Microfluidic Tissue Engineering and Bio-Actuation

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Bio-hybrid technologies aim to replicate the unique capabilities of biological systems that could surpass advanced artificial technologies. Soft bio-hybrid robots consist of synthetic and living materials and have the potential to self-assemble, regenerate, work autonomously, and interact safely with other species and the environment. Cells require a sufficient exchange of nutrients and gases, which is guaranteed by convection and diffusive transport through liquid media. The functional development and long-term survival of biological tissues in vitro can be improved by dynamic flow culture, but only microfluidic flow control can develop tissue with fine structuring and regulation at the microscale. Full control of tissue growth at the microscale will eventually lead to functional macroscale constructs, which are needed as the biological component of soft bio-hybrid technologies. This review summarizes recent progress in microfluidic techniques to engineer biological tissues, focusing on the use of muscle cells for robotic bio-actuation. Moreover, the instances in which bio-actuation technologies greatly benefit from fusion with microfluidics are highlighted, which include: the microfabrication of matrices, biomimicry of cell microenvironments, tissue maturation, perfusion, and vascularization.

1. Introduction

Over the last few decades, nature has inspired scientists to develop a multitude of advanced materials that have been useful in the realization of dynamic systems, like motile machines and robots. By observing active mechanisms in biology, it has been possible to design and generate technologies that partially or fully comprise compliant and deformable materials.[1] These technologies will promote the evolution of soft machines that are able to interact safely with natural environments and humans, and to adapt effectively to complex, dynamic situations. Such soft machines have been constructed and programmed to achieve different motion abilities and undertake various tasks.[2,3]

Despite the rising interest in soft technologies, inanimate materials cannot efficiently replicate the structural and functional complexity of biological systems, which consist of living cells that display an exceptional level of functional integration. In fact, a single cell is endowed with intrinsic mechanisms for actuation, sensing, repair, communication, information, energy storage, and energy use. As such, even bioinspired soft robots cannot fully replicate living beings’ dynamics or physiological functions (e.g., environmental perception, communication, self-healing, and energy conversion). This issue could be addressed by directly incorporating living entities (cells and tissues) into man-made systems to generate bio-hybrid technologies. Robots that arise from the synergetic combination of living entities and functional deformable materials (which are typically used in soft robotics) are then referred to as ‘bio-hybrid robots’ (or ‘bio-robots’).[4,5] In these systems, biological modules perform specific functions in the machine. This specialization renders the robot’s performance and behavior more similar to that of living creatures.[6,7] In addition, bio-hybrid machines inherit the properties of living materials, which can contribute to their compliant environmental integration (such as reactivity to external stimuli and adaptation to environmental variations, as well as, degradability and biocompatibility).

Nevertheless, there are several technical challenges in using living cells as engineering materials. One major issue is that the cells need an appropriate environment to survive and be functional. So far, scientists have been able to maintain cells in a cell culture environment using fluids with a specific nutritive and gaseous content.[8] However, conventional static cell culture can only mimic the features of certain biological microenvironments (such as, the vascular beds or biological barriers) to a limited extent. Consequently, systems have been developed to culture cells under dynamic fluidic conditions, with the aim of mimicking the dynamics of biological fluids, such as, the interstitial fluid, blood, and cerebrospinal fluid.[9–12]

Microfluidics is a versatile technology that arises from the technological synergy between micro-electromechanical systems and fluidic mechanics. This technology allows one to convey, separate, or otherwise process fluids with high spatiotemporal resolution.[11,13,14] By using micro-pneumatic systems (like liquid pumps, gas valves, etc.) and microfluidic structures (chips), one can precisely control fluid volumes in the range of nanoliters to picoliters. Such precision control has led to...
tremendous advancements in several research areas, particularly in environmental, manufacturing, and biological sciences. Notably, microfluidics has been applied to inkjet printing, air and water quality control, biomolecular analysis, and personalized medicine. In the micro-domain, fluids behave differently, as physical microfluid states are dominated by surface tension, energy dissipation, and fluidic resistance. Microfluidics aims not only to understand how fluidic microsystems diverge in their behavior from fluids considered in larger domains, but also to exploit these peculiarities for new uses. In this sense, microfluidics supports miniaturization, as microfluidic devices aim to reduce large systems to micro-scaled models while keeping the same complexity. As a result of its miniaturization potential, microfluidics is often applied to lab-on-a-chip technologies, such as devices in which cells or microscaled tissues are grown in dynamic flow culture.

