintaining constant contact with ice. After combining Lifeink 200 Collagen with stromal cells, the bioink was transferred into a cartridge equipped with a piston and placed into the BIOX temperature-controlled printhead set at 5°C.

After 15 minutes of resting for the GelMA C-based bioink, the bioprinting process can begin.

3. Results

The primary objective of this research is to create an in vitro tumor microenvironment using 3D bioprinting. To achieve this, a compartmentalized structure was designed employing two distinct bioinks: one dedicated to reproducing the tumor microenvironment and the other to simulating the stromal microenvironment. The use of the BIOX 3D bioprinter from Cellink, equipped with temperature-controlled printheads, allows the bioinks to be maintained at optimal, controlled temperatures during printing. In this chapter, the topics of structural optimization from a technical perspective will be addressed, focusing on various challenges and solutions related to dimensions, structural design, and specific details during the printing process. Subsequently, the focus shifted to biological challenges and the selection of the most suitable biomaterial for the intended purpose. Therefore, development from a biological perspective will be explored, with an analysis of cell-cell and cell-extracellular matrix interactions.

3.1 Optimization of design and fabrication procedure

During the study, several technical challenges were encountered. First, issues related to the 3D bioprinter itself had to be addressed, including the integration of the UV module for photocrosslinking and the precise control of the printhead's movements across different spatial coordinates. Additionally, factors such as the selection of the optimal nozzle and the definition of the structure's geometric parameters, such as its size and complexity, were thoroughly analysed.

Initially, constructs ranging from two to five layers were tested, but a two-layer structure was ultimately selected. This decision was motivated by practical considerations, smaller constructs are easier to observe under a microscope, and by a desire to reduce material waste. Accordingly, the construct dimensions were fixed at 4 mm × 4 mm, since larger formats (e.g., 20 mm × 20 mm) were too cumbersome for microscopic analysis and led to excessive material consumption, while smaller formats (e.g., 2.5 mm × 2.5 mm) did not allow adequate compartmentalization during printing.

Various printing parameters, including extrusion speed, extrusion pressure, movement settings, and the selection of the UV module, were tested and optimized to achieve reliable and reproducible constructs.

3.1.1 Evaluation of construct dimensions

This section evaluates the selection of the optimal construct size based on various considerations.

The 20×20 mm construct has several drawbacks: its dimension lengthens the fabrication time, complicates examination through microscopy and leads to excessive bioink consumption (and cells consequently), making it less practical for routine analysis. Moreover, compared to the total dimension of the construct, the key region (core – shell interface) to analyse the tumor-stroma interaction would occupy only a small portion. In contrast, the 2.5×2.5 mm construct although it minimizes material waste, compromises proper compartmentalization, which is essential for accurately mimicking the tumor microenvironment. The 4×4 mm construct strikes an optimal balance, because it is sufficiently large for detailed microscopic observation, while also ensuring effective compartmentalization. The comparison, as shown in *Figure 30*, shows the three dimensions tested for the structure.

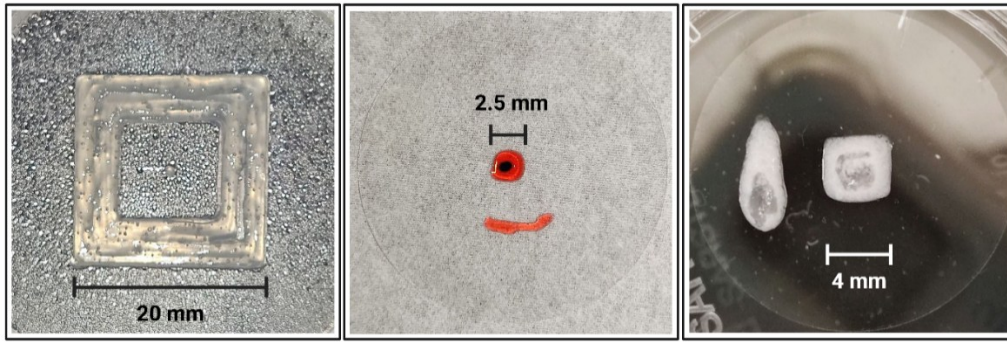


Figure 29: The three images illustrate printed constructs with dimensions of 20×20 mm, 2.5×2.5 mm, and 4×4 mm, respectively.

As for the third dimension, we want the construct to be composed of a composed of a continuous layer of the shell material to isolate the model from the rigid glass substrate and a second layer consisting of both the tumor and the stromal material. We set the height of the first layer to correspond to the 80% of the nozzle diameter to ensure the proper deposition on the glass support. The second layer has a thickness equal to the nozzle diameter. Given these parameters, assuming the use of a 25G nozzle, the final dimensions of the construct will be $4 \text{ mm} \times 4 \text{ mm} \times 0.450 \text{ mm}$. Figure 31 graphically represents the final construct dimensions used in all the experiments conducted in this study.

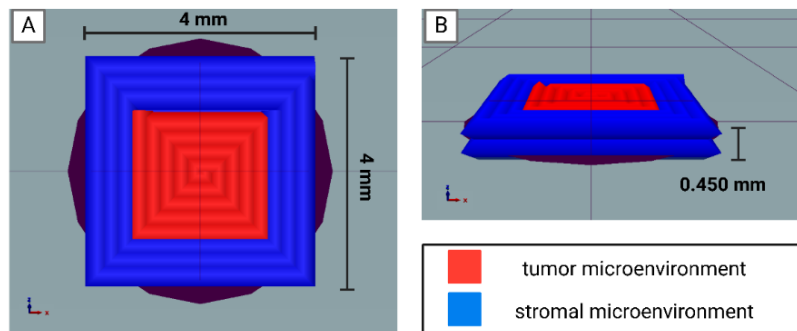


Figure 30: Schematic representation of the final 3D construct used in all experiments. The structure consists of two layers with a base dimension of 4×4 mm and a total height of 0.450 mm.

3.1.2 Refining the design based on thickness considerations

The design of the structure to be reproduced was defined and optimized throughout the study. Specifically, during several experimental tests, it was observed that the volume of bioink extruded during printing was significantly higher than expected. To further investigate and verify this phenomenon, specific extrusion tests were performed to accurately assess the actual thickness of the printed lines.

A G-code consisting of parallel lines was created to facilitate thickness measurement. These tests were conducted using different materials, including GelMA C and Lifeink 200 Collagen, employing a standard 25G nozzle (nominal inner diameter of 0.250 mm). Within both bioinks used for the extrusion tests, cells were incorporated to evaluate the extrusion properties most similar to the final fabrication situation. For printing with GelMA C, UV-shielding cartridges and nozzles were utilised since, following deposition, UV modules were applied according to the protocol reported in *Table 7*. Conversely, no UV modules were used for Lifeink 200 Collagen, as this material is not sensitive to photocrosslinking processes. After the printing process, the deposited lines were analysed under the microscope, measuring the thickness of the deposited lines to evaluate extrusion accuracy. Given the nominal nozzle diameter of 25G (0.250 mm), an equivalent line thickness of 0.250 mm was expected for both materials. However, as illustrated in *Figure 31*, the actual thickness obtained was significantly greater than anticipated, and the values are reported in *Table 9*.

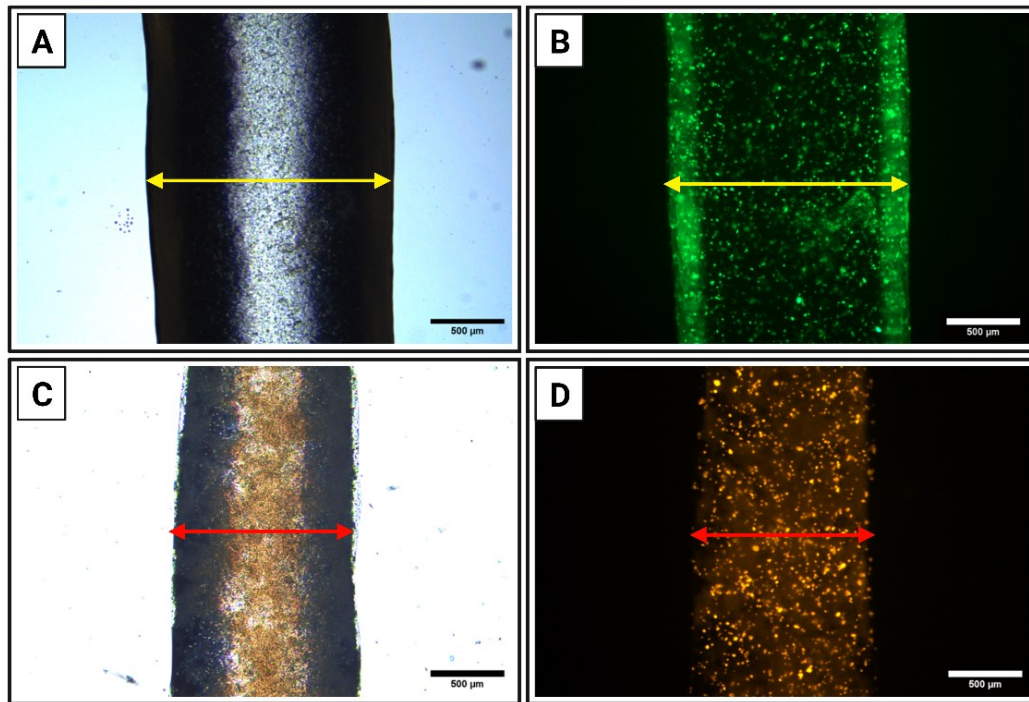


Figure 31: Microscopic images of bioink lines containing different cell types. (A) GelMA C with tumor cells in brightfield; (B) GelMA C with tumor cells in green fluorescence; (C) Lifeink 200 Collagen with stromal cells in brightfield; (D) Lifeink 200 Collagen with stromal cells in red fluorescence. The images were acquired using an optical microscope. The measured thickness of each bioink line is highlighted with arrows and reported in the table 9.

Bioink	Thickness [mm]
GelMA C with tumor cells (A-B)	$1,6557 \pm 0,0332$
Lifeink 200 Collagen with stromal cells (C-D)	$1,2344 \pm 0,0274$

Table 9: The table presents the measured thickness of bioink lines for two different conditions: GelMA C with tumor cells and Lifeink 200 Collagen with stromal cells. The thickness was determined using microscopy images (A–B and C–D, as reported in Figure 31) and the values are expressed as mean $\pm$ standard deviation.

This result highlighted the need to further optimize the structural design by reducing the total number of printing loops, thus creating a less compact structure. The problem could be solved by decreasing the extrusion pressure; however, tests were done, and it was noted that a homogeneous and continuous extrusion was not obtained, which was not the desired result. For this reason, using Repetier-Host software, the G-code was manually modified by halving the initial number of loops while maintaining the overall dimensions of the structure (4x4 mm). This reduction effectively addressed the issue of over-extrusion, resulting in a more precise and uniform deposition of bioink.

As can be seen in *Figure 32*, the problem of over-extrusion is very evident and has an impact on the final structure, as the aim is to achieve a compartmentalized structure, in particular to obtain a suitable spatial organisation of the system components.

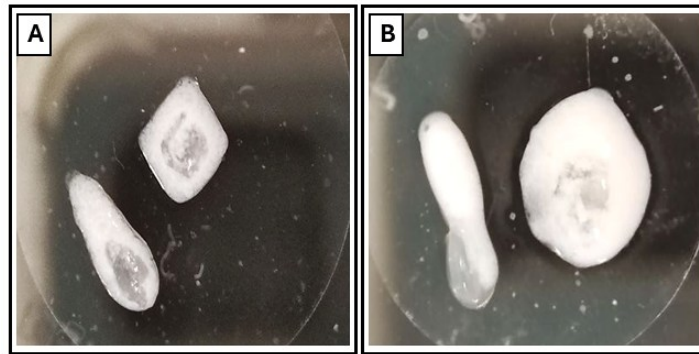


Figure 32: In the figure, a clear difference can be observed between an optimised structure (A) and a non-optimised one (B). (B) A clear over-extrusion of the material can be seen, and the dimensions are much larger and irregular. (A) In the optimised structure, it can be seen that the dimensions are true to reality and are contained.

