8 Microfluidic-Driven Biofabrication and the Engineering of Cancer-Like Microenvironments

Carlos F. Guimarães, Luca Gasperini, and Rui L. Reis

Abstract

Despite considerable advances in cancer research and oncological treatments, the burden of the disease is still extremely high. While past research has been cancer cell centered, it is now clear that to understand tumors, the models that serve as a framework for research and therapeutic testing need to improve and integrate cancer microenvironment characteristics such as mechanics, architecture, and cell heterogeneity. Microfluidics is a powerful tool for biofabrication of cancer-relevant architectures given its capacity to manipulate cells and materials at very small dimensions and integrate varied living tissue characteristics. This chapter outlines the current microfluidic toolbox for fabricating living constructs, starting by explaining the varied configurations of 3D soft constructs microfluidics enables when used to process hydrogels. Then, we analyze the possibilities to control material flows and create space varying characteristics such as gradients or advanced 3D micro-architectures. Envisioning the trend to approach the complexity of tumor microenvironments also at higher dimensions, we discuss microfluidic-enabled 3D bioprinting and recent advances in that arena. Finally, we summarize the future possibilities for microfluidic biofabrication to tackle important challenges in cancer 3D modelling, including tools for the fast quantification of biological events toward data-driven and precision medicine approaches.

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8.1 Introduction

The past couple of decades have been marked by remarkable advances in the engineering of living tissues. More than ever, it is nowadays established that the architecture of biological tissues, their physical and mechanical characteristics, are important modulators of cellular responses and contribute to the overall tissue and organ functionality—to a similar extent to that of biochemical cues [1]. This notion applies to healthy tissues, but also diseased ones, such as cancer, where changes in the architecture of the tissue, extracellular matrix composition, and consequent mechanics play critical outcomes in cellular responses, from early proliferation to late metastasis [2]. Therefore, when attempting to approach and miniaturize living tissues for creating important research models, which are capable of recapitulating critical physiological responses, it is essential to reconstruct the tissue microenvironment and approach its 3D complexity to derive relevant responses, such as predicting the outcome of a certain drug in cancer cell invasion.

In biofabrication, a more recent area of the global tissue engineering field, there has been continuous development of technologies which allow for constructing complex cell/material structures with increasing level of detail and complexity. In the field of bioprinting, for example, the latest advances have enabled the creation of human-sized organ constructs such as the heart [3], or even other 3D structures in a matter of seconds [4]. Even though bioprinting presents an important advance to recreate tissues or even approach whole organs, it entails a resolution which is not yet fine enough to reproduce the intricacies of very fine biological environments, namely those of the cancer microenvironment [5–7]. Therein, differences at the single-cell level can be found, with a variety of cellular entities and extracellular matrix components, interacting in a very small niche, which gradually grows and evolves toward a more mature cancer tissue. As such, creating smaller structures that still encompass the 3D characteristics of cancer environments can take advantage of microfluidic technologies and their finer resolution capacities.

In microfluidic conditions, liquids such as hydrogel precursors flow in very small-sized channels, where turbulence is extremely low and thus fluids tend to maintain their trajectory without typically mixing. This characteristic can be employed to translate multiple precursor flow 3D configurations into hydrogel shapes by taking advantage of crosslinking precursors upon extrusion, using a varied toolbox of hydrogel crosslinking techniques [8–12]. By manipulating materials and cells at very small scales, microfluidics enables for a whole set of possibilities for biofabrication. Unlike typical on-chip technologies that attempt at recreating the physiology of tissues and organs within plastic chips in dynamic cultures, this

chapter will mostly explore microfluidics as a direct biofabrication tool to create independent structures with advanced 3D complexity at very small scales.

Starting with an overview of how microfluidic flows can be combined with hydrogel technologies to create soft, 3D structures that can approach the mechanics of living tissues and integrate cells, within structures such as cell-laden fibers, droplets, and combinations of such. Then, we will discuss how the quick and easy manipulation of different flows can be used to create space-varying compositional characteristics within fabricated structures, such as gradient-like transitions that can approach those transitions typically found in healthy and diseased tissues, or be leveraged toward high-throughput, single-sample screening approaches. After, we will discuss how flows alone can be used to create complex 3D architectures within microfluidic-biofabricated structures, from hundreds of micrometers to near single-cell dimensions, approaching the organization of several living microenvironments, namely those of cancer in early invasive stages. We will then explore how additional complexity can be obtained by combining different technologies with microfluidic biofabrication, namely bioprinting for the gradual assembly living constructs with complex shapes due to microfluidic-enabled manufacturing.

By providing an overview of the current microfluidic biofabrication toolbox, this chapter exposes the opportunities and current needs within the field of cancer-like environment engineering. We outline a clear set of strategies that can be used to imbue purely 3D, soft, microfabricated constructs with material and cellular architectures that can approach important characteristics of living cancers. By allowing to do so in fast, standardized, and affordable ways, microfluidic biofabrication is likely to grow in the next few years and overcome other technologies when cellular-level resolution is required, such as the recreation of truly physiological cancer microenvironment models.

8.2 Microfluidics: A Versatile Tool for 3D Hydrogel Processing

Microfluidic techniques are designed to manipulate liquids of various viscosities, and different available techniques are best suited to process liquids of different nature. When the liquid is a hydrogel precursor, typically a water-based solution of specific polymers, some conditions induce its sol–gel transition or, in other terms, the crosslinking of the dissolved polymers and hardening of the liquid into a hydrogel [13]. These conditions depend on the gel-forming solution and determine which is the most suitable microfluidic technique to process them into a 3D hydrogel. These gel-forming polymers are categorized, sometimes improperly, into two main families based on their sol–gel mechanism: those that form gels physically and those that form gels chemically.

The family of polymers that physically forms gels include those polymers that do so thanks to noncovalent (ionic and weak interactions) bonds between the polymeric chains. Alternatively, chemically crosslinked polymers form a gel by strong covalent bonds between the chains. Some physically crosslinked polymers are thermoresponsive polymers that rearrange due to temperature variation into

insoluble structures. Gelatin and collagen belong to this family, and the former presents an upper critical solution temperature above which gelatin and water are miscible and the gel is formed by cooling a warm solution below the critical temperature, which is around 35 °C [14]. Collagen, regardless of being the precursor of gelatin, form gels in the opposite way and presents a low critical solution temperature close to physiological conditions, above which forms a gel. The formation of gels by temperature variation can be a relatively slow process, especially when strong cooling or heating is not allowed [15]. This limitation is particularly evident when cells are present in the solution because of their sensibility to drastic variation in temperature that affects their viability. For this reason, polymers such as collagen and gelatin are well suited for droplet-based microfluidic techniques that allow the necessary time for them to harden.

With this technique, the droplet is formed thanks to a microfluidic setup that comprises a junction of two or more channels containing different phases. The water-based droplet-forming phase is forced into the hydrophobic continuous phase at the junction, then the shear stresses applied by the continuous phase break the stream of the water-based solution, forming a droplet. This process leads to high-throughput formation of highly monodisperse and separate droplets of liquids in an immiscible phase[16]. The outlet channel can be connected to a tube of variable length and can be treated at different temperatures than the temperature of the starting solutions. The separation of the droplet is ensured by the presence of surfactants that avoid coalescence, thus allowing enough time for the gel droplet to form, and then be collected by various means such as centrifugation or filtration. A similar technique is typically used to produce droplets of photo crosslinked polymers.

Photocrosslinkable polymers are chemically crosslinkable polymers containing functional groups along the backbone that are sensible to radical chemical reactions that form covalent bonds among the polymeric chains. These polymers can be obtained by chemical modification of natural polymers, such as hyaluronic acid [17] or can naturally have this characteristic, such as collagen using riboflavin as a photoinitiator [18]. Here, the microfluidic setup for the production of 3D hydrogels is similar to the one used for the thermoresponsive polymers because also this photocrosslinking process tends to be relatively slow [19]. In fact, a light of high intensity that makes the formation of gel quicker is not optimal because it could also harm cells. The droplet-forming solution contains a photoinitiator that forms a radical reactive species when exposed to light of a specific wavelength, so the outlet channel or tube where the droplet flow is exposed to light and the hardened droplets can be collected. Here, the material that makes the outlet should be transparent to the specific wavelength that excites the photoinitiator into the radical (e.g., fluorinated ethylene propylene for UV light). While droplets based techniques are optimal for thermoresponsive and photocrosslinkable polymers, they still require a careful selection of the materials used for the microfluidic system.