In the context of bio-hybrid machines, microfluidics could be used to enhance the development and performance of their biological phase. As it has been extensively demonstrated in 3D cell models, microfluidics allows scientists to investigate cellular processes and microenvironments. For example, a multitude of 3D hydrogel-based microfluidic models have been developed to create more in vitro physiological biomimetics for the study of cell differentiation, signaling, and migration. The approaches for fabrication and maturation of 3D tissue models within microfluidic platforms substantially contributed to the engineering of tissues with high structural and functional biomimicry. Due to this potential, microfluidics might be able to synergize with the 3D engineered tissue in bio-hybrid robots by sustaining and enhancing their survival and functions (Figure 1). Importantly, microfluidics converges with the engineering of biological tissue due to its various capabilities (Figure 1). In fact, microfluidics enables fluid perfusion/flow in microchannels, while tuning the biochemical gradients, biomechanically stimulating the cells, and culturing various types of them in one platform. In the future, this ability will allow for a highly biofidelic replication of the cell microenvironments, which will result in better tissue generation and maturation in vitro. Microfluidics can also be used to produce micro-engineered tissues. Since this method of tissue manufacturing is highly reproducible and continuous, it could be used for high throughput productions of biological building blocks. One advantage of this would be that the assembly of such blocks might generate larger tissue constructs with a high level of structural and cellular complexity. Furthermore, microfluidics might be able to control, with microprecision, the fluidic dynamics that are involved in culturing large tissue volumes, ensuring fine control over cell survival and a long lifespan of whole constructs in vitro.

These achievements in tissue engineering would greatly advance bio-hybrid robotics. The first results in this direction will most likely be reported in the field of bio-actuation. Actuation is the robotic function that accounts for most examples of biological realization, and several bio-actuators that consist of cells with specialized contractility, namely, muscle cells, are already available. These bio-actuators have exhibited motion and locomotion capabilities, both of which are useful to robotics. Various designs for bio-actuators have been reported (Figure 1), which include micro-actuators incorporating small cell clusters or large bio-hybrid devices integrated with functional muscle tissue. The living muscle component is combined with flexible synthetic materials of diverse morphology to create planar sheet-like actuators, rolled linear actuators, or 3D tissue actuators kept under tension while hanging from supporting pillars.

The present review aims to survey the contribution of microfluidics to tissue engineering and to describe bio-actuation for soft robotics and biomedical applications, in order to highlight the instances in which bio-actuation could prosper thanks to microfluidic approaches. These areas of interest refer to the control of the fluids in multicellular assemblies and engineered tissues, and they include: Microfabrication; fluidic mimicry for cell stimulation and replication of cell microenvironments; tissue perfusion and vascularization. First, we will briefly introduce the reader to the convergence between microfluidics and soft robotics. Then, we will discuss the contribution of microfluidics to tissue engineering, especially in relation to the simulation of the cell microenvironment; tissue manufacturing; and implementation of vascularization and perfusion systems. Furthermore, we will report on the recent progress in the microfluidic culture of muscle cell-based constructs that can be used as bio-actuators. Finally, we will anticipate the most promising directions for future research in microfluidic culture of functional muscle tissue.