As anticipated, the G-code was modified manually, making the structure more compact and reducing the over-extrusion problem. *Figure 33-A* shows the non-optimized structure with a high number of turns, while *Figure 33-B* shows the optimized structure with half the number of turns.

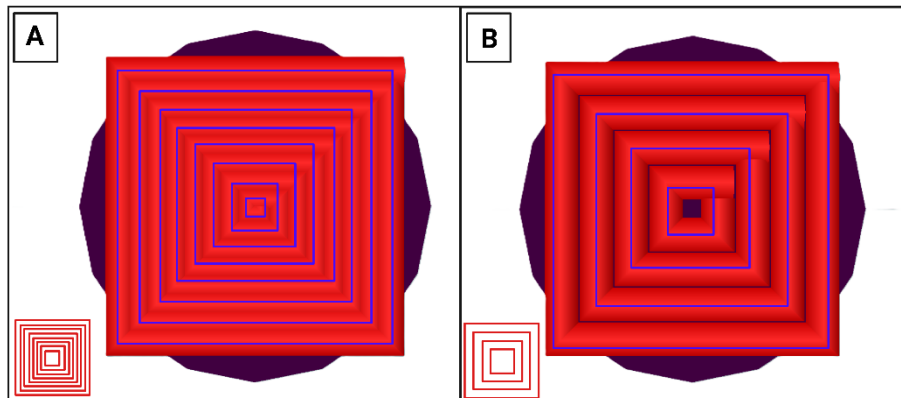


Figure 33: (A) The image illustrates the initial structure design with an excessive number of repetitions, and consequently, an excessive number of loops. In contrast, (B) presents the optimized structure design, where the number of loops has been halved.

In addition to this functional modification, improvements were also made to the construct printing methodology. Initially, each individual layer consisted of a series of concentric, closed, and separated squares, as shown in *Figure 35-A*. This approach relied on the default trajectories generation of the slicing software. However, this created problems during extrusion: interruptions of the bioink flow at the end of each square were observed causing a non-homogeneous deposition and voids construct volume (*Figure 34-B*)

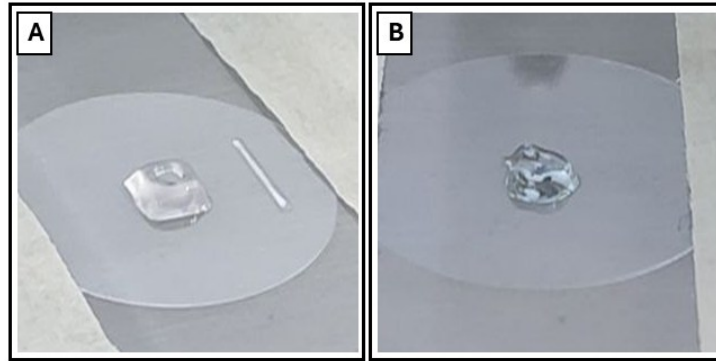


Figure 34: (A) The image shows the optimally printed construct, with the design described in figure 35-B; (B) shows the not optimally printed construct, which follows the design described in Figure 35-A.

To overcome this issue, a continuous printing approach was adopted optimizing the gcode. Bioink is extruded continuously, starting from the outer vertex and progressing inward towards the central area, without interrupting the flow. This method ensures more uniform bioink deposition significantly reducing, simultaneously, the printing time.

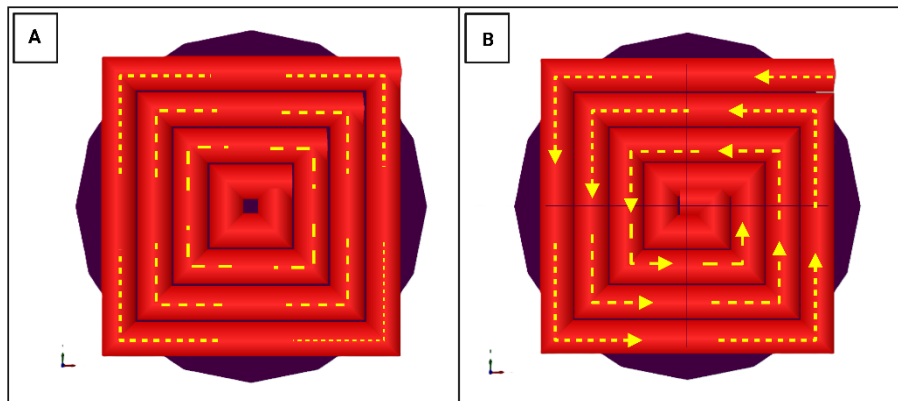


Figure 35: (A) The image shows the structure design with a pattern of independent concentric squares. (B) presents the optimized design, featuring a continuous and uniform concentric pattern progressing from the outer to the inner zone.

Once the structural design was optimized, the final scheme adopted in all subsequent experiments is shown in *Figure 35-B*.

3.1.3 Printing strategy adjustments

A further optimization was performed in the management of the two printheads during the printing process. Layer by layer 3D printing indeed is based on the alternating activation of the two printheads required to deposit the bioinks. However, while testing the fabrication, we observed several drawbacks:

1. temperature sensitive materials undergo premature gelation in the nozzle while the tool is inactive
2. UV crosslinked materials undergo intra-nozzle gelation when UV source is activated

The first issue is related to lack of the thermoregulation of the nozzle. This creates a thermal difference between the temperature-controlled printhead and the nozzle itself that causes the formation of a gelled volume which compromise the previously printed layer. This happens both due to the extrusion test phase before printing and also due to the printheads changes required in the fabrication of the structure.

The second issue is caused by the UV exposure applied after printing each layer. In the case of photosensitive materials such as GelMA C, the biomaterial at the nozzle tip undergoes UV exposure thus crosslinking and forming a blob that must be removed before continuing the printing process.

To prevent these issues, an extrusion test with both tools can first be performed before starting the printing process. Subsequently, printing is initiated with the first tool, which operates correctly due to its monitored temperature. Then, a pause is introduced both to test the pressure of the second tool and to clear the gelated material in order to allow the proper printing with the second tool.

3.2 Optimization of the TME biomaterials

One of the main objectives of this study is to identify biomaterials capable of mimicking the microenvironment in which tumor and stroma interactions guide the tumor proliferation. For this to happen the biomaterial should guarantee high viability, migration and proliferation of the embedded cells. Moreover, a cell-mediated remodelling of the material should be observed throughout the experiment to allow the investigation of the cell-ECM interactions that happens in vivo.

We place special emphasis in the analysis of the behaviour of fibroblasts which are crucial to evaluate the biomaterial performance. Their morphology is highly dynamic and responds to interactions with the extracellular matrix and surrounding cells. Initially, fibroblasts exhibit a circular or spherical morphology, but upon contact with properly optimized extracellular matrix-mimicking gels and with

the surrounding cells, their shape change into a more elongated and stretched conformation. This transition, from a spherical to a stretched morphology, serves as a key indicator that fibroblasts are in an environment conducive to cellular growth. For this reason, an in-depth study of cellular behaviour was conducted, evaluating both cell–cell interactions and cell–matrix interactions.

Regarding the cell lines, initially, murine 4T1-GFP breast cancer cells were used as tumor model along with human WI38 fibroblasts. Later, the cell lines were modified: we kept the 4T1-GFP cells for the inner core, while we switched to Tomato-tagged mouse adult fibroblasts (MAF) to match the murine origin of the tumor compartment and streamlining the imaging through microscopy.

A key advantage of using exclusively murine tumor and stromal cells, rather than a combination of cells from different species, is the enhanced biological consistency of the model. When the cells originate from the same species, interspecific differences are significantly reduced; for example, signalling pathways, growth factors, and membrane interactions are more similar, allowing for a more faithful reproduction of the physiological and pathological mechanisms observed *in vivo*. Furthermore, using murine cells for both the tumor and stromal compartments promotes more realistic cell–cell and cell–matrix interactions, as the cells can communicate more effectively on a molecular level. [46]

Throughout the study, cell concentrations were also varied to assess their impact on construct formation. In parallel, different biomaterials for the bioprinted structure were evaluated by analysing both their biocompatibility with cells and their printability, specifically, the ease of extrusion and the stability of the construct post-printing.

In the following experiments, the focus was primarily on GelMA C, Matrigel, and Lifeink® 200 Type I Collagen, discussing how the selection and sometimes the materials themselves evolved over time. In fact, both positive and negative data guided subsequent choices, ultimately allowing for optimization of the printing process and improvement of biological interactions.

Once the initial challenges were overcome, many parameters remained fixed, such as the number of layers, the construct dimensions, and the bioprinter used (BIOX), while other variables, including the type of biomaterial, the cell lines, and their concentrations, were adjusted as needed.

The following sections will detail the experimental results and the technical considerations that informed these choices.

3.2.1 GelMA C and Matrigel for TME modelling

In the experiment described in this section, two different bioinks were prepared: one to simulate the tumor microenvironment and another to simulate the stromal microenvironment. The overall structure represents the desired compartmentalization of TME.

The bioink simulating the stromal microenvironment was formulated with GelMA C, incorporating WI38 fibroblasts. In contrast, the tumor microenvironment was prepared using Matrigel, incorporating 4T1-GFP tumor cells, as outlined in *Table 10*.

The reason why we chose these materials is related to the need to obtain a “core” with a composition as similar as possible to the basement membrane, while the external part, the “shell”, had to be easily bioprintable and at the same time had to have characteristics similar to the stromal compartment. For these reasons, the latter had to contain a collagen component, which in our specific case was obtained using GelMA C.

The detailed preparation of both bioinks is described in section 2.6.1.

Biomaterial	Cell line	Compartment
GelMA C (90%)	WI38	Stromal
Matrigel	4T1-GFP	Tumoral

Table 10: Summary of the stromal and tumor microenvironments, detailing the biomaterial and cell line used for each condition.

For this experiment, the preparation of the GelMA C-based stromal bioink follows the procedure detailed in section 2.5.1, "*Preparation protocol for bioink based on GelMA C at 90% concentration*", where the material should be diluted at a 1:10 ratio relative to the total bioink volume. Meanwhile, the preparation of the Matrigel-based tumor bioink follows the procedure described in section 2.5.2. Finally, the cell concentration of both bioinks is reported in *Table 11*.

Cell line	Concentration	Compartment
WI38	150.000 cells/ml	Stromal
4T1-GFP	500.000 cells/ml	Tumoral

Table 11: Cell concentration for the stromal and tumor microenvironments.

3.2.1.1 Bioprinting process

The optimization of the bioprinting process was conducted to define the optimal fabrication parameters required by the two materials tested. The resultant parameters are reported in *Table 12*.

Compartment	Stromal	Tumoral
Bioink	GelMA C and WI38	Matrigel and 4T1-GFP
Nozzle	UV-shielding 25 G	Normal 25G
Printhead temperature	24 °C	5 °C
UV module	yes	no
Printbed temperature	15 °C	15 °C
Printing speed	2 mm/s	2 mm/s
Pression	30-33 kPa	30 kPa

Table 12: Summary of the printing parameters for the stromal and tumoral microenvironments.

As reported in *Table 12*, the GelMA C-based bioink requires photocrosslinking; therefore, the UV module is applied according to the protocol described in *Table 7*. The UV module is activated at the end of the GelMA C layer printing process to induce crosslinking within the biomaterial, ensuring a stable and durable printed structure. On the contrary, Matrigel does not require UV treatment.

During the printing phase, as described in section 3.1.3, blob formation occurs at the nozzle tip of the inactive tool. To address this issue, the printing process is temporarily paused, and an extrusion test is performed to calibrate the correct pressure, which is then set in the protocol. Once this step is completed, printing resumes and carried out to completion. After printing, the structure is incubated for approximately one hour before adding cell medium. The construct is then maintained in the incubator until the end of the observation period, which, in this experiment, lasted 8 days.