For the fabrication of hydrogel droplets, the system must be hydrophobic so that the oil can efficiently wet the channels of the microfluidic setup to avoid contact between the droplets and the system. The materials that make the system should be

compatible with the oil used, a suitable and well-performing surfactant should be present, be compatible with the oil, and should favor the formation of water droplets in oil. Considering two commonly used surfactants, for example, Tween and Span, the former is characterized by a high HLB number (Hydrophilic, Lipophilic Balance, is an index of the solubilizing properties of emulsifiers) thus favoring the formation of oil in water droplets, oppositely the latter has a low HLB number and favors the formation of water droplets in oil [20, 21].

Droplet techniques, due to their nature and in particular being emulsion-based, are challenging when used to process ionically crosslinkable materials, which represent an important family of biopolymers that include alginate and gellan gum. These polymers contain carboxyl groups along their backbone that carry a net negative charge. In the presence of positive ions, they form insoluble complexes due to the complexation of those groups that are responsible for the solubility of the polymer in water. Emulsion-based microfluidic techniques are less straightforward to employ with these polymers due to the challenges in using dissolved ions in these systems. The positive ions typically derive from the dissolution of salts in water that should be placed in contact with the polymeric solution to obtain the gel. To do this, it is possible to follow a more complex approach and fabricate two different droplets, one containing the polymer and the other containing the salts, which coalesce forming a gel before collection. Another approach is to use a hardening bath containing the dissolved salts. When the suspension of water droplets and oil reaches the bath, the droplets can separate from the oil due to differences in density so that they can reach the water solution containing the salts. During this process, the droplets must pass through the oil-hardening bath interface that acts as a barrier that can deform the droplets or can block them if the difference in density is limited. Overall droplet-based techniques are interesting approaches for the fabrication of 3D hydrogels that are highly monodispersed in size. The size can vary from submicron to some hundred microns based on the viscosity of the solution used, the size of the channels, the flow rates of the oil, and the gel-forming solution. Furthermore, given their round shape and sub-needle size, the hydrogels can be easily handled with a pipette and can be injected if needed. Moreover, recent advances in microfluidic droplet fabrication are even opening new possibilities for increasing their 3D complexity, such as the creation of inner architectures using airflow (Fig. 8.1) [22].

Other than droplets, microfluidic techniques can be used to produce fibers and those techniques are generally referred to as continuous flow microfluidics. These techniques are wet spinning techniques, where a microfluidic chip is used to extrude a polymeric solution into the hardening bath. These techniques are simpler than droplet-based techniques if applied to those materials that rapidly crosslink in the presence of a hardening bath, and for this reason, they are well suited to process ionically crosslinkable materials. Here, when the solution exits the chip, the surface in contact with the bath quickly crosslinks forming a fiber, which can then sink or float based on the density ratio between gel-forming solution and bath. The fiber can be collected easier than droplets because the oil is not present and post processing steps to remove it are not needed.

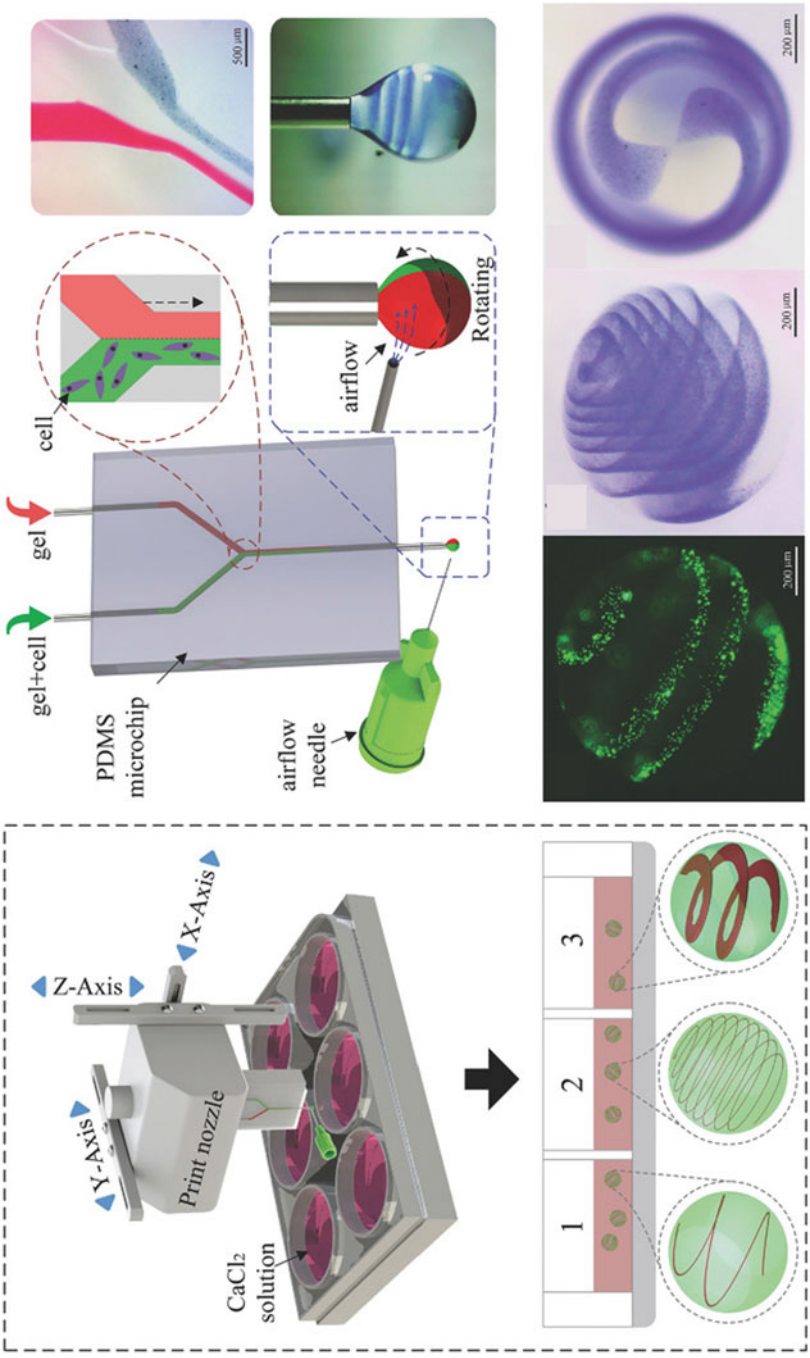

Fig. 8.1 Airflow-assisted generation of microfluidic droplets with controlled inner 3D architectures. Reprinted with permission from [22]

Oppositely to droplet techniques, continuous flow is challenging when used to process polymers that do not crosslink rapidly since the gel-forming solution does not have enough time to form the gel and ends up being dissolved into the bath. While droplet and continuous flow microfluidic are different, they share the same microfluidic concept related to how fluids behave in microchannels, that is, they flow in laminar conditions. In laminar conditions, fluids have a tendency not to mix so that it is possible to design a microfluidic chip with two inlets that meet at a junction, thus obtaining two fluids flowing side by side in the outlet. Similarly, it is possible to design a junction so that one fluid flows on the inner portion of the outlet and one on the outer part forming a coaxial flow [23].

More complex designs that encompass more fluids are possible, for example, obtaining two fluids side by side enwrapped in the third liquid. One or more fluids, e.g., the fluid generating the shell in the previous example, can be a polymeric solution that forms solid hydrogels in specific conditions. Those conditions can be triggered on-demand to solidify one or more of the streams to obtain outside the chip a micro-hydrogel with the same spatial distribution of the generating fluids. The outcome is a hydrogel fiber with different regions recognizable in its cross-section. The amount of space that these regions will occupy in the fiber is determined by the flow rate of the liquid they were made from. By changing the flow rate of the fluids, for example, one can obtain a coaxial fiber with a big core and a thin shell or a small core and a thick shell by inverting the initial flow rates. This size distribution can be obtained into a single fiber with a constant diameter using programmable flow pumps that linearly change the flow so that one side of the fiber can have a small core and the other side a bigger core with a thinner shell.