2. Microfluidics in Soft Robotics

Over the years, microfluidics has found applications in various fields, and very recently its synergistic combination with soft robotics has also been proposed. In soft robotic systems, microfluidics could transport and deliver vital reagents and fuels. In addition, the fluid dynamics in microfluidic systems can cause the physical deformation of soft structures with a high spatial resolution, thus improving their control during motion. In fact, microfluidics enabled actuation systems in which pneumatic pressure can be applied and regulated through microlevel control. As opposed to most microfluidic applications, where the geometries of the microchannels ideally remain steady during operation, microfluidic soft actuators should be based on microchannels that are inherently dynamic. In addition, microfluidic control can elicit a fine-grain deformation of compliant and deformable materials that constitute dynamic machines like soft robots. Recently, soft robots with bio-inspired designs controlled with microfluidic circuits have been realized. The first autonomous robot composed solely of soft materials had an octopus-inspired design and was powered by a chemical reaction regulated through an integrated microfluidic platform. Briefly, a microfluidic logic, fabricated via soft lithography, was used to autonomously control the fluid flow and the catalytic decomposition of an on-board propellant fuel supply. The gas generated from the fuel decomposition was inflated in the fluidic network downstream of the reaction sites and enabled the robot actuation. In such a work, microfluidics showed promise for the development of onboard configurations and power control in soft robots. Later, another group proposed a spider-shaped robot made out of several layers of silicone elastomer and built...
through a soft lithography technique. Each layer was then cut and bonded together to create a monolithic structure representing the robot body in its entirety. Importantly, a network of vessel-like, hollow microfluidic channels was embedded and interwoven into the layers. By pumping liquid or air, the channels worked as actuators that caused structural deformations of the robot in an origami-like folding process. A permanent deformation was achieved by injecting a photo-curable resin and then cross-linking it with UV light. This work highlighted the potential of microfluidics in providing soft actuator structures that can be used for reversible or permanent deformations. Interestingly, in another case, microfluidics was used to imbibe sensorial abilities in soft robots. Modeling via finite-element method and experimental observations instructed the integration of sensing in soft robots with microfluidic networks that were characterized by dynamic channel geometries of predictable dimensions. In fact, since the microchannel dimensions in microfluidic soft robots change during operation, the properties of the fluid transport occurring in the system can also vary. The prediction of such features enabled soft fluid
transport systems with deterministic deformation, which could be applied to touch and actuation sensing in soft actuators. Soft microfluidics thus can collocate multiple functionalities and eliminate the need for extra structural elements (such as sensors) in robotics applications.

3. Microfluidics in Tissue Engineering

Cells cannot only grow in adherent monolayers but also in various 3D configurations that lead to the generation of living tissue models in vitro.[57,58] For instance, tissue models can originate from self-assembled cell aggregates, pellets, or constructs obtained by seeding cells onto supporting matrices composed of biocompatible materials, termed ‘scaffolds.’[59–62] Thus far, tissue models and their underlying tissue engineering techniques have been predominantly applied to biomedicine.[63] This led to advancements in the development of research platforms for fundamental biology and drug discovery and in the generation of clinical grafts for tissue repair and replacement.[64] However, the majority of currently available 3D cell culture models do not yet efficiently mirror the structural complexity and functional integration of actual tissues.[65] Among the unmatched features are the heterocellular composition and the presence of a functional vasculature. In general, tissue engineering struggles with combining different cell types as these typically require different biochemical environments. Although in the last years huge efforts have been dedicated to build circulatory systems within engineered tissues, models at the millimeter scale are difficult to integrate with appropriate vasculature-like systems and connect to effective micro-physiological systems. However, running meso or macro-scaled tissue culture under static culture conditions poses challenges for the creation of the appropriate cell microenvironment and the cell survival, as, in such settings, the convective transport of culture medium is dominant and physicochemical gradients cannot be created (Figure 1). Also, the freshness of culture media frequently varies, since manual media exchange does not occur with continuity, but rather at discrete time points. For these reasons, conventional static culture techniques are inefficient at maintaining cells with limited fabrication efficacy. In fact, for the construction of such devices, the cells can grow in supporting materials mimicking the ECM, like hydrogels, and be subjected to stable concentration gradients of metabolites.[103] Cell-laden hydrogel micro-units (like micro-capsules, films, and fibers) can replicate physiologically relevant 3D heterocellular micro-environments with unique ECM composition. Flexible gel can be used to entrap these micro-units, grouping them within deformable and biodegradable matrices that can be exposed to biochemical gradients.[104,105] For example, cell-containing films (‘biopapers’) can be stacked within microfluidic systems to control the spatial distribution of bioactive molecules (e.g., metabolites or drugs).[104,105]