3.2.1.2 Considerations and experimental outcomes

As described in section 3.2, the main objective of this study is to identify the most suitable combination of biomaterials to ensure optimal cell-cell and cell-extracellular matrix interactions. For this reason, Matrigel was chosen as the ECM for the tumor microenvironment. Matrigel is a solubilized basement membrane matrix, extracted from murine tumors, whose composition promotes cell adhesion, proliferation, and differentiation across various cell types, including tumor cells [45].

During the observation period, it was demonstrated that Matrigel allows tumor cells to proliferate, leading to an exponential increase in the tumor area over time, as shown in *Figure 36*.

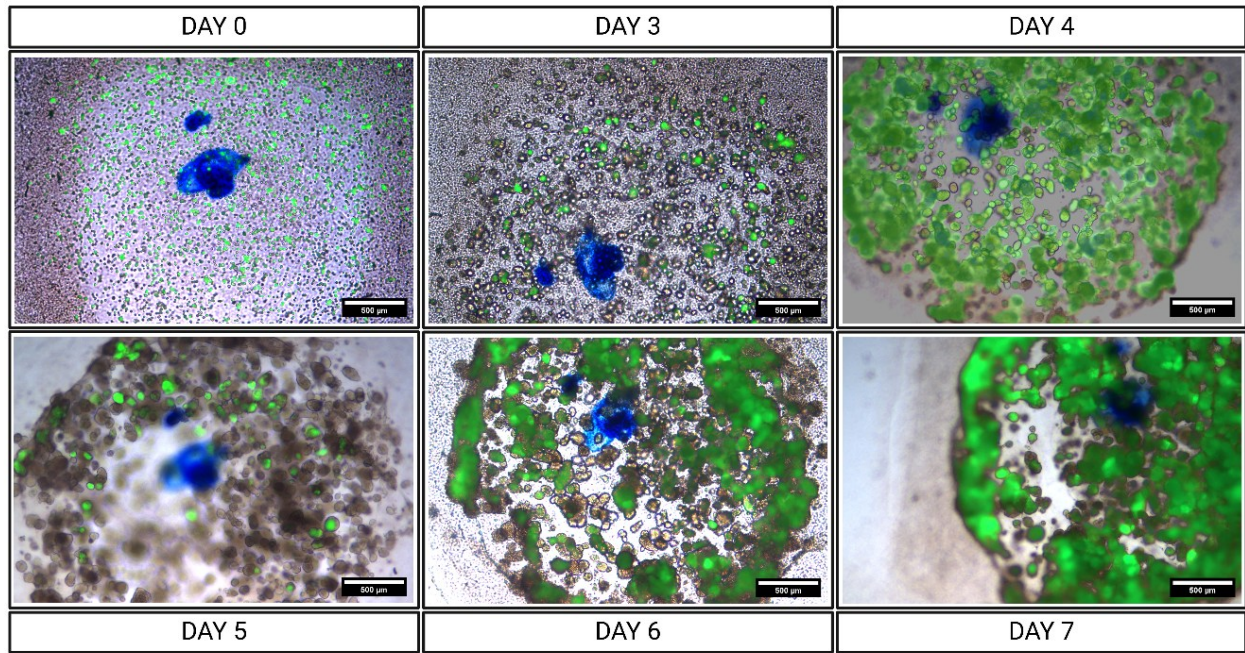


Figure 36: Images showing the merge between brightfield and green fluorescence channels, where 4T1-GFP cells appear fluorescent in green.

However, despite its high biocompatibility with tumor cells, the printability of this biomaterial is significantly limited. Specifically, extrusion issues emerged due to its high temperature sensitivity: the biomaterial tends to gel rapidly, making the printing process challenging and poorly reproducible. Additionally, no biologically stable bonds were formed between Matrigel and GelMA C, resulting in Matrigel detaching from the rest of the structure in most cases (Figure 37)

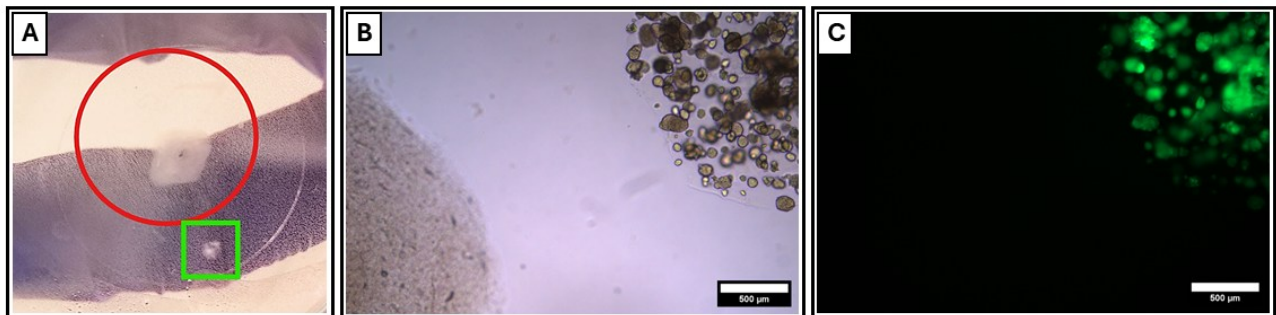


Figure 37: the images show that the Matrigel core is detached from the GelMA C shell structure. (A) The two structures (Matrigel in green and GelMA C in red) are detached. (B) A brightfield image of the structure where GelMA C is on the left and the Matrigel is on the right. The same image is acquired in fluorescence (B) to highlight the Matrigel with the tumour cells in green.

Regarding the stromal microenvironment, some biological challenges were also encountered. Fibroblasts did not exhibit the typical morphological transition over time; in particular, their shape remained spherical rather than stretched (Figure 38). This phenomenon could be related to cell

concentration, suggesting that an increase in cell concentration might be a potential improvement. Alternatively, the lack of morphological adaptation could depend on the biomaterial used. Since fibroblast behaviour is a key aspect of this study, it is crucial to identify the cause of this lack of morphological response.

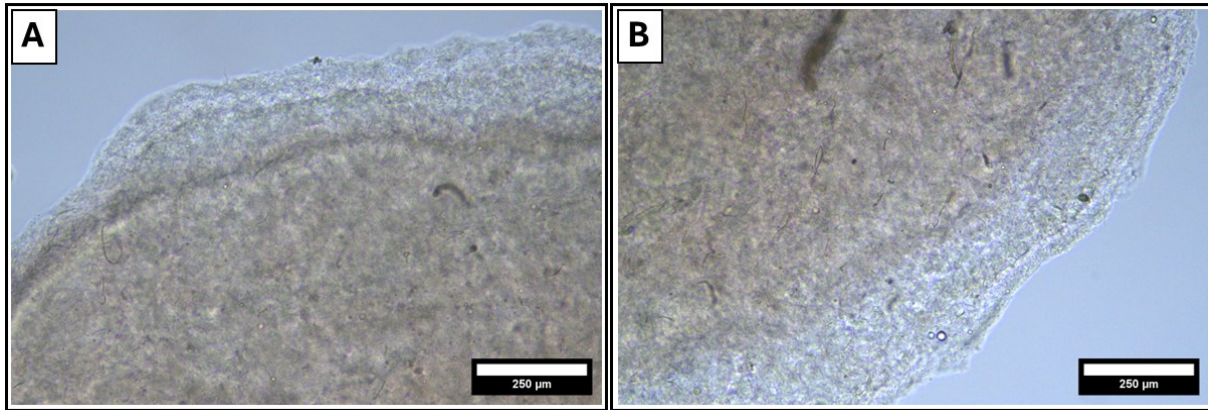


Figure 38: The figure shows the structure at observation day 8, with more detail on the behaviour of the fibroblasts, showing that they are in the spherical conformation. The acquired images show a focus of the upper left area of the structure (A) and the lower right area (B)

Unlike Matrigel, GelMA C has demonstrated excellent bioprinting properties, being easily extrudable and less sensitive to temperature variations. Based on these considerations, future developments will focus on optimizing tumor microenvironment printability by replacing Matrigel with GelMA C, ensuring a compartmentalized and reproducible structure. Additionally, to promote fibroblast morphological adaptation, an increase in cell concentration is proposed for both cell lines.

3.2.2 GelMA C for TME modelling

To address the limitations observed in the first experiment, here we focus on improving the core compartment printability. Starting from the results obtained in the first experiment we decided to test the replacement of the Matrigel-based bioink with GelMA C. Consequently, both bioinks used are formulated with GelMA C but contain two different cell lines: WI38 stromal cells and 4T1-GFP tumor cells, as reported in Table 13. The detailed preparation of both bioinks is described in section 2.6.2.

Biomaterial	Cell line	Compartment
GelMA C (90%)	WI38	Stromal
GelMA C (90%)	4T1-GFP	Tumoral

Table 13: Summary of the stromal and tumor microenvironments, detailing the biomaterial and cell line used for each condition.

For this experiment, the preparation of the two GelMA C-based bioinks follows the procedure detailed in section 2.5.1, "*Preparation protocol for bioink based on GelMA C at 90% concentration*", where the material should be diluted at a 1:10 ratio relative to the total bioink volume.

Finally, the cell concentration of both bioinks is reported in *Table 14*.

Cell line	Concentration	Compartment
WI38	1.500.000 cells/ml	Stromal
4T1-GFP	1.500.000 cells/ml	Tumoral

Table 14: Cell concentration for the stromal and tumor microenvironments.

3.2.2.1 Bioprinting process

The printing procedure, described in section 3.2.1.1, is the same as in the previous experiment. However, in this case, the UV module is activated for both bioinks, as they are both GelMA C-based and require photocrosslinking after printing each layer. The UV module used is the one reported in *Table 7*, while the set parameters are detailed in *Table 15* and the G-code was optimized accordingly.

Before starting the bioprinting process, an extrusion test is always conducted to assess the printing pressure, which may vary depending on the bioink and its biomaterial properties. The optimized printing parameters are reported in *Table 15*.

Compartment	Stromal	Tumoral
Bioink	GelMA C and WI38	GelMA C and 4T1-GFP
Nozzle	UV-shielding 25 G	UV-shielding 25 G
Printhead temperature	24 °C	24 °C
UV module	yes	yes
Printbed temperature	15 °C	15 °C
Printing speed	2 mm/s	2 mm/s
Pression	24-27 kPa	24-26 kPa

Table 15: Summary of the printing parameters for the stromal and tumoral microenvironments.

As described in section 3.1.3, blob formation occurs at the nozzle tip of the inactive tool primarily because of the activation of the UV module. In this experiment, this drawback is worsened by the fact that both the inks are GelMA C-based. For this reason, an additional pause was introduced between the first and the second layer of the printing process. Therefore, while the printing process is temporarily paused, an extrusion test is performed to fine-tune the optimal pressure, which is then

recorded in the protocol. Once this step is completed, printing resumes and continues until the entire structure is completed.

After printing, the structure is incubated for approximately one hour before adding the cell medium. The structure is then maintained in culture in the incubator until the end of the observation period, which, in this experiment, lasted 7 days.

3.2.2.2 Considerations and experimental outcomes

The printability of a biomaterial is a fundamental aspect to achieve high-quality bioprinting and fabricating a compartmentalized structure. In the previous experiment, it was demonstrated that GelMA C presents excellent printability properties and good biostability even with minimal temperature variations. For this reason, it was considered an alternative bioink to Matrigel. In fact, as reported in section 2.1.1, several studies have highlighted that the presence of CNFs incorporated into the GelMA solution significantly enhances the bioprintability of the material.

Therefore, to improve and ensure the reproducibility of 3D bioprinting, it was necessary to address this issue from the outset, searching for a biomaterial that would enable the correct biofabrication of the designed structure while minimizing bioprintability challenges. However, while biofabrication-related difficulties have been resolved, new biological challenges have emerged.