Finally, it is worth mentioning that certain approaches aim at combining discrete droplet generation with continuous hydrogel fiber spinning. A recent work has shown that GelMa droplets generated by oil–water separation could be integrated into a continuous stream of alginate, originating a hydrogel fiber with highly packed cellular spheroids (Fig. 8.2). This approach represents a very interesting alternative to fabricating single cancer cell droplets or spheroids, as the hydrogel fiber serves as a support for improving the manipulation of several droplets at once, making it easier for applying different culture treatments (e.g., anticancer drugs) as well as analyzing (e.g., fixing, staining, and imaging).

8.3 Microfluidic Real-Time Control of 3D Construct Composition

Using microfluidics-based techniques for processing hydrogel materials presents also unique opportunities to control the composition of constructs, namely, to obtain gradient-like distributions. Gradients are interesting architectures for tissue engineering and biofabrication for two main reasons. The first, is that biological tissues present natural gradients formed at interfaces such as tendon–bone or cartilage–bone interfaces [1], a characteristic which is very important when attempting to engineer, e.g., osteochondral tissues [25]. The second, is that gradients are able to integrate

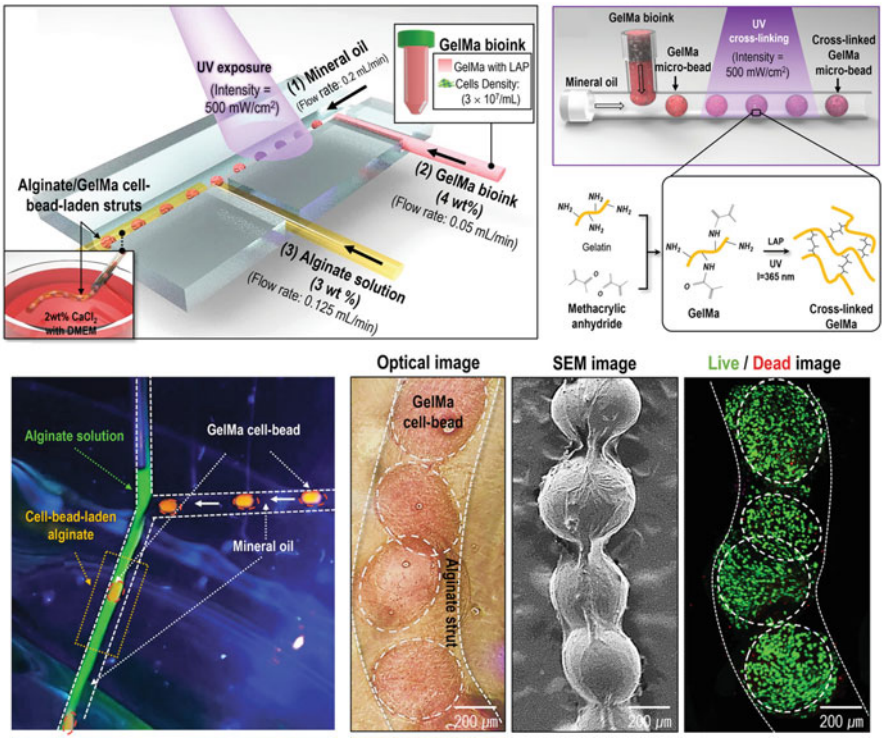

Fig. 8.2 The combination of microfluidic droplet generation and continuous flow fiber fabrication enables the creation of cell-bead-laden alginate fibers, which can transport highly cellular, packed spheroid-like structures within a single support fiber. Reprinted, with permission, from [24]

a full spectrum of conditions in a single sample, serving as very powerful platforms for high-throughput screening cell–material interactions [26]. In this regard, gradually changing material composition, cellular density, or ECM molecule distribution are important characteristics of 3D niches that must be optimized for engineering cancer microenvironments, in which microfluidic biofabrication has enabled unique advances.

For some time, microfluidic mixer chips have been used to manipulate liquid hydrogel precursors and establish gradients ranging between two extreme conditions, coupled with different crosslinking strategies, such as photopolymerization of PEG hydrogels [27]. Moreover, the manipulation of hydrogel precursors for gradient formation can be simultaneously combined with cell encapsulation in such chips, creating not just surfaces for cell adhesion but gradient-like 3D environments where cell responses can be studied, as a function of crosslinking density, polymer concentration, or even cellular density, in order to optimize the engineering of the tumor microenvironments [28]. In that work, researchers have shown that this platform was able to successfully present glioma cells with varying tumor microenvironment relevant characteristics, such as

extracellular matrix density, mechanics, and glioma cell density, where in situ analysis could be performed at the molecular and genomic levels, such as the expression of genes of interest (VEGF and HIF-1) or the secretion of MMPs. Even though these gradient-forming chips present interesting opportunities for library building and 3D biomaterial screening, the engineered environments are still confined to the chip and limited by its size and the possibilities for post-fabrication manipulation of the construct. Typically, these are also analyzed in a limited number (e.g., three or four) regions, which reduces the overall throughput of the technique. To overcome these limitations, researchers have developed techniques to fabricate 3D gradients using microfluidic biofabrication to derive individual out-of-chip constructs, which can be further manipulated and yield higher levels of throughput.

In such an approach, researchers have combined microfluidic-driven precursor mixing with wet spinning to fabricate cell-laden 3D hydrogel fibers with compositional gradients, which were able to integrate a gradient of 3D hydrogel stiffness, used to screen stem cell differentiation triggering, but representing an equally important characteristic to assess for cancer microenvironment engineering [29] (Fig. 8.3). In this work, the team has also demonstrated that a similar approach could be combined with multiple crosslinking stimuli to fabricate multi-material, multi-crosslinking gradients, where further responses ranging from adhesion to proliferation and mechanotransduction could be studied. In previous work, 3D hydrogel fibers were also shown to be interesting platforms for quickly engineering 3D tumor-like environments, where different cancer cell: macrophage ratios in proximity could be adjusted for mimicking different cancer stages [10].

Droplet-like microgels have also been explored as an alternative to continuous fibers for gradient fabrication. A team has demonstrated that droplets could be fabricated from different precursors using microfluidic water/oil emulsions and UV-crosslinking, then aggregating the resulting microgels to create patterns or gradients. By annealing the microgels, the researchers showed that a continuous 3D microgel scaffold could be deposited with gradients in stiffness or biodegradability. Mesenchymal stem cells were then cultured to screen their adhesion and morphology with the varying 3D microenvironmental characteristics [30] (Fig. 8.4). This approach may be further combined with recently developed microgel jamming and printing technologies [31], where 3D gradients can be assembled in more complex shapes for approaching the architectures of tissues or, for example, different compartments relevant to approach the cellular heterogeneity of the cancer microenvironment.

The cancer heterogeneity is not only related to architecture and mechanics but also to the presence of distinct cell populations and subpopulations, where varying numbers of cells and ratios between, e.g., cancer, stromal, and immune cells come into play as in any other functional organs [32]. In that regard, microfluidic-driven platforms have also been applied, albeit to a lower extent, to cellular and cell density gradient studies. By manipulating hydrogel precursors with suspension cells similarly to the previously discussed results, researchers have also demonstrated how intricate cellular niches, such as those of hematopoietic stem cells, can be studied in a high-throughput fashion, by creating cellular gradients ranging from pure