In recent years, microfluidics has been widely applied to tissue engineering, as it allows for the spatial control of fluids in micrometer-sized channels, the engineering of co-culture systems, the control of signaling biochemical gradients, the implementation of perfusion/flow, and the mechanobiological stimulation of cells.[31] Moreover, the miniaturization, reaction acceleration, and automation typically found in microfluidic devices has also helped scientists to study and manufacture various biological systems in vitro. In particular, microfluidic devices have some technical advantages (like large-scale integration and flexible customization) that make them perform well in the following three types of tissue engineering: Simulating cellular microenvironments for better tissue development, engineering biomaterials to fabricate tissue-mimicking structures, and perfusing large tissue constructs.[16] In fact, microfluidics-based techniques can replicate the environmental conditions that allow the biological constructs (ranging from cell clusters and spheroids to highly structured biological tissues) to evolve optimally.[106] Furthermore, microfluidics can serve as a production line of micro-engineered units, which are needed to form tissues with an elevated architectural and cellular complexity.[107–109] Finally, the control of the fluid microdynamics can be applied to engineer perfusion systems that enable cell survival within large volumes of constructs (Figure 2, top right). Of interest for this application are hydrogels-based microfluidic chips, in which the capillaries are patterned directly into the polymeric matrix (Figure 2, bottom).[110–114] One strategy for hydrogel patterning is laser ablation, as focalized short-pulsed lasers can track micrometer-sized cavities in various hydrogels and generate intricate microfluidic networks.[115,116] In situ techniques that avoid manual handling have been optimized to inscribe networks within 3D cell culture models.[117] As another option, hydrogel-based microfluidic systems with customized perfusion patterns can be prepared by templating the channels through temporary bioinks.[118] In microfluidic hydrogels, the cells can grow within the biocompatible hydrogel matrices and take advantage of the fluidic conduction enabled by the micro-channels,[117] or alternatively the hollow channels can be filled with cells that then grow according to the patterned scheme (Figure 2, bottom).[118]

Hydrogels have a high content of water, and higher biocompatibility and degradability as compared to conventional chips’ constituents, like the PDMS.[120] For these reasons, they have been proposed as substitutes for traditional body materials of biomedical microfluidic chips.[121] Despite the wide applicable potential of hydrogel chips, their realization is still confronted with limited fabrication efficacy. In fact, for the construction of such devices, soft wet formulations of hydrogels have to remain intact following various procedures, including template printing, patterning, molding, and crosslinking. In recent years, the attempts to combine high printing resolution and...
### Table 1. Examples of different microfluidic organ-on-a-chip models.