Specifically, it was observed that tumor cells located in the central area of the bioprinted structure did not proliferate over time, and the tumor microenvironment remained contained (*Figure 39*), unlike what occurred with the Matrigel-based bioink, where sustained cell proliferation was clearly observed over time.

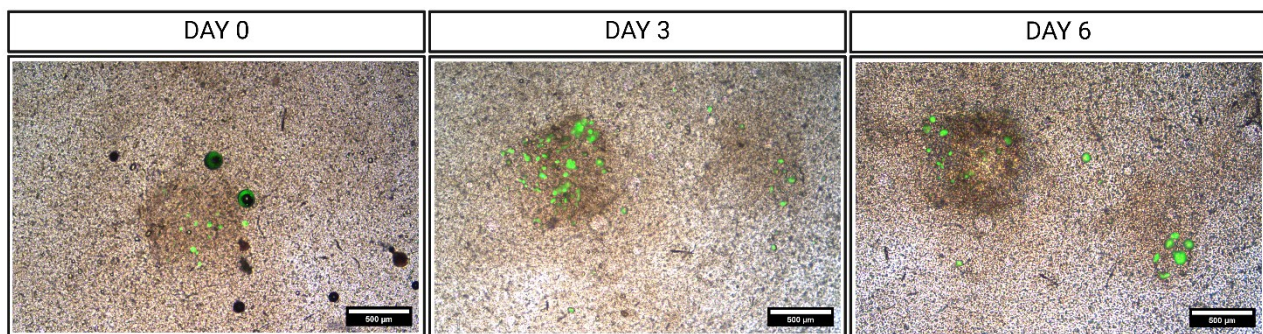


Figure 39: Optical microscope images obtained by merging brightfield and green fluorescence images, where 4T1-GFP cells appear fluorescent in green.

The same issue was encountered in the stromal microenvironment, as a bioink capable of promoting fibroblast stretching has not yet been identified. In fact, over the course of several days, WI38 cells

maintained their initial spherical conformation without transitioning to the expected stretched morphology (Figure 40).

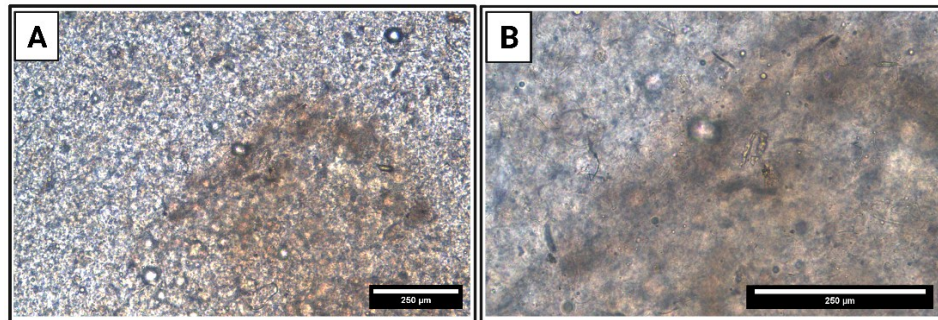


Figure 40: Brightfield optical microscope images showing a magnification of the structure at 10X (A) and 20X (B), with a particular focus on fibroblasts in their rounded morphology.

At this point, it is possible to rule out the hypothesis that increasing cell concentration could influence cell behaviour. It was initially assumed that increasing cell concentration, particularly of fibroblasts, might promote a morphological shift, enabling the cells to stretch during the observation period. However, this phenomenon did not occur, and fibroblasts continued to exhibit their spherical conformation.

A possible explanation for the lack of cell activity could be related to the cell-extracellular matrix interaction. In this experiment, as in the previous one, GelMA C-based bioinks were used with a concentration of 90% of the total bioink volume as reported in the bioink datasheet. It is likely that this high concentration resulted in an excessively rigid matrix, which hindered or prevented cell proliferation.

A potential future development could involve reducing the concentration of GelMA C-based bioink, lowering it from 90% to 70%, following the protocol described in section 2.5.1, "*Preparation protocol for bioink based on GelMA C at 70% concentration*", where the material must be diluted at a 3:10 ratio relative to the total bioink volume. Additionally, the increase in cell concentration and the results demonstrating the lack of cell activity confirm that the latter is not due to cell concentration, but rather to another factor, probably the bioink concentration itself. For this reason, in future experiments, we investigated a diluted bioink in terms of printability and cell viability.

3.2.3 Optimizing GelMA C concentration for TME modelling

In this experiment, we replicated the previous conditions as for what it concerns the printing procedure. However, investigated the reduction of the GelMA C concentration in each bioink. Specifically, the concentration is decreased from 90% to the current 70%.

Two distinct GelMA C-based bioinks are prepared, each containing a different cell line to recreate the desired compartmentalization. Specifically, one bioink incorporates WI38 stromal cells, while the other contains 4T1-GFP tumor cells, as reported in *Table 16*.

The detailed preparation of both bioinks is described in section 2.6.2.

Biomaterial	Cell line	Compartment
GelMA C (70%)	WI38	Stromal
GelMA C (70%)	4T1-GFP	Tumoral

Table 16: Summary of the stromal and tumor microenvironments, detailing the biomaterial and cell line used for each condition.

For this experiment, the preparation of the two GelMA C-based bioinks follows the procedure detailed in section 2.5.1, "*Preparation protocol for bioink based on GelMA C at 70% concentration*", where the material must be diluted at a 3:10 ratio relative to the total bioink volume.

The cell concentration of both bioinks is reported in *Table 17*.

Cell line	Concentration	Compartment
WI38	500.000 cells/ml	Stromal
4T1-GFP	500.000 cells/ml	Tumoral

Table 17: Cell concentration for the stromal and tumor microenvironments.

3.2.3.1 Bioprinting process

For this experiment we used the same bioprinting procedure described in section 3.2.2.1. The UV module used is the one reported in *Table 7*, while the set parameters are detailed in *Table 18*.

The optimized parameters obtained in the extrusion test performed before the printing process to assess the printing pressure are reported in *Table 18*. The pressure ranges shown in the table are rather wide; this is probably due to the fact that, during the preparation of the bioink, a sufficiently homogenous mixing was not carried out. As a result, some areas of the cartridge contained more diluted bioink than others. In addition, the presence of any 'blobs may cause a localized increase in the pressure required for extrusion.

Compartment	Stromal	Tumoral
Bioink	GelMA C and WI38	GelMA C and 4T1-GFP
Nozzle	UV-shielding 25 G	UV-shielding 25 G
Printhead temperature	24 °C	24 °C
UV module	yes	yes
Printbed temperature	15 °C	15 °C
Printing speed	2 mm/s	2 mm/s
Pression	22-32 kPa	16-25 kPa

Table 18: Summary of the printing parameters for the stromal and tumoral microenvironments.

As was observed in the previous experiment, gelling of the biomaterial was noted at the nozzle tip. For this reason, a pause was again introduced to do an extrusion test during printing. After printing, the structure is incubated for approximately one hour before adding the cell medium. It is then returned to the incubator until the end of the observation period, which, in this experiment, lasted 8 days.

3.2.3.2 Considerations and experimental outcomes

To investigate the role of the biomaterial concentration on the stretching of the embedded stromal cells we tested different variations in the GELMA bioink formulation. The purpose of this analysis was to determine which experimental condition best supported cell viability and to identify the scenario in which fibroblasts spread more effectively over time.

Specifically, three different GelMA C-based bioinks were analysed:

1. *Sample 1:* Bioink with 90% GelMA C in cell suspension,
2. *Sample 2:* Bioink with 70% GelMA C in cell suspension,
3. *Sample 3:* Bioink with 70% GelMA C in cell suspension and Type I collagen rat tail.

The first case reflects the bioinks used in previous experiments, the second is essential for confirming the results of the current experiment, while the third represents an alternative attempt to combine two bioinks to create an optimized matrix. Collagen was included because it mimics the composition of the stromal compartment, with the intent to enhance cell–biomaterial interaction. The *Figure 41* presents brightfield optical microscope images of the three tested bioink types, captured at different time points during the observation period.

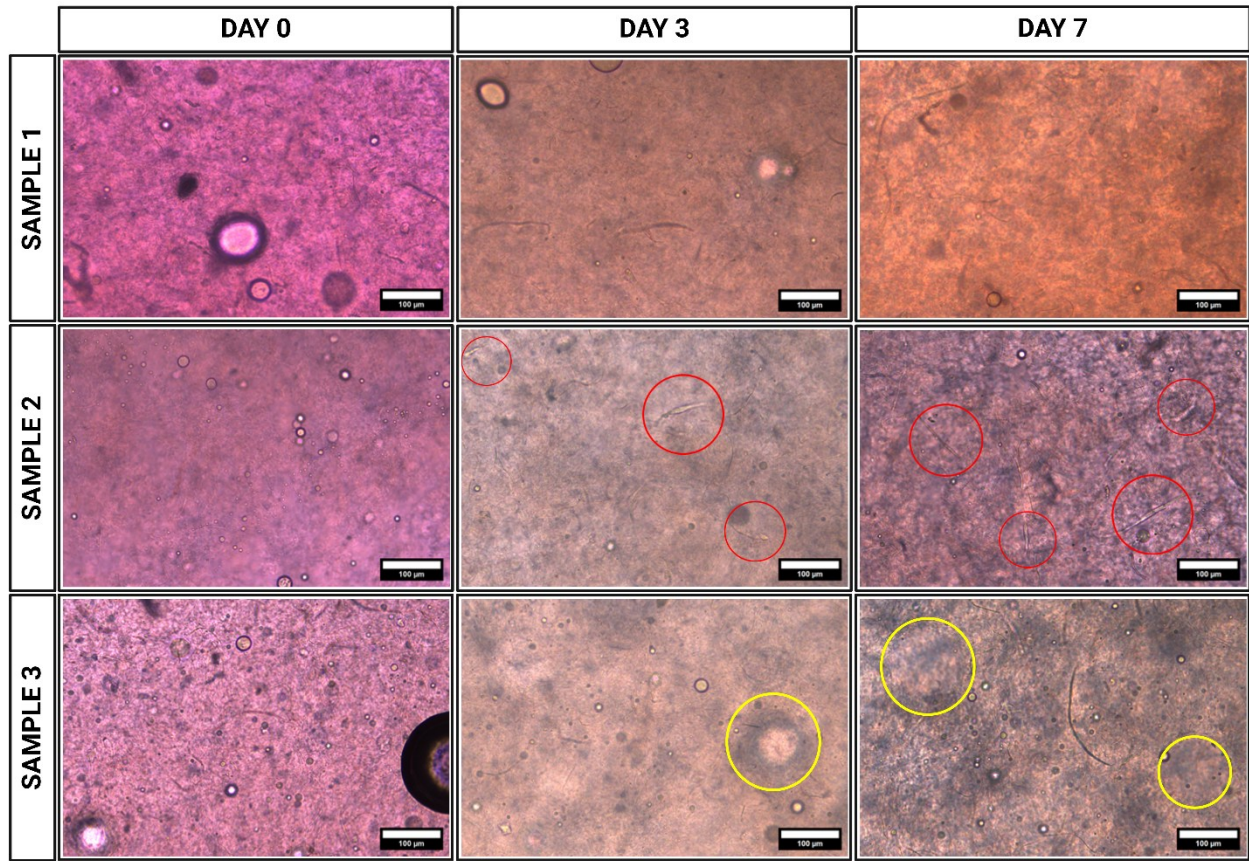


Figure 41: Brightfield images at 20X magnification showing the three observed bioinks. Observation time points: Day 0, 3, and 7. Sample 1 corresponds to the first bioink type, Sample 2 to the second bioink type, and Sample 3 to the third bioink type. The yellow circles highlight areas where blobs are present, while the red circles indicate stretched fibroblasts.

In *Sample 1*, the GelMA C concentration is 90%; as demonstrated in previous experiments, under these conditions, fibroblasts do not stretch. In fact, throughout the observation period, they retain their initial spherical conformation.