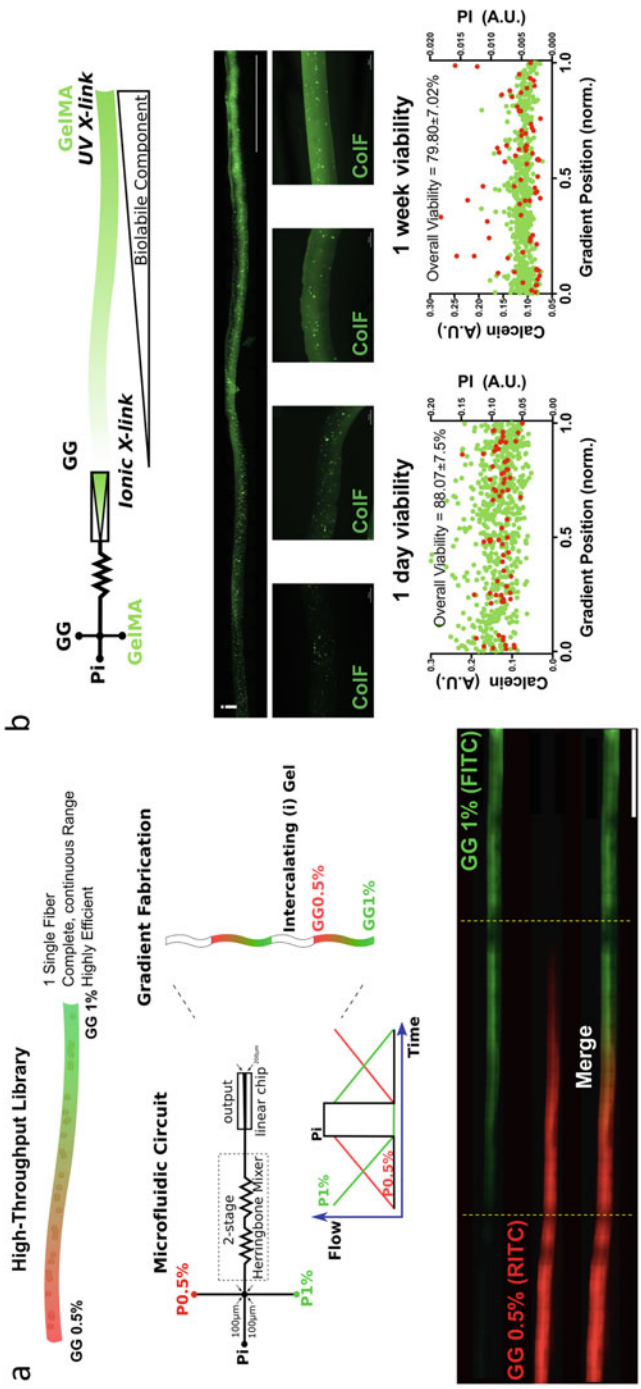

Fig. 8.3 High-throughput fabrication of 3D hydrogel fiber gradients with single (**a**) and multi-material (**b**) configurations for automated single-cell screening. Adapted with permission from [29]

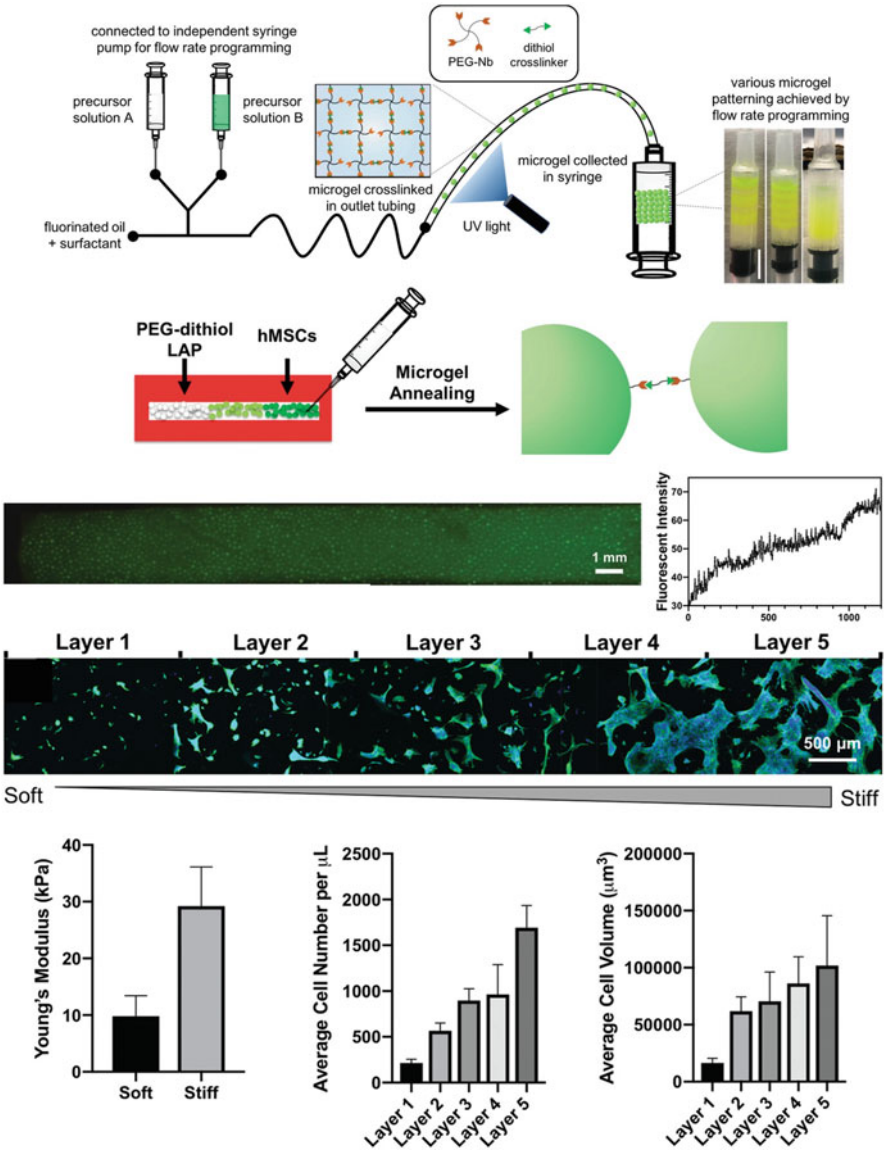

Fig. 8.4 Microfluidic-driven generation of hydrogel droplets and microgel annealing for creating 3D gradients suitable for cell culture and high-throughput screening. Adapted with permission from [30]

hematopoietic progenitor cells to pure osteoblast populations [33]. More recently, 3D gradients of vascular density were also fabricated via microfluidic mixing to study the effect of angiocrine cues on stem cell behavior [34]. Similar approaches can be leveraged to cancer microenvironment engineering, where the cancer cell

niche and cell–cell interactions can be studied and optimized quickly taking advantage of broad ranges of ratios between cancer cells, vasculature, stromal cells (e.g., cancer-associated fibroblasts), and immune cells (e.g., macrophages or T-cells).

Moreover, other types of cancer hallmarks can be approached in a high-throughput fashion for screening drugs or modelling physiologically relevant responses, such as hypoxia. Typically, cancers are characterized by highly dense environments with deficient vasculature, where certain regions of the tumor tissue experience low oxygen levels (hypoxia), which in turn lead to altered metabolism, and trigger signaling cascades such as those promoting angiogenesis [35]. Indeed, hypoxic conditions can alter the response and resistance of cancer cells to drugs, and the absence of such physiologically relevant parameters in 3D cancer models may lead to wrongful conclusions when testing new therapeutic strategies. Microfluidic chips and platforms present interesting opportunities to tackle this scenario by allowing for the creation of gradients also in oxygen concentration [36]. Researchers have shown that oxygen levels ranging from 0 to 20% could be obtained in a single chip, where cancer spheroids could be cultured in gradually changing levels of oxygen, demonstrating how varying oxygen levels could alter the metabolic activity of cancer and immune cells, as well as differences in the success of the anticancer drugs Doxorubicin and Tirapazamine [37]. The team demonstrated that lower oxygen levels (hypoxia) led to increased resistance to both drugs, highlighting the need for approaching physiological conditions when miniaturizing cancers for drug testing.

Lastly, it is important to refer that microfluidic-biofabricated gradients are not only important as fundamental and applied research tools, but these may also be more closely interfaced with clinical settings for patient-specific, personalized, and precision medicine approaches. A recent work has demonstrated how patient-derived tumor xenografts of glioblastoma could be integrated into gradients of brain-mimicking stiffness, showing how varying 3D mechanics affected cellular proliferation and, particularly, regions with increased stiffness led to increased resistance to the drug temozolomide [38]. Even though the work does not employ microfluidic techniques, it clearly demonstrates the importance of creating physiologically relevant platforms for assessing patient-derived cell responses to treatments, namely going beyond traditional 2D plates with nonphysiological stiffness. By further combining this knowledge with the high-throughput and speed of microfluidic-driven biofabrication, future platforms may enable a much faster and personalized approach to therapies, where patient cells can be quickly employed for in vitro therapeutical studies, also requiring lower amounts of cells and materials due to the unique microfluidic miniaturization capabilities.