| Target organ | Cells | Microfluidic platform | Major findings | Application | Ref. |
|--------------|-------|-----------------------|----------------|-------------|------|
| Liver        | Human hepatoma cells | Microfluidics-based bilayer device with a discrete chamber modeled upon the liver’s tissue architecture. | Preserved cell proliferation and hepatic functions (serum protein synthesis and metabolism). | Drug screening and toxicology | [68] |
|              | Primary rat hepatocytes | Biochip with microfluidic perfusion with a suspended membrane as a cell substrate that mimicked the space of Disse. | Microfluidic flow induces defined expression of Zonula occludens (ZO)-1, transferrin, Asialoglycoprotein receptor 1, Multidrug resistance-associated protein 2, and enhanced formation of hepatocyte microvilli. | Study of the human hepatocellular physiology. | [69] |
|              | Hepatocytes co-culture | Microfluidic device with liver-lobule-like hexagonal tissue-culture chambers and separate seed-feed network. Each chamber contains a central outlet. | Stabilized albumin secretion and urea synthesis, retention of cell morphology and functionality, tissue-like structure and bile-canaliculi network formation. | Modeling of patient-specific hepatic disease, drug screening, toxicity studies, and integration in multi-organ human-on-a-chip systems. | [70] |
|              | Non parenchymal cells | Microfluidic chip with flow recirculation through collagen-coated polystyrene scaffolds seeded with cells. | 3D microfluidic system permissive to hepatitis B virus (HBV) infection. Recapitulation of the HBV life cycle, innate immune and cytokine responses co-culture with non-parenchymal cells. | Investigation of viral diseases; drug screening. | [71] |
| Lung         | Primary human alveolar epithelial cells | Microfluidic chip with compartmentalized polydimethylsiloxane (PDMS) microchannels and an extracellular matrix (ECM)-coated porous PDMS membrane. The platform applies vacuum to the side chambers to cause mechanical stretching of the membrane and simulate breathing tissue dynamics. | Mechanical strain enhances toxic and inflammatory responses to silica nanoparticles, nanoparticle uptake, epithelial, and endothelial uptake of nanoparticles. | Replicate the lung to assess the biological functions of cells, and nanotoxicological testing. | [72] |
|              | Microvascular endothelial cells | Microphysiological system ("breathing lung-on-a-chip" microdevice). | Reconstruction of the 3D microarchitecture, mechanical activity, and integrated physiological function of the alveolar–capillary interface processes. | Screening of environmental particulates and modeling of complex human disease. | [73] |
|              | Primary human lung alveolar epithelial cells | Microfluidic microchip integrating a stretchable membrane made of collagen and elastin. | Replication of the native structure of the alveoli, and reconstitution of the air-blood barrier. Expression of alveolar epithelial cell markers are expressed, preservation of barrier integrity for up to 3 weeks. | Replication of lung pathophysiology and drug screening in personalized medicine. | [74] |
|              | Primary human lung endothelial cells | Compartmentalized microfluidic chip recreating air–liquid interface and cyclic breathing motions. | Integration of a 3D porous GelMA hydrogel with an inverse opal structure and a high structural similarity to native human alveolar sacs. Preservation of cell functions. | Pathophysiological modeling and research. | [75] |
| Gut          | Primary human alveolar epithelial cells | Microdevice with two microfluidic channels separated by a porous flexible membrane. | Replication of the intestinal micro-environment (fluid flow, peristalsis, and microbial flora). | Recreate the gut microphysiology for transport, absorption, and toxicity studies. | [76] |
|              | Human colon carcinoma cell line (Caco-2) | Microfluidic chip with three PDMS layers (for reservoirs, interconnectivity, and tissue culture). | Incorporation of a collagen scaffold to combine 3D structure and fluidic shear. Improvement in gut functions (absorptive permeability of gut epithelium and enzymatic activity). | Study of gut physiology and effects of different tissue environments on gut cells. | [77] |
|              | Microfluidic chip with three parallel hollow microchannels The central channel is split into an upper (lumen) and lower (capillary) channel by an ECM-coated porous PDMS membrane. | Culture of multiple commensal microbes. Mechanical stimulation induces intestinal bacterial overgrowth and inflammation. Mimetism of ileus and inflammatory bowel disease. Immune cells and lipopolysaccharide endotoxin stimulate production of proinflammatory cytokines. | Analysis of microbiome and intestinal pathophysiology. | | [78] |
| Target organ                        | Cells                                                                 | Microfluidic platform                                                                 | Major findings                                                                                                                                                                                                 | Application                                                                 | Ref. |
|------------------------------------|----------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------|------|