In *Sample 2*, however, significant differences can be observed. In this case, the bioink contains 70% GelMA C, a considerably lower concentration. From day 3, fibroblasts begin to change their conformation, becoming progressively more stretched, with a more pronounced effect observed on day 7.

In *Sample 3*, where the bioink consists of 70% GelMA C combined with 30% cell medium and Type I collagen rat tail, fibroblasts appear stretched as in the previous case. However, some opaque areas are visible in the images, likely due to blob formation within the matrix. These areas are highlighted with yellow circles in the images. This suggests that a phase separation between collagen and GelMA C. For this reason, the mixture between collagen and GelMA C was not used form this test onward, as the formation of blobs within the matrix creates heterogeneity in the extracellular matrix.

To further investigate the biological performance of the bioink formulations, a *Live/Dead assay* was performed to assess the viability of WI38 fibroblasts. The detailed protocol of the assay is described in section 2.6.2, "*Influence of GelMA C concentration on fibroblast viability and matrix interaction*".

The graphs in *Figure 42* show that on day 1, the highest viability is recorded in *Sample 2*, with a percentage of 73%. This value decreases very slowly over time, reaching 68% on day 7. *Sample 1*, on the other hand, showed low viability at day 1, but an increased viability in day 7. This proves that high concentration GelMA C is not cytotoxic per se. The effect on cells, which remain rounded and with limited interaction with the biomaterial could be attributed to the fact that such high concentration and thus stiffness is excessive for cells to remodel the material and acquire a spindle like shape. As opposed to *Sample 1* and *2*, *Sample 3* is showing low viability. This result combined with the heterogeneity observed before pushed us to discontinue the use of this material.

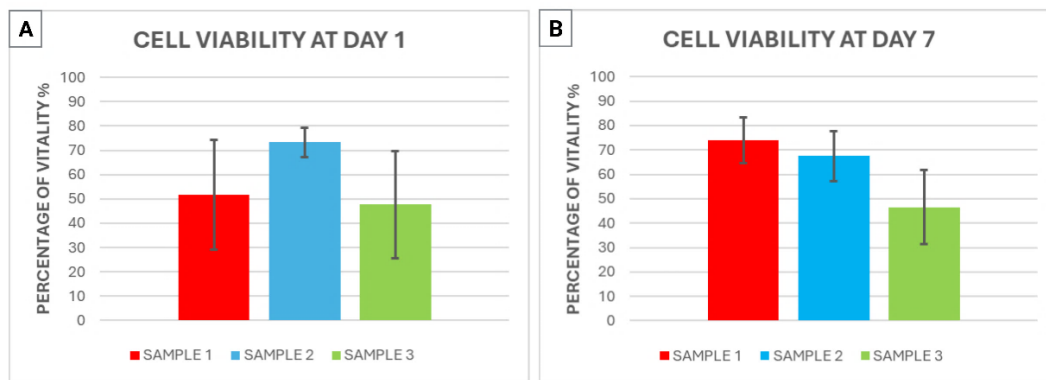


Figure 42: Graphs showing the evolution of WI38 cell viability on Day 1 (A) and Day 7 (B). The data were obtained using the “Live/Dead” assay on the three test samples, followed by cell counting.

In terms of stromal cell spreading, fluorescence images acquired during the *Live/Dead assay* show a gradual elongation and stretching over time, a phenomenon particularly evident on day 7 (*Figure 43*). This result demonstrates that the concentration used in *Sample 2* is optimal for promoting cell-matrix interaction.

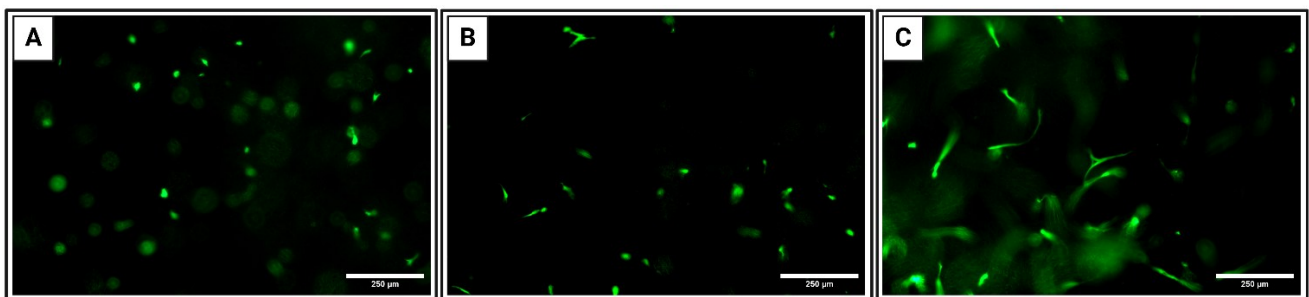


Figure 43: Fluorescence images showing live fibroblasts (green) in Sample 2 during the observation period: (A) Day 1, (B) Day 4, (C) Day 7.

Based on the observed results, it can be concluded that the reduction in GelMA C concentration in the different bioinks led to significant improvement in the cell-material interactions, helping to address previously observed issues. It was observed that a lower GelMA C concentration promoted cell spreading over time. Specifically, fibroblasts appeared more stretched and exhibited a morphological transformation, shifting from a rounded to a spindle-like morphology which is the one observed *in vivo*.

However, limitations still remain in the tumor cells-stromal cells interaction which seems to be inadequate to allow a proper observation of the *in-vivo* dynamics. A possible reason for this may be related to the cell line used.

For this reason, the use of a different cell line is proposed: MAF are therefore implemented in the model which are adult murine fibroblasts originated from the same specie as 4T1-GFP cells. This approach could reduce variability and promote greater cell-cell and cell-matrix interactions. As described in section 3.2, a key advantage of using exclusively murine tumor and stromal cells, rather than a combination of cells from different species, is the enhanced biological consistency of the model, significantly reducing interspecific differences.

3.2.4 Murine adult fibroblasts as stromal cells for TME modelling

This experiment aim is the investigation of the role of the murine fibroblasts in the stromal cell-tumor cells interaction. The printing conditions were kept as the one described in section 3.2.3. Specifically, both the tumor compartment as well as the stromal microenvironment are recreated with the diluted GelMA C-based bioink. The key difference lies in the different cell line used to simulate the stromal environment: MAF, as reported in *Table 19*.

The detailed preparation of both bioinks is described in section 2.6.2.

Biomaterial	Cell line	Compartment
GelMA C (70%)	MAF	Stromal
GelMA C (70%)	4T1-GFP	Tumoral

Table 19: Summary of the stromal and tumor microenvironments, detailing the biomaterial and cell line used for each condition.

For this experiment, as in previous ones, the preparation of the two GelMA C-based bioinks follows the procedure detailed in section 2.5.1, “*Preparation protocol for bioink based on GelMA C at 70% concentration*”.

The cell concentrations of both bioinks are reported in *Table 20*.

Cell line	Concentration	Compartment
MAF	5.000.000 cells/ml	Stromal
4T1-GFP	5.000.000 cells/ml	Tumoral

Table 20: Cell concentration for the stromal and tumor microenvironments.

3.2.4.1 Bioprinting process

The bioprinting process follows the same methodology described in section 3.2.3.1. The UV module used is the one listed in *Table 7*, while the printing parameters are specified in *Table 21*.

Before initiating the bioprinting process, an extrusion test is performed to assess the printing pressure, which may vary depending on the bioink composition, cell concentration and biomaterial properties. The optimized parameters are reported in *Table 21*.

Compartment	Stromal	Tumoral
Bioink	GelMA C and MAF	GelMA C and 4T1-GFP
Nozzle	UV-shielding 25 G	UV-shielding 25 G
Printhead temperature	24 °C	24 °C
UV module	yes	yes
Printbed temperature	15 °C	15 °C
Printing speed	2 mm/s	2 mm/s
Pression	19-20 kPa	14-27 kPa

Table 21: Summary of the printing parameters for the stromal and tumoral microenvironments.

At the end of the printing process, the construct is placed in the incubator for one hour. The culture medium is then added, and samples are kept in the incubator throughout the observation period, which, in this experiment, lasted 8 days.

3.2.4.2 Considerations and experimental outcomes

To facilitate the monitoring of the experiment, we used murine adult fibroblasts derived from genetically modified mice expressing “tdTomato”, a constitutively orange fluorescent protein that enabled their immediate visualization under a fluorescence microscope, as reported in section 2.2. This was possible thanks to the collaboration with the Stefano Piccolo’s group. Thanks to this fluorescent protein, fibroblasts in the stromal microenvironment are readily visible through fluorescence imaging as shown in *Figure 44*.

During the observation period, we were able to observe that fibroblasts in the stromal microenvironment progressively stretched, especially towards the end of the observation period, a phenomenon attributed to the 70% GelMA C concentration in the bioink, as demonstrated in the previous study.

Moreover, the use of murine-origin cells, both for the stromal and tumor microenvironment, has promoted better cell-cell interaction within the 3D structure, thanks to their biological affinity, as they share the same origin. Furthermore, it was observed that tumor cells finally started to proliferate over time and, more importantly, that they migrate toward the stromal compartment. This is probably due to the dual optimization implemented: the lower concentration of the GelMA C bioink and the choice of murine cells for the stromal compartment.

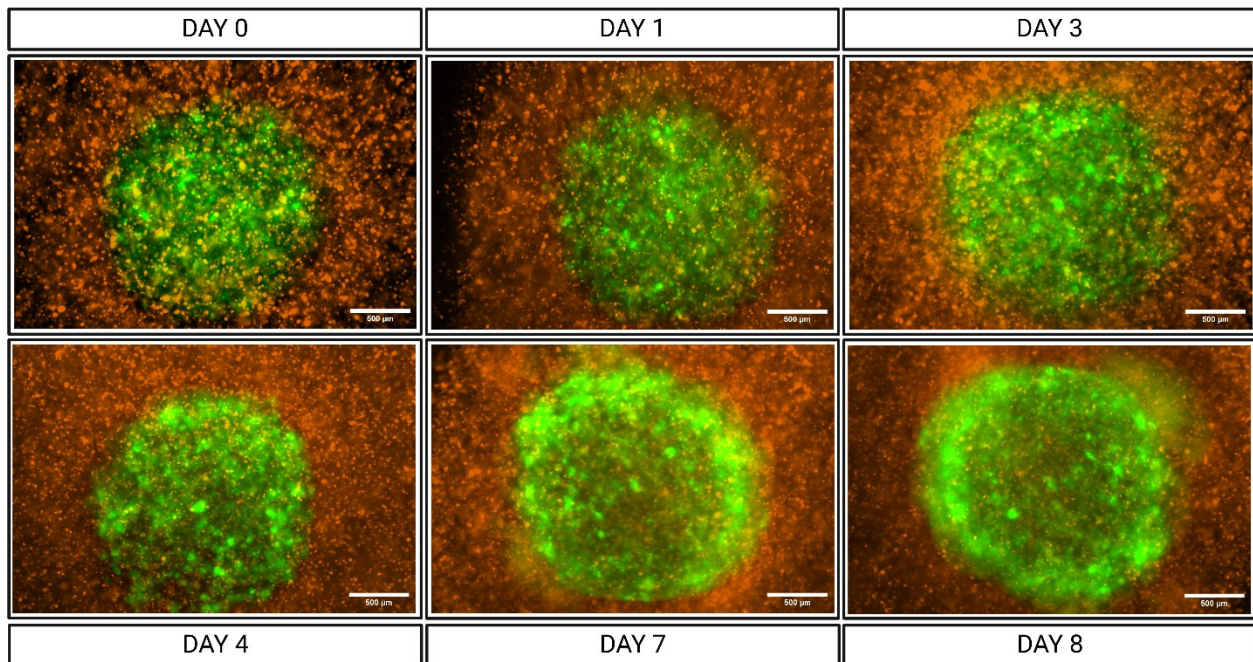


Figure 44: Fluorescence optical microscopy images showing the behaviour of 4T1 cells (green) and MAF cells (red) over time. The images were acquired in the central region, where the tumor core (4T1, green) is in the inner area, surrounded by the stromal compartment (MAF, red) in the outer area.