8.3.1 Discrete Generation of Individual Microfluidic Segments

Even though the previous section focuses on continuous microfluidic structures, it is also important to discuss the possibility to create individualized segments within

microfluidic-fabricated structures, which can be separated by inert gaps and function as an array of 3D environments.

Indeed, the fluid tendency to not mix with microchips can be exploited to fabricate vertically segmented fibers using a microfluidic chip containing a junction. The junction can be a simple T or Y junction or can have a more advanced geometry able to connect more channels together. The junction is made in such a way that the fluids coming from different inlet channels can join in one common exit channel. By applying pressure on one or more channels, only the fluids from those channels will flow to the exit channel. By stopping the application of pressure on those channels the flow will stop. By pressurizing other channels new and different fluids will flow in the exit channel pushing forward the fluid already present in the exit channel. By repeating this process, the exit channel is filled with different fluids, such fluids do not mix (or with minimal mixing) so that different compartments along the path of the exit channel can be identified. After this, some or all the fluids composing the segments of interest are hardened so that the compartments can keep separated. The segments may not be perfectly shaped cylinders due to the rheological nature of the generating fluids. Fluids that behave as Newtonian fluids develop a parabolic speed profile inside the channel and as such the segment generated by these fluids may have a parabolic profile at the bases [39]. Oppositely, a fluid following a non-Newtonian power law model develops a different speed profile-forming cylinders with a flatter base in the middle and a parabolic profile on the sides. The final product of this technique is a fiber composed of segments that can be composed of different materials.

This feature can be exploited to obtain single segments, making this technique an oil-less alternative approach to droplet microfluidic. The advantage of this technique is the absence of oil that simplifies the fabrication (see Sect. 2) and cylinders are formed rather than spherical objects. Spheres are the geometrical shape that includes the highest amount of mass in the lowest amount of surface and this is how water-based droplets minimize the surface in contact with the oil. Oppositely, cylinders present a higher surface-to-volume ratio, which favors the diffusion of nutrients and metabolites when cells are encapsulated [40]. To obtain single segments there are two main approaches that can be followed: the use of a sacrificial gelling agent or the use of a non-gelling agent as one of the segments. In the first case, a fiber is formed and a sacrificial gelling agent can be degraded and removed. One example is a fiber composed of gellan gum and alginate segments, where the alginate can be removed by enzymatically accelerated degradation using alginase or by using chelating agents that do not affect gellan gum [41, 42]. Considering the second case, segments are formed directly by using any solution with a similar viscosity that one of the segments of interest that does not form gels in the hardening bath, such as hyaluronic acid. When extruded, the non-gelling phase dissolves in the hardening bath while the gelling phase hardens forming cylindrical gels that can be collected for further use.

8.4 Microfluidic Flow-Based Generation of 3D and Cancer-Like Architectures

As initially outlined, the shapes and architectures present within living tissues are as important as their bulk composition and play an important role in the consequent mechanical properties and biological events. Characteristics such as ECM orientation, tissue anisotropy, and the presence of multiple compartments with different cellular and ECM compositions are critical to approach and model living tissues [1]. In 2D surfaces, oriented topographies and their impact on cellular behavior have been explored for a long time [43–45], but their translation to 3D systems is not so straightforward. Typically, to introduce orientation and shape in 3D hydrogels, there is a need to use composite systems where nanoparticles [46] or microgels [47] are aligned using externally-driven methodologies such as magnetic fields, to create 3D orientation and introduce shape control in hydrogels. Alternatively, the process of hydrogel crosslinking can also be combined with the manipulation of ice crystal formation to induce a certain degree of control in pore dimension and orientation [48]. Even though the discussed technologies present high versatility and can be employed for introducing 3D shapes and topographies within constructs, these require multiple steps and component manipulation to implement and control structure within the fabricated hydrogels. However, recent studies have demonstrated that by taking advantage of microfluidic flows alone, it is possible to create organization within hydrogel precursors pre-crosslinking, which, if maintained upon crosslinking, can lead to varied and interesting architectures at very small dimensions.

One interesting approach is that of leveraging chaotic hydrogel flows with different precursors mixing and swirling together due to the presence of helical elements in microfluidic channels within a print head [49, 50]. Researchers have demonstrated how this approach could combine continuous, high-throughput wet spinning with the orientation of separate compartments of different hydrogel precursors (between alginate and alginate-gelatin methacryloyl (GelMA) blends), resulting in 3D hydrogel microfibers with intrinsic 3D architectures, generated by flows alone and without the need for any additional entities (Fig. 8.5). The researchers demonstrated that chaotic flows enabled spinning fibers with incremental numbers of semi-parallel GelMA filaments within alginate ones, and these 3D hydrogel pockets were single handedly capable of promoting muscle cell alignment and muscle fiber-like maturation [50]. Previously, the team also applied a similar strategy of chaotic flows to create densely packed cellular structures, enabling, e.g., the creation of constructs where different degrees of intimacy between cancer cells and healthy cells could be obtained [51]. The combination of both concepts presents interesting opportunities for the high-throughput fabrication of fibers where multiple compartments can mimic the interaction between cancer cells and other microenvironment entities, approaching and miniaturizing important processes and providing very interesting platforms for therapy testing.

Indeed, the creation of hydrogel microfibers is a particularly powerful approach for cancer modelling. Using less chaotic, more organized 3D flow-focusing hydrogel biofabrication, it was also demonstrated how a single microfluidic setup could be

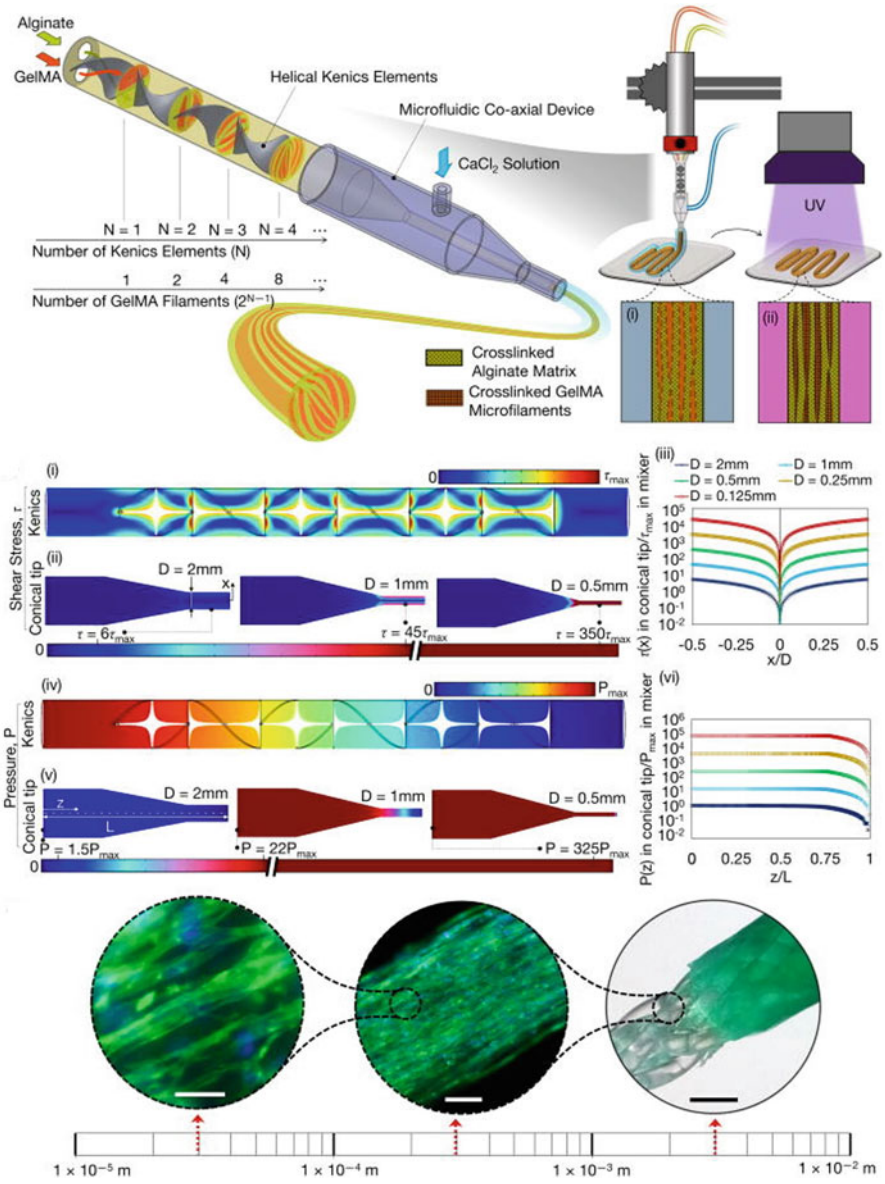

Fig. 8.5 Chaotic generation of hydrogel fiber architectures with hydrogel compartmentalization and 3D cell/hydrogel alignment. Reprinted with permission from [50]