However, this model still fails to replicate the cell-mediated stroma remodeling characteristic of the tumor microenvironment. Ideally, fibroblasts creating a shell around the tumor cells should be observed. However, this does not seem to happen, as fibroblasts appear randomly distributed along the outer zone throughout the experimental period (*Figure 45*).

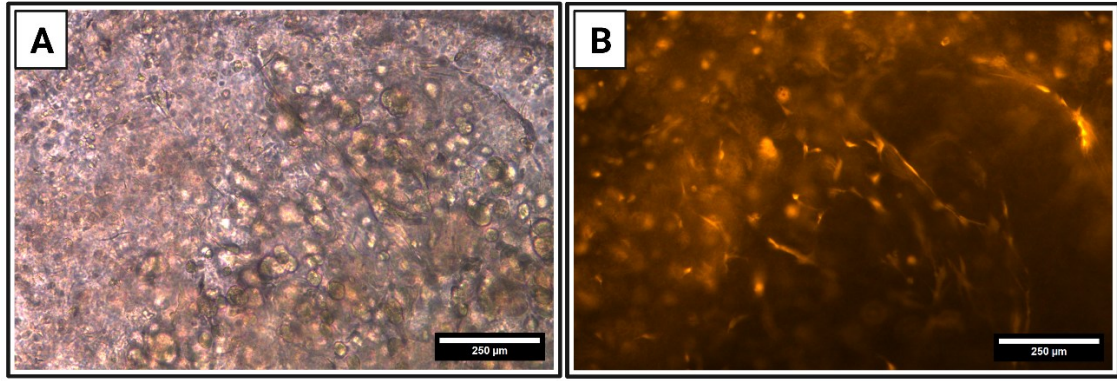


Figure 45: Optical microscopy images showing fibroblast behaviour at day 3 at 10X magnification. The image was acquired at the interface between the stromal and tumor environments. The lower right area appears dark because, in that region, the tumor environment begins, which is not visible in red fluorescence. (A) displays a brightfield image, while (B) shows the fluorescence image in red, where the MAF cells are visible in an elongated condition.

This behaviour is likely influenced by the presence of GelMA C, which, while facilitating cellular stretching, might partially restrict the ability of fibroblasts to arrange correctly the biomaterial on a macroscopic level, preventing the formation of a structured shell.

Based on this experiment, it can be concluded that the challenge of reproducing an ideal tumor microenvironment has been successfully addressed, as a 70% GelMA C matrix has been identified that effectively interacts with tumor cells. Conversely, for the stromal microenvironment, further study is needed. To tackle this limitation and to simulate more accurately the stromal compartment, we decided to modify the stroma-mimicking biomaterial replacing GelMA C with pure Lifeink® 200 Type I Collagen, incorporating MAF cells.

3.2.5 GelMA C and Lifeink 200 Collagen for TME modelling

This experiment builds upon the developments achieved so far. Diluted GelMA C bioink was used for the tumor compartment and MAF as stromal cells, which demonstrated to be key to allow the observation of the cell-cell interactions. The cells used in this experiment and the biomaterials employed are listed in Table 22.

The detailed preparation of both bioinks is described in section 2.6.3.

Biomaterial	Cell line	Compartment
Lifeink® 200 Type I Collagen	MAF	Stromal
GelMA C (70%)	4T1-GFP	Tumoral

Table 22: Summary of the stromal and tumor microenvironments, detailing the biomaterial and cell line used for each condition.

For this experiment, as in previous ones, the preparation of the GelMA C-based bioink for the tumoral microenvironment follows the procedure detailed in section 2.5.1, “*Preparation protocol for bioink based on GelMA C at 70% concentration*”. Meanwhile, the preparation of the Lifeink® 200 Type I Collagen-based bioink follows the procedure described in section 2.5.4.

The cell concentration of both bioinks is reported in *Table 23*.

Cell line	Concentration	Compartment
MAF	5.000.000 cells/ml	Stromal
4T1-GFP	5.000.000 cells/ml	Tumoral

Table 23: Cell concentration for the stromal and tumor microenvironments.

3.2.5.1 Bioprinting process

The printing process is very similar across all the experiments described so far. A key difference lies in the type of cartridge used: specifically, for a GelMA C-based bioink, which requires crosslinking, a UV-shielding cartridge is necessary, whereas Lifeink® 200 Collagen does not require crosslinking and can therefore be used with a standard cartridge.

Before starting the bioprinting process, an extrusion test is conducted to assess the printing pressure, which varies depending on the bioink and its biomaterial properties. The optimized parameters are reported in *Table 24*.

Compartment	Stromal	Tumoral
Bioink	Lifeink® 200 Collagen and MAF	GelMA C and 4T1-GFP
Nozzle	Normal 25G	UV-shielding 25 G
Printhead temperature	5 °C	24 °C
UV module	no	yes
Printbed temperature	15 °C	15 °C
Printing speed	2 mm/s	2 mm/s
Pression	17-19 kPa	15-21 kPa

Table 24: Summary of the printing parameters for the stromal and tumoral microenvironments.

As indicated in *Table 24*, the GelMA C-based bioink requires photocrosslinking; therefore, the UV module is applied following the procedure outlined in *Table 7*. The UV module is activated at the end of the GelMA C layer deposition to initiate crosslinking within the biomaterial, ensuring structural integrity and long-term stability. In contrast, Lifeink® 200 Collagen does not require UV exposure.

As in previous experiments, printing phase is paused to perform an extrusion test, which serves to check the appropriate pressure and clear any clogs at the nozzle tip.

After printing, the 3D construct is incubated for approximately one hour before the cell medium is added. The structure is then placed in the incubator and maintained under controlled conditions until the end of the observation period, which, in this experiment, lasted 8 days.

3.2.5.2 Considerations and experimental outcomes

The compartmentalized structure was successfully achieved in this experiment. Based on the findings from previous experiments, it was observed that MAF cells elongate, similar to what was seen with the GelMA C-based bioink, but in this case, the effect is much more pronounced. In fact, the presence of collagen enhances cell-matrix interaction, leading to fibroblasts appearing stretched as early as day 1 as can be observed in *Figure 46*. This represents a significant difference compared to previous studies and confirms that collagen plays a crucial role in cellular behaviour, as detailed in section 1.2.2, "*The role of Collagen in TME remodelling*".

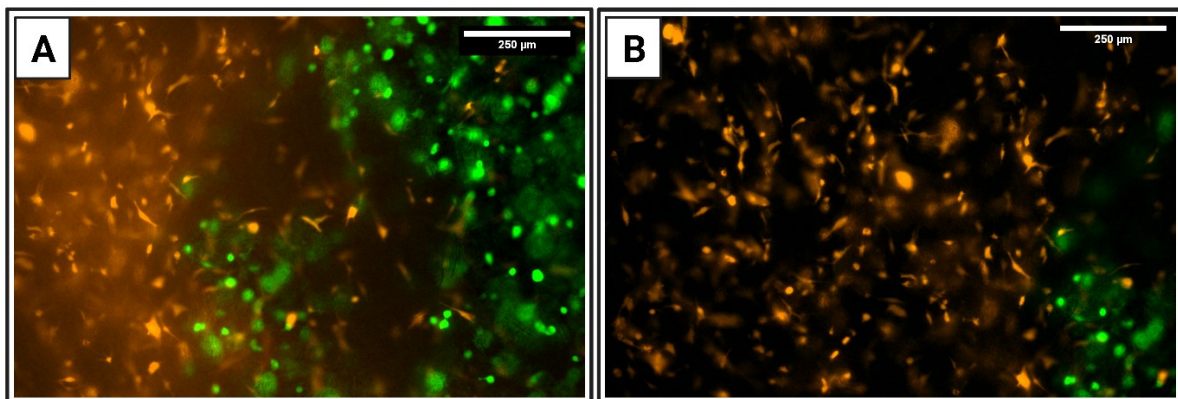


Figure 46: The two images (A) and (B) show the interface between MAF cells (orange fluorescence) and 4T1-GFP cells (green fluorescence) at day 1.

Beyond the rapid elongation of MAF cells, the expected behaviour was also observed: over the observation period, MAF cells arranged themselves uniformly within the structure, forming the characteristic core-shell organization. Additionally, fibroblasts exhibited a directional alignment, contributing to the formation of an aligned shell surrounding the tumor compartment.

In *Figure 47*, the compartmentalized construct is shown across different days, starting from the day of printing up to the final observation day, corresponding to day 8. The figure presents the most significant acquisitions, highlighting the biological behaviour that developed over time.

In all images, a clear separation between the two compartments is evident. Initially, the cell concentration appears limited; however, as the days progress, cell growth becomes increasingly noticeable. This is observed in both the stromal and tumoral environments, as indicated by the enhanced fluorescence signal intensity over time.

On day 0, MAF cells exhibit a spherical conformation. By day 1, a morphological shift is already apparent, as the cells begin to adopt the characteristic spindle-like morphology. The stretching of fibroblasts continues to increase throughout the observation period. Notably, starting from day 7, fibroblasts tend to align directionally, ultimately forming a shell-like structure around the tumor environment. This rearrangement of the stromal cell is accompanied by a cell-mediated remodeling of the collagen bioink of the outer compartment.

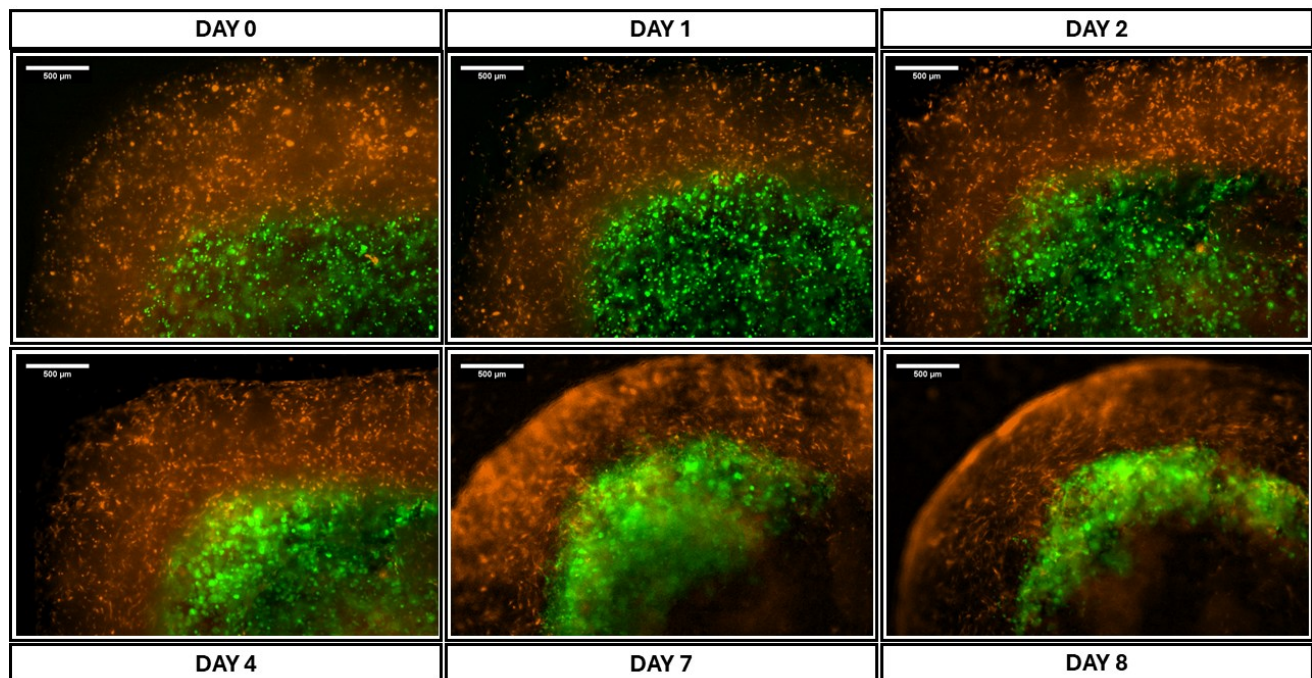


Figure 47: Fluorescence images showing MAF cells (red fluorescence) and 4T1 cells (green fluorescence) at different observation days.

Confocal microscope images were acquired. The images are presented in *Figure 44*, where it can be observed that on day 1, fibroblasts show an initial change in their morphology, and a clear separation between the stromal and tumoral environments. By day 3, fibroblasts begin to assume a fully stretched shape and an initial alignment along the tumor-stroma interface. A significant shift is noticeable on days 7 and 8. As previously observed in optical microscope images, fibroblasts (MAF cells) align directionally, forming a shell-like structure around the tumoral environment. On day 8, the fluorescence signal is markedly intensified, which is attributed to the increased cell concentration due to proliferation.

This serves as evidence that the chosen biomaterials in which the cells are embedded are suitable to assure high cell viability and proliferation as well as to observe and investigate the dynamics of the cell-matrix interactions.

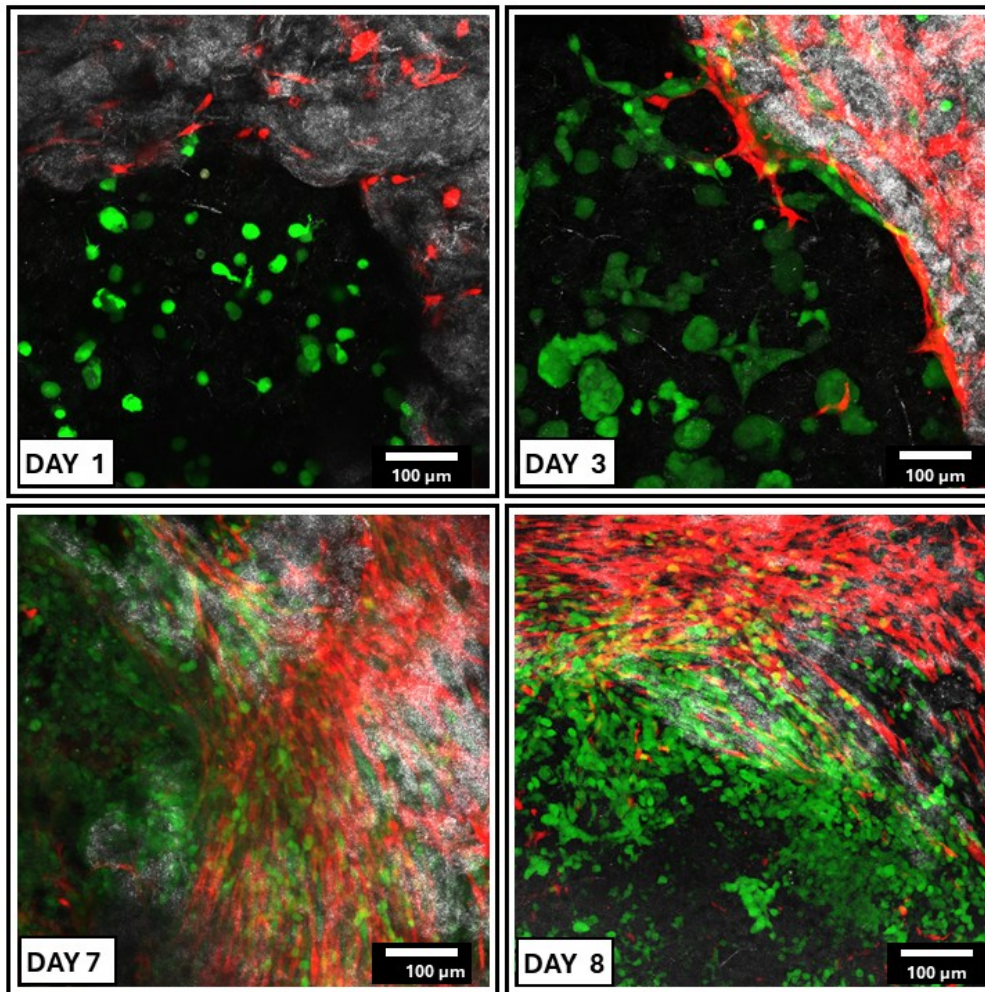


Figure 48: The figure presents images acquired using a confocal microscope, showing different observation days. MAF cells (red fluorescence), 4T1-GFP cells (green fluorescence), and collagen are visible.

Regarding the tumoral microenvironment, it proliferated over time, as observed in previous experiments. However, an interesting finding emerged: around day 7 of observation, the tumoral cells appeared to migrate outward, positioning itself at the interface between the stromal and tumoral regions (*Figure 49*).

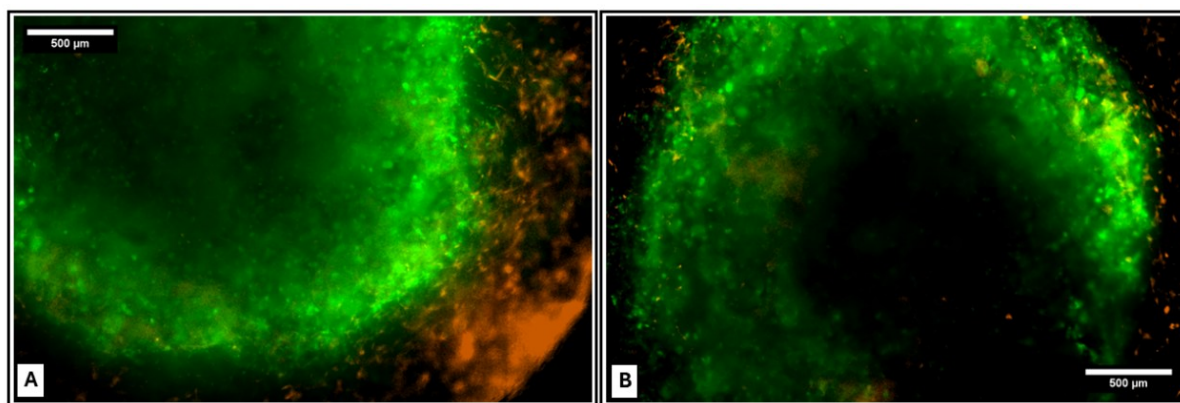


Figure 49: The images were acquired using an optical microscope, focusing on the central region of the structure to better observe the tumor environment. The images were acquired on day 7 (A) and day 8 (B), showing 4T1 cells (green fluorescence) and MAF cells (red fluorescence). It can be observed that the tumor cells migrate towards the outer region, forming a shell-like arrangement.

To determine whether this phenomenon is driven by the presence of Lifeink 200 collagen or by interactions with MAF cells, meaning whether it results from cell-cell interactions or cell-matrix interactions the experiment has been repeated without incorporating MAF cells into the stromal microenvironment.

3.2.6 Cell-free Lifeink 200 Collagen for stromal compartment

The primary objective of each study is investigating the mechanism of cellular migration from the tumor region. Specifically, the role of MAF on the tumor cells invasion through the stromal outer layer was investigated. To pursue this aim we fabricated a compartmentalized structure with a cell-free stromal compartment constituted of high concentration collagen bioink.

The cells used in this experiment and the biomaterials employed are listed in *Table 25*.

The detailed preparation of both bioinks is described in section 2.6.3.

Biomaterial	Cell line	Compartment
Lifeink® 200 Type I Collagen	-	Stromal
GelMA C (70%)	4T1-GFP	Tumoral

Table 25: Summary of the stromal and tumor microenvironments, detailing the biomaterial and cell line used for each condition.

For this experiment, as in previous ones, the preparation of the GelMA C-based bioink for the tumoral microenvironment follows the procedure detailed in section 2.5.1, “*Preparation protocol for bioink based on GelMA C at 70% concentration*”. Meanwhile, the preparation of the Lifeink® 200 Type I Collagen-based bioink follows the procedure described in section 2.5.4.

The cell concentration of both bioinks is reported in *Table 26*.

Cell line	Concentration	Compartment
-	-	Stromal
4T1-GFP	5.000.000 cells/ml	Tumoral

Table 26: Cell concentration for the stromal and tumor microenvironments.

3.2.6.1 Bioprinting process

The bioprinting process follows the same procedure as described in section 3.2.5.1.

Before initiating the bioprinting process, an extrusion test is performed to evaluate the printing pressure, which varies based on the bioink and its biomaterial properties. Optimized printing parameters are reported in *Table 27*.

Compartment	Stromal	Tumoral
Bioink	Lifeink® 200 Collagen	GelMA C and 4T1-GFP
Nozzle	Normal 25G	UV-shielding 25 G
Printhead temperature	5 °C	24 °C
UV module	no	yes
Printbed temperature	15 °C	15 °C
Printing speed	2 mm/s	2 mm/s
Pression	8-16 kPa	20-21 kPa

Table 27: Summary of the printing parameters for the stromal and tumoral microenvironments.

As indicated in *Table 27*, the GelMA C-based bioink requires photocrosslinking; therefore, the UV module is applied following the procedure outlined in *Table 7*. In contrast, Lifeink® 200 Collagen does not require UV exposure. As in previous experiments, printing is paused to perform an extrusion test, which serves to clear any clogs at the nozzle tip due to UV irradiation.

After printing, the 3D construct is incubated for one hour before the cell medium is added. The structure is then placed in the incubator until the end of the observation period, which lasted 8 days.

3.2.6.2 Considerations and experimental outcomes

The aim of this experiment was to investigate the role of collagen in the tumor microenvironment and assess whether its presence influences the migration of 4T1 cells toward the stromal area.

Optical microscope images were acquired to compare the results with those obtained in the previous experiment, described in section 3.2.5.2.

As shown in *Figure 50*, over the observation period the tumor cells migrated toward the collagen shell similarly to what was observed in the experiment with MAF cells suggesting that the migration of 4T1 cells may be directly influenced by the presence of collagen.

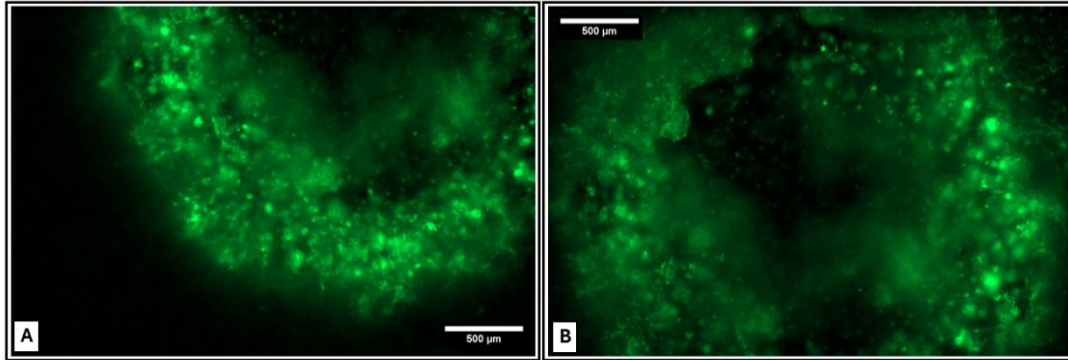


Figure 50: The images were acquired using an optical microscope, focusing on the central region of the structure to better observe the tumor environment. The images were acquired on day 7 (A) and day 8 (B), showing 4T1 cells (green fluorescence). It can be observed that the cells migrate towards the outer region, forming a shell-like arrangement.

To better understand the tumor cell invasion mechanism and the role of collagen in this phenomenon, images were acquired using a confocal microscope (*Figure 51*), which allowed for a more detailed observation with higher magnifications. Additionally, reflection microscopy was exploited for the visualization of the collagen fibers. This technique makes it possible to study the structure and organization of collagen, providing crucial insights into the extracellular matrix and cellular interactions, which is the focus of this study.