employed together with the tuning of hydrogel precursor viscosity and the organization of flows within the chip, to create a plethora of multi-compartment hydrogel fibers with very small dimensions down to sub-50 μm diameters [52] (Fig. 8.6a). These unique microfibers were validated as suitable platforms to mimic and miniaturize important biological organizations, namely those present in cancer.

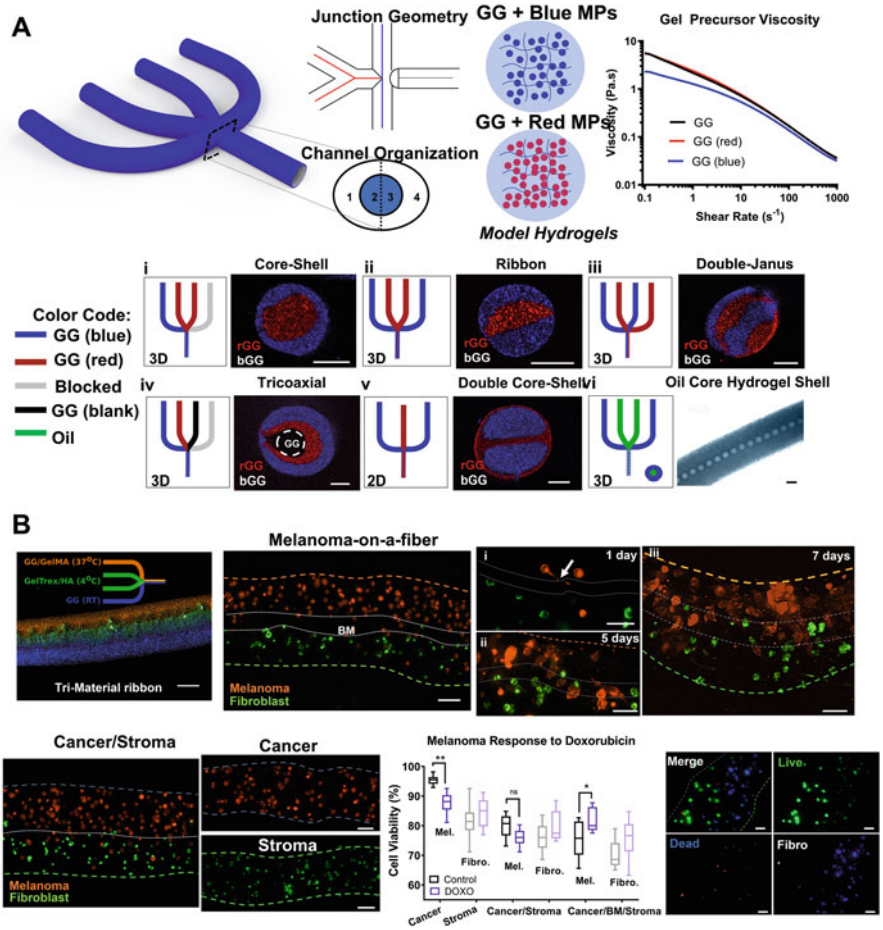

Fig. 8.6 3D Flow-focusing hydrogel biofabrication of multi-architecture hydrogel microfibers (**a**) and ribbon-like engineering of melanoma-on-a-fiber models (**b**). Adapted with permission from [52]

By exploring a specific shape, called ribbon-like, the team demonstrated that cancer/basement membrane/stroma compartments could be organized in a parallel manner, encapsulating melanoma cells in the cancer compartment, fibroblasts in the stromal one, and a thin basement membrane separating both, recreating the first steps of cancer cell invasion, potentially preceding metastasis (Fig. 8.6b). The results demonstrated that the basement membrane invasion started as early as 1-day post-fabrication, becoming more and more evident as the cancer cells overtook the construct invading toward the stroma. Furthermore, the team showed that the complexity of the 3D construct directly affected the outcome of cancer cell responses to doxorubicin, as their resistance to the drug increased with higher model complexity. These results evidence how the recapitulation of tumor microenvironments in

their multiple dimensions is important to obtain relevant responses in in vivo cancer models [52].

Moreover, it is also important to consider that modelling the 3D complexity of cancer microenvironments needs to be coupled with advances that enable the efficient analysis of ongoing biological processes, to derive clear and quantifiable data that can be used for next generation testing platforms and precision medicine approaches. In this regard, microfluidic biofabrication and microfiber compartment architectures may also present exciting opportunities by interfacing the engineering of 3D microtissues with advances in hydrogel optical fibers [53–56]. Very recent work has demonstrated that cytocompatible, polysaccharide-based hydrogel fibers could take advantage of co-centrical layers in order to clad a cell-laden fiber core with lower refractive index layers, to transport and maintain cancer cells while simultaneously enabling the guiding of light [57] (Fig. 8.7a). In these living optical fibers, the team demonstrated that light-cell interactions could transport information regarding cellular events, such as metabolic activity, proliferation, and protein expression. By taking advantage of this process, the study demonstrated how the growth of cancer fibroids (fiber-like organoids) could be tracked over time via fast, nondestructive optical analysis, directly converting the complex process of cancer 3D proliferation to directly quantifiable optical data. This quantification was then leveraged to quickly screen and identify inhibitory thresholds of the anticancer drug cisplatin, easily pinpointing the concentration level at which the drug successfully inhibited 3D cancer growth (Fig. 8.7b). The capacity to perform the digitalization of biological events presents exciting new avenues for the generation of biological data from cancer in vitro 3D models and paves the way for faster personalized medicine testing and precision, data-driven approaches.

Overall, microfluidic biofabrication and, particularly, the continuous, high-throughput spinning of hydrogel fibers has presented very interesting technological advances, ranging from the creation miniaturized microenvironments, with living tissue-like architectures, to the tackling of new challenges in the conversion of biological events into quantifiable data. Even though these hydrogel structures have evolved to integrate significant complexity within single fibers, the combination of microfluidic biofabrication with bottom-up, additive manufacturing approaches such as bioprinting, presents further possibilities for increasing dimensions, and obtaining further complex biological constructs and models, as discussed ahead.