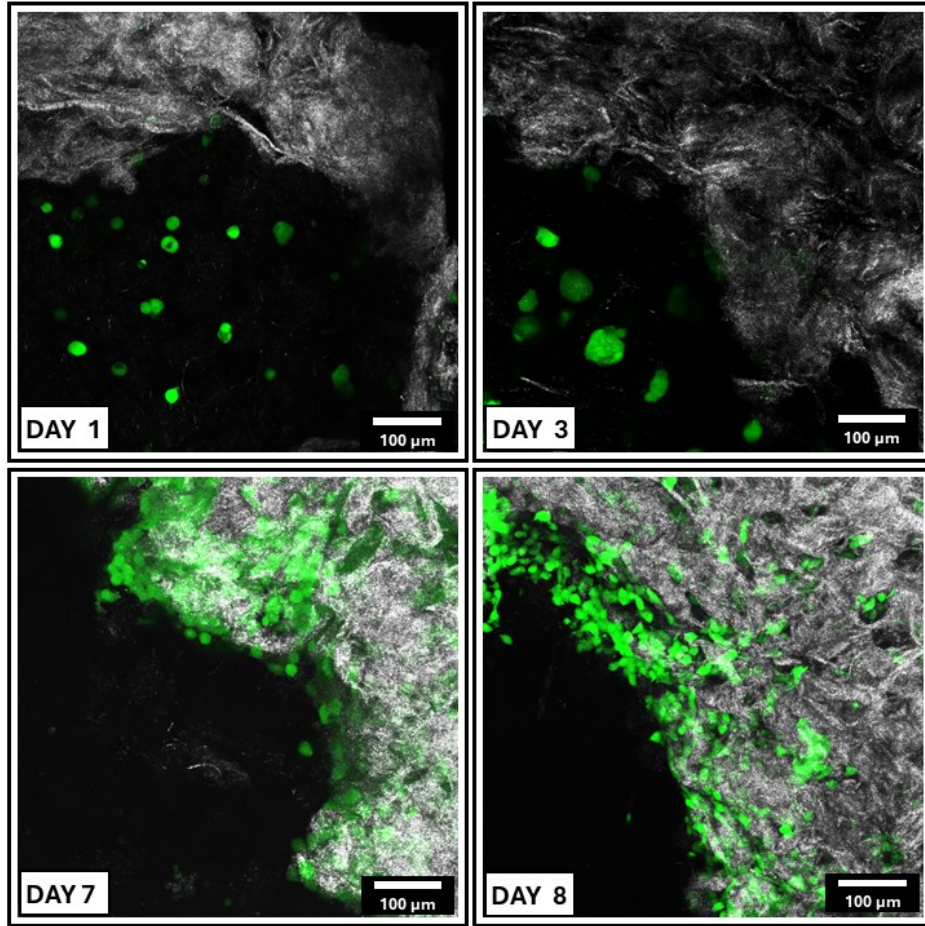


Figure 51: The figure presents images acquired using a confocal microscope, showing different observation days. 4T1-GFP cells (green fluorescence), and collagen are visible.

The behaviour of tumor cells is similar to that observed in the previous experiment. Although stromal fibroblasts were not present on the outer section of the structure, over time 4T1-GFP cells migrate towards the stromal area. Specifically, starting from day 7, cell migration towards the stromal area becomes clearly visible and by day 8, tumor cells infiltrate through it. This result demonstrates that tumor cells tend to migrate radially from the initial tumor core and that the presence of collagen promotes cell-matrix interaction.

Moreover, the most noticeable difference can be observed when comparing Day 8 between *Figure 51* and *Figure 48*. In the absence of MAF, tumor cells invade the stromal environment, attempting to settle within the collagen fibers. Conversely, in the condition where MAFs are present, fibroblasts exhibit an interesting behaviour: they form a directional barrier that hinders tumor cell migration, attempting to block their entry, ultimately leading to a core-shell organization. The characterization of the collagen fibre network and its space and time-dependent remodelling is therefore key to investigating the interactions between cells and the matrix, and to understand tumor progression. [47]

The structural remodelling was also observed at a macroscopic level. Indeed, from the acquired images, it is evident that over the observation days, the construct progressively decreased in size as shown in *Figure 52*.

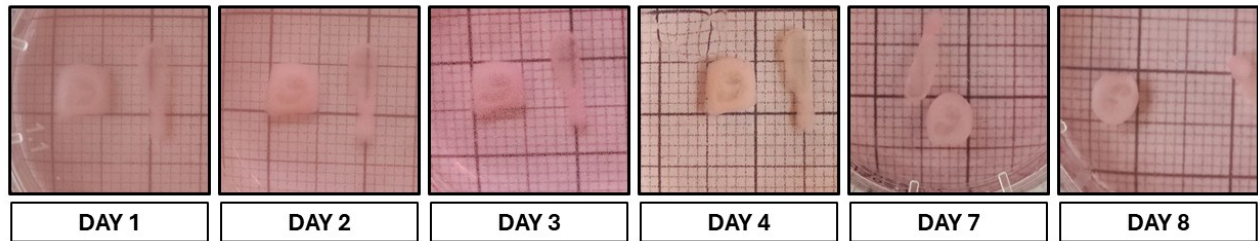


Figure 52: Images acquired with a camera to visualize the construct dimensions over the observation days, from day 1 to day 8.

This phenomenon is due to microscopic remodelling, which also manifests at a macroscopic level. A clear reduction in size can be observed over time, and the data have been plotted in a graph, as shown in *Figure 53*.

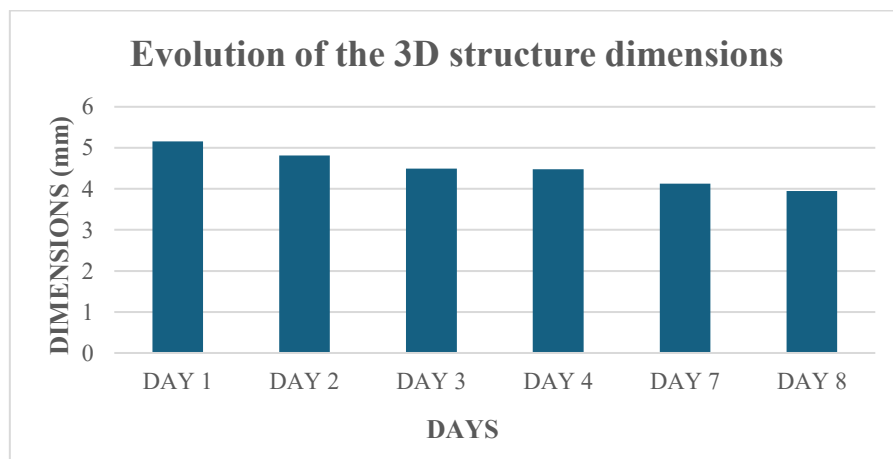


Figure 53: Graph representing the variation in construct dimensions over time during different observation days. The reported values represent the average of the measurements acquired and refer to the length of the construct, corresponding to one side of the printed square structure.

The structure dimensions were measured based on the constructs preserved during the observation days (*Figure 48*). The analysis was performed using ImageJ software, which allowed for obtaining the measurements reported in the *Table 28*.

DAYS	DAY 1	DAY 2	DAY 3	DAY 4	DAY 7	DAY 8
DIMENSION	5.154 ±	4.8147 ±	4.4928 ±	4.4815 ±	4.1286 ±	3.949 ±
[mm]	0.1616	0.1859	0.1637	0.099	0.8342	0.8629

Table 28: Mean construct dimensions with standard deviation over different observation days. Values are expressed in the format mean ± standard deviation.

4. Conclusions

The objective of this thesis was to develop a compartmentalized structure capable of simulating the tumor microenvironment. To achieve this, extrusion-based bioprinting was employed, allowing for the creation of a three-dimensional construct through 3D bioprinting, designed to replicate the desired microenvironment. The approach allowed the creation of two distinct compartments, one inner core simulating the tumoral and one outer shell to mimic the stromal tissue, allowing the investigation of cell-matrix and cell-cell interactions.

During the development of this thesis, several technological and biological challenges were encountered and progressively resolved. One of the first issues was determining the optimal dimensions for the bioprinted structure. An optimized geometry was selected suitable both to achieve precise separation of the compartments and for microscopic observation while minimizing excessive material waste. Furthermore, an optimization of the printing trajectories was required to achieve high shape fidelity and high consistency of the printing process.

From a biological perspective, a crucial aspect was the selection of the ideal biomaterial and cell lines. The aim was to find a trade-off between biocompatibility, bioprintability and physiological relevance of the model. Initially, Matrigel, chosen for its excellent biocompatibility, was discarded due to its low thermal stability, which compromised its bioprintability, and due to the delamination between the the inner and core compartments during the observation period. To improve printability GelMA C was tested for both compartments. However, the results showed that, despite the good bioprintability, fibroblasts did not exhibit the characteristic spindle-like morphology, and tumor cells displayed limited proliferation.

To overcome these issues, the concentration of GelMA C in the bioink was optimized so to improve cell viability and cell–matrix interactions. In this regard, a *Live/Dead assay* performed to evaluate the biological performance, showed that a GelMA C-based bioink at 70% concentration exhibited a higher cell viability compared to the datasheet suggested condition with 90% concentration. In fact, it was observed that fibroblasts under this condition changed their morphology into an elongated shape, which represents the *in vivo* like condition. However, the tumor cells and stromal cells interactions were still limited.

To address this problem, it was hypothesized that the difference in cellular origin between fibroblasts and tumor cells might have an influence in the compartment's interactions. Indeed, initially employed stromal cells were a human healthy fibroblasts cell line (WI38). To reduce the interspecies difference with murine cancer cell line (4T1), it was decided to replace human fibroblasts with tdTomato tagged murine adult fibroblasts (MAF). This change resulted in greater tumor proliferation, likely due to the

presence of cells from the same origin. However, a key phenomenon characteristic of the in-vivo published observation was still missing in the in-vitro model: the cell-mediated stromal matrix remodelling and stromal cell rearrangement along the tumor boundary.

To overcome this limitation, GelMA C in the stromal environment was replaced with Lifeink 200 Collagen. This modification significantly improved the results: tumor cells proliferated over time, and fibroblasts acquired the spindle-like morphology, transitioning from a spherical to an elongated shape. Furthermore, a cell-mediated modification of the structure was observed, with fibroblasts arranging themselves directionally over time, promoting the formation of a tumor-surrounding dense shell.

To better understand the mechanism underlying this phenomenon, a comparative experiment was conducted, replicating the model without the addition of MAF cells. An interesting behaviour was observed: in the presence of MAF cells, 4T1-GFP cells attempted to invade the stromal environment but were hindered by fibroblasts. Conversely, in the absence of MAF cells, 4T1-GFP cells migrated without any biological resistance. The replacement of GelMA C with Lifeink 200 Collagen promoted tumor cell migration, while fibroblasts played a key role in forming a physical and biological barrier.

In conclusion, this thesis demonstrated that the interaction between tumor and stromal compartments is strongly influenced by cell-matrix and cell-cell interactions.

The development of a bioprinted TME system of this kind is a significant advancement in the field, providing a fundamental tool for in vitro studies of tumour behaviour and establishing a solid foundation for investigating the interactions between the stromal and tumour microenvironments. While the model developed in this study replicates the microenvironment of a murine mammary carcinoma, the same techniques, incorporating specific cell lines, can be applied to other tumour types, thereby expanding the potential for use in various research contexts.

These results pave the way for future research aimed at better understanding the role of extracellular matrix components and cell-cell interactions in tumor progression.

In addition, 3D bioprinted TME models have the potential to go beyond biological understanding and could be developed into platforms for personalised drug testing in the future. Finally, a further improvement to enhance the predictive accuracy of chemotherapeutic regimes bioinks could be formulated using patient-derived ECM to more effectively recapitulate patient-specific tumor microenvironment.

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