8.5 Microfluidic-Enabled Bioprinting

So far, we have been discussing the possibilities for microfluidic-fabricated structures such as fibers or droplets to integrate a broad arrange of characteristics encompassing important materials, shapes, and cues within inner architectures that can mimic relevant biological environments. Even though these constructs can be seen as the final model, they can also serve as building blocks, which can further be assembled toward larger, more complex 3D structures. In particular, this process can

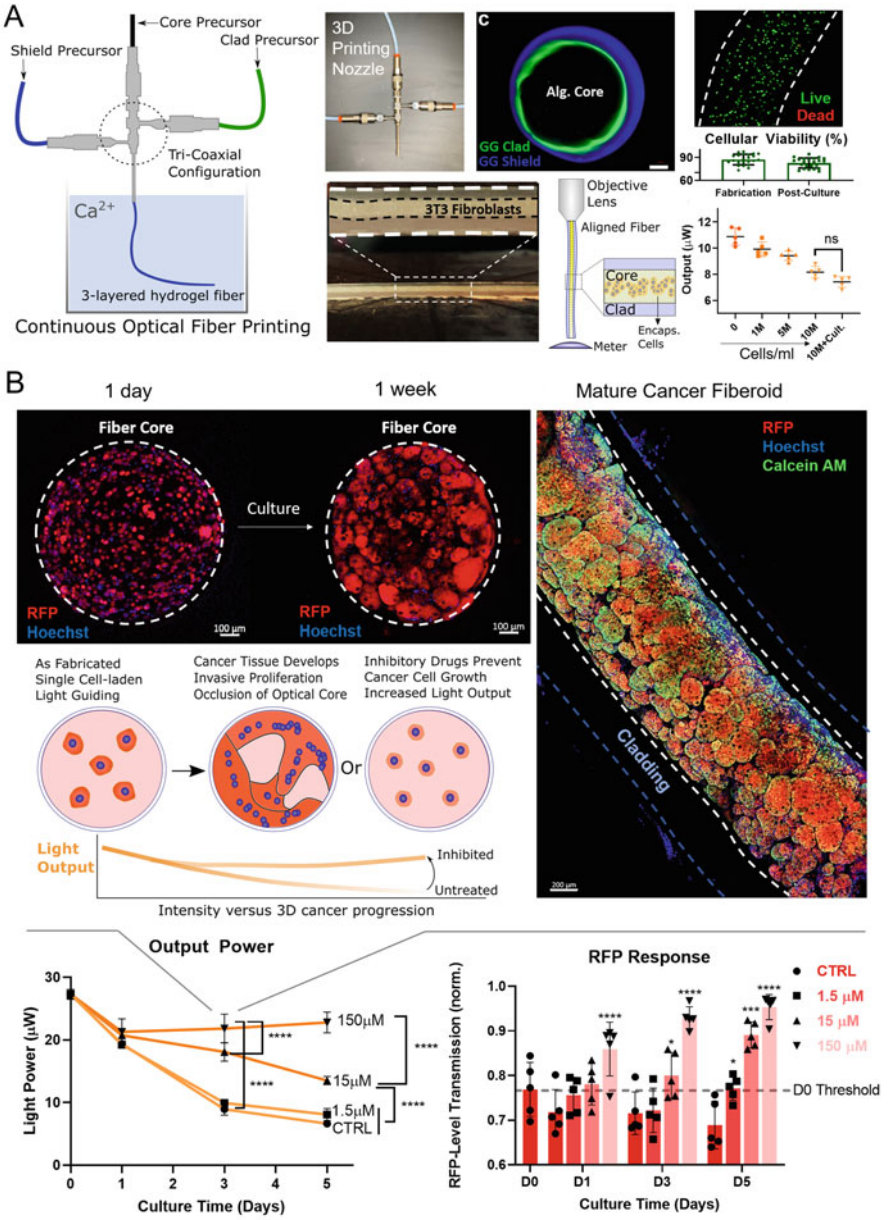

Fig. 8.7 Biofabrication of living optical fibers based on multi-layered polysaccharide hydrogel fibers (**a**), and the conversion of 3D cancer fibroid growth to directly quantifiable optical data for drug inhibitory threshold discovery (**b**). Adapted with permission from [57]

be approached by leveraging 3D bioprinting principles to deposit and assemble microfluidic biofabricated building blocks [58, 59].

An earlier example is that of using microfluidics to obtain two side-by-side, non-mixing flows, printing them as single fibers with two compartments, frequently named as Janus Fibers. This type of structure, unlike single-composition fibers, enables close contact between, e.g., different cell populations [60]. For example, researchers 3D printed constructs with dual fibers containing fibroblasts and muscle cells, each in a different Janus-fiber compartment, assembling the construct and demonstrating improved in vivo integration when compared to a uniform hydrogel construct [61]. With the advances in microfluidic-driven bioprinter devices, the dual inlet chips evolved to more complex configurations, where multiple materials can be controlled as well as their crosslinking, employing independent channel pressures [62]. This type of approach has been explored to create 3D muscle tissue models that responded physiologically to a variety of biochemical stimuli [63]. Similarly, microfluidic bioprinting was used to create renal models, where core-shell configurations could be manufactured to approach renal tubules, with dimension and compartment size resolutions which are typically hard to approach with classical 3D printing nozzles [62]. These technological advances may further improve previously reported multi-material 3D printing approaches, where a single nozzle connected to a variety of hydrogel precursors can be controlled to alternate between deposited material on-demand, enabling, e.g., the creation of vascularized 3D models, which would be very interesting for approaching cancer tissue vascularization modelling [64]. More recently, the integration of microfluidic-fabricated microgels within hydrogel inks has also been demonstrated as an interesting approach to obtain heterogeneous constructs with pockets of cell-laden hydrogels surrounded by an environment of a different bioink [65]. Even though the authors did not focus on cancer applications, this strategy can be very interesting to obtain micro-tumors surrounded by a distinct cellular environment in a biphasic composition that can be printed in an arbitrary shape (Fig. 8.8).

Other than taking advantage of microfluidics to create complex, multi-compartment, but continuous fiber composition, the field of bioprinting has recently explored the capacities to obtain space-varying compositions. Microfluidics has been used to combine and mix different inputs, timing it with the 3D bioprinting deposition to obtain not only single fiber gradients but gradual composition changes in whole 3D printed constructs. By developing a custom print head where a coaxial extrusion nozzle received material from a passive microfluidic mixer, which was connected to the inlets, researchers have shown how different bioinks could be deposited individually or together at the same time. By uniformly mixing inlet material before extrusion, the researchers were able to deposit layers with gradually changing composition toward approaching the osteochondral (bone cartilage) transition [66]. In a more recent work, a similar concept was explored where a custom setup connected different material inlets to a chaotic mixer, and then to an outlet [67]. Researchers demonstrated how light-based crosslinking could then be used to crosslink multiple hydrogel layers that could contain intricate gradients of composition, in different shapes (Fig. 8.9). The team then used this process to fabricate 3D

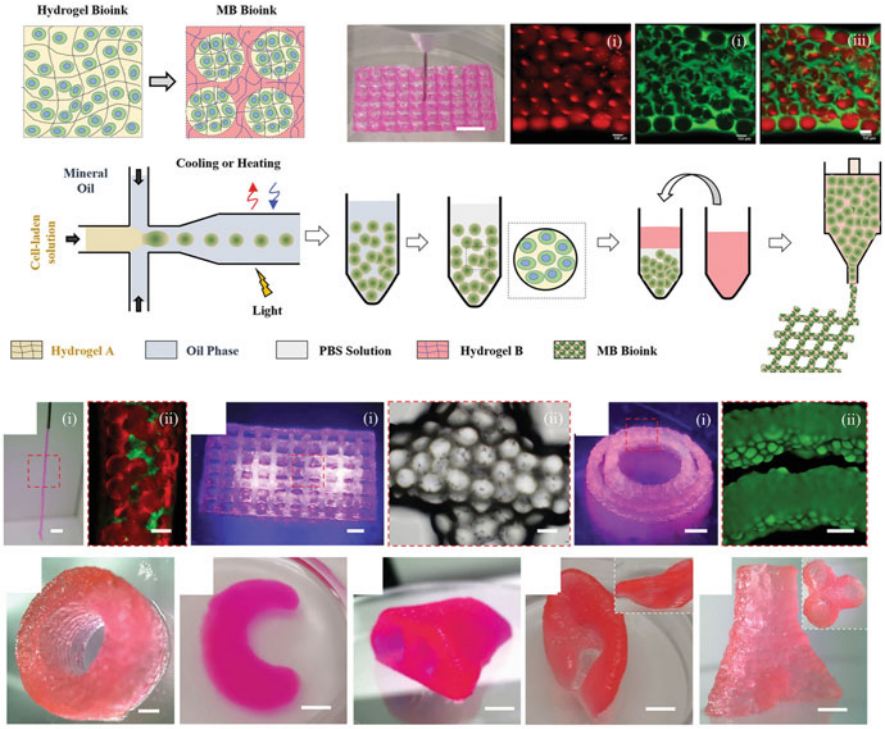

Fig. 8.8 3D Printing of heterogeneous bioinks via the combination of microfluidic biofabricated microgels within a uniform bioink blend for the integration of 3D cell-laden hydrogel depots within bioprinted constructs. Adapted with permission from [65]

cancer cell density models, where the number of cancer cells decreased radially from the center, approaching the dense characteristic of highly hypoxic tumor centers. They also demonstrated the capacity to create complex vascular structures with gradually changing configuration and channel dimensions (Fig. 8.9). The combination of both types of models would be also extremely relevant to approach cancer vascularization and the hypoxic dynamics behind angiogenesis, blood vessel growth, and potential metastatic disease.

Another interesting combination at the interface of microfluidics, bioprinting, and biofabrication, is the creation of intricate 3D architectures within chips. This can be approached through different manners, namely by bioprinting structures directly inside microfluidic chips, such as vascular channels that can then be perfused in dynamic culture conditions, among other examples [68]. However, bioprinting directly within a microfluidic chip presents limitations, as either the printing resolution is not fine enough to create complex microfluidic architectures or, alternatively, the resulting chip presents very large dimensions and diverges from the main purposes of having a microfluidic setting. To overcome this, researchers have also

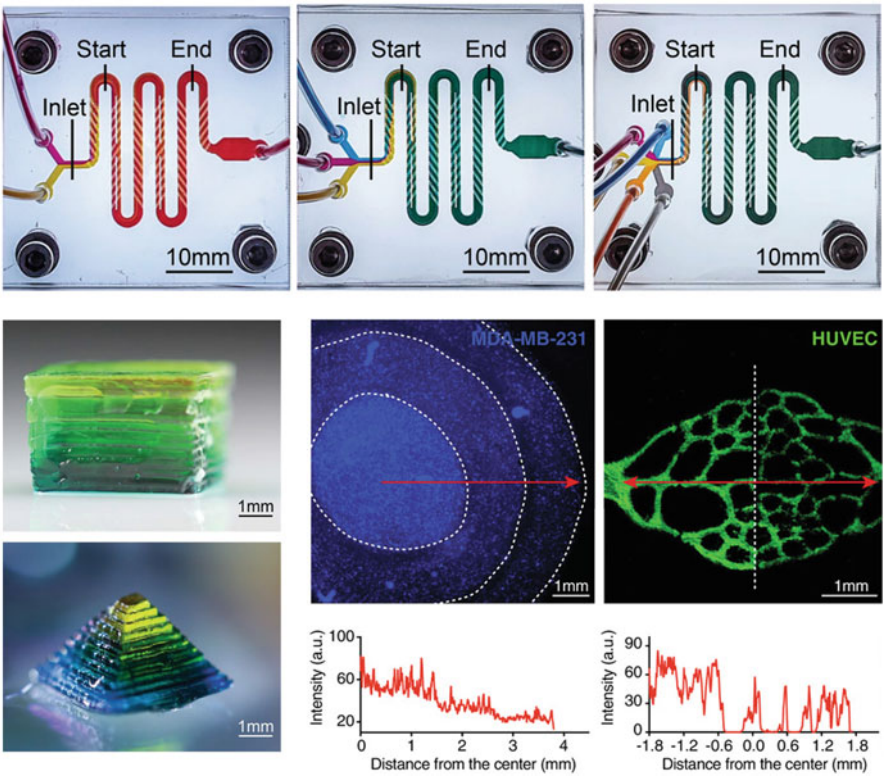

Fig. 8.9 Microfluidic-enabled bioprinting for the assembly of complex 3D constructs with gradients in layer composition, and their application to density-varying breast cancer and complex vascular networks biofabrication. Adapted with permission from [67]

developed maskless lithography, exploring the way materials flow within microfluidic conditions, and then "locking" them in a particular 3D configuration using light-based crosslinking [69]. Among other applications, this approach allowed for the creation of small tumor environments with a randomly distributed vascular-like network, all limited to an area of around 10 mm^2, which represents a very small size within which relevant 3D shapes could be miniaturized without the restraints of having to physically print them.

Overall, microfluidics has enabled very interesting advances in the field of 3D bioprinting by allowing the controlled deposition of different fiber shapes, as well as quickly altering between different inks in real printing time to create gradients and approach important transitions of living tissues both within health and disease contexts. It will be interesting to see how some of these advances come together soon, e.g., how the combination of printed 3D cancer models may incorporate the advances in printing of blood vessel networks to recapitulate important events behind metastatic disease, which represents the highest disease burden scenario of

most cancers. Similarly, the creation of more complex, multi-cellular constructs may provide important platforms to understand the complex cancer microenvironment crosstalk, as well as model the effect of next generation, microenvironment-disruptive therapeutics.

8.6 Conclusions

Microfluidic techniques present unique opportunities to miniaturize important characteristics of biological environments in fabricated structures, ranging from individual droplets to continuous fibers. The capacity to manipulate hydrogel precursors as liquids within low turbulence settings, allied with the broad toolbox that exists in hydrogel crosslinking on-demand, microfluidic biofabrication is primed to lead the field of biofabrication at the smallest of scales and highest level of 3D resolution. Indeed, microfluidic biofabrication has enabled important advances in the miniaturization of multi-compartment 3D constructs, as well as the particularly important space-varying composition creation, either from a high-throughput screening perspective or to simply recapitulate the complexity of living tissues.

In the specific case of cancer, the complexity of the diseased tissue and its similarity to an organ on its own requires a paradigmatic shift in the way it is modelled in vitro, namely to integrate the multi-cellular dynamics of the microenvironment, as well as the ECM characteristics such as the typical fibrotic responses [70]. In this regard, microfluidic biofabrication has enabled important breakthroughs in the creation of complex multi-cellular, multi-material, and multi-compartment 3D architectures which can enable, e.g., closely monitoring cancer/stroma and basement membrane invasion dynamics. Indeed, the creation of 3D shapes within structures such as hydrogel fibers represents important advances to model 3D cancer environments, but a further challenge remains: the way to translate ongoing biological events into quantifiable, comparable data.

Indeed, models are only useful if their complexity can be matched by means to extract data, where advances such as the integration of optical, electrical, thermal, and similar means of analyzing biological constructs such as 3D hydrogel fibers [57] or spheroids and organoids [71] will play an ever-growing role in future in vitro models. As the ways to analyze engineered constructs evolve, so does the amount of data that can be generated in brief amounts of time. This data will create unique opportunities for mining, analyzing, and creating large 3D biology model databases, where its interface with machine learning and other artificial intelligence algorithms may expand in silico modelling informed on 3D in vitro constructs. Simultaneously, those technologies can also be explored to drive the optimization of microfluidic biofabrication parameters, resulting in improved models, and so on and so forth in successive synergistic iterations [72, 73].

Finally, the combination of microfluidic biofabrication with approaches that typically function at slightly different dimensions, such as 3D bioprinting, is also providing important advances where the powerful real-time material manipulation via microfluidics can be combined with 3D material deposition to create larger

constructs. Thus, characteristics such as multi-compartment fibers and space-varying compositions can be translated to 3D printed constructs. This area can still evolve toward the unique combination of multiple structures, such as cancer hypoxic 3D environments and bioprinted vascular beds, leading to a significant step forward in the understanding of multi-entity events, such as those involved in metastatic disease [74]. Furthermore, it is still important to mention that microfluidic fabrication has also been employed for some time at smaller dimensions, namely for the fast fabrication of nanoparticles of different dimensions for drug delivery purposes [70, 75, 76]. Even if not so straightforward, it would be interesting to see advances where microfluidic nanosynthesis could be combined with biofabrication platforms to assess, for example, the interaction and distribution of nanoparticles in complex 3D cancer models as well as their therapeutic efficacy. These models are primed to partially replace in vivo animal studies while remaining closer to human physiology using human-derived cell sources.

After decades of cancer research and the development of anticancer therapeutics, the societal burden of the disease is still among the highest, and several cancers are extremely hard to treat, especially those undergoing metastasis. Uncovering the intricate 3D complexity of the disease and the multi-entity interactions that contribute to the development and prognostics of cancer may likely hold the key for next generation therapeutics. In this context, the unique capacity of microfluidic biofabrication to miniaturize 3D biological environments in high-throughput fabrication and analysis setups is primed to open new avenues for cancer research by enabling unprecedentedly complex, easily adaptable models. Combining these models with tools for the direct quantification of biological events and data analysis is likely to unlock a whole new frontier in precision, data-driven cancer research, and medicine.

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