| Skin                               | Primary human intestine epithelium                                   | Microfluidic chip with two parallel microchannels for epithelial and vascular culture | Epithelial cells’ polarization and formation of villi-like projections. Cells’ apical surfaces are exposed to an open lumen and interface with endothelium. Chip’s transcriptome closely resembles human duodenum.                     | Studies of gut physiology (nutrition, metabolism, infection, drug pharmacokinetics, and personalized medicine).                                      | [79] |
| Skin                               | Human intestinal microvascular endothelium                           | Microfluidic chip with three PDMS layers, two PET porous membranes, and three separate channels with four vertically stacked cell layers.                        | Biochemical induction of skin inflammation and edema testing model using our skin chip. Alterations of the endothelium permeability and expression of proinflammatory cytokines.              | Disease modeling or toxicological testing of cosmetics or drugs.               | [80] |
| Skin                               | Human epidermal keratinocytes (HaCaT)                                | Multi-chamber microfluidic chip with interchangeable lids and insets, which allow one to switch from the bioreactor setup to the analysis system set up.          | Realization of a full-thickness skin equivalent with a fibrin-based dermal matrix. Microfluidic culture improved epidermal morphogenesis and differentiation, and enhanced barrier function. | Drug screening and toxicological applications.                                | [81] |
| Skin                               | Human skin fibroblasts (CRL-1634)                                    | Microfluidic device with a PDMS microfluidic channel and a porous membrane.             | Generate in vivo-like tubular cell micro-environments. Enhanced cell polarization, cytoskeletal reorganization, and hormone-induced molecular transport.          | Modeling of the renal tubule system for drug screening and advanced tissue engineering. | [82] |
| Kidney                             | Primary rat inner medullary collecting duct (IMCD) cells             | Microfluidic device with two adjacent channels separated by a porous membrane, mimicking the apical ‘luminal’ and basal ‘interstitial’ space.              | Enhanced epithelial cell polarization and primary cilia formation; increased albumin transport, glucose reabsorption, and brush border alkaline phosphatase activity. First drug (cisplatin) toxicity study using primary cells in a microfluidic ‘kidney-on-a-chip’ platform. | Human-relevant renal toxicological studies.                                   | [83] |
| Kidney                             | Primary human kidney proximal tubule epithelial cells                | Microfluidic device with two closely opposed, parallel microchannels separated by a laminin-coated, porous PDMS membrane.                            | Cell differentiation of human into podocytes exhibiting maturity markers and foot processes. Production of glomerular basement-membrane collagen, and differential clearance of albumin and insulin. Replication of adriamycin-induced albuminuria and podocyte injury. | Drug development and personalized medicine applications.                     | [84] |
| Kidney                             | hiPSCs Human glomerular microvascular endothelial cells              | Microfluidic kidney-glomerulus-on-a-chip platform with two closely opposed, parallel microchannels separated by a laminin-coated, porous PDMS membrane. | Cell differentiation of human into podocytes exhibiting maturity markers and foot processes. Production of glomerular basement-membrane collagen, and differential clearance of albumin and insulin. Replication of adriamycin-induced albuminuria and podocyte injury. | Drug development and personalized medicine applications.                     | [85] |
| Kidney                             | Human renal proximal tubular epithelial cells (RPTECs)              | Three-layer microfluidic chip containing a microfluidic drug concentration gradient generator and a flow-temperature controlled platform for cell culture. | Enhanced cell growth and mimetism of drug nephrotoxicity. Decrease in cisplatin-induced nephrotoxicity upon cimetidine addition.                                 | Nephrotoxicity screening and drug development.                                | [86] |
| Kidney                             | Human peritubular capillary endothelial cells (PCECs)                | Microfluidic chip containing cylindrical hollow tubes of physiological size, parallel and closely packed in a collagen I matrix.                          | Increased cell proliferation and decrease in F-actin stress fibers density in dilated tubes. Interplay between tightly packed renal tubules.                         | Kidney pathophysiology.                                                      | [87] |
| Brain                              | Pluripotent human (NTERA2) cells                                    | Brain-on-chip platform with different cell culture spaces separated by a perforated membrane.                                                   | Expression of mature axonal and dendritic markers. Features of the blood–brain barrier environment (zonula occcludens tight junctions, and increased trans-endothelial electrical resistance). Cell migration in response to chemotactic cues. | Modeling of neural differentiation and maturation; analysis of complex tissue behaviors. | [88] |
| Brain                              | Human fetal neural progenitor cells (hNPCs)                         | Organoid-on-a-chip platform with two parallel culture chambers separated by a central channel with trapezoid pillar array structures for interconnection. | Enhanced 3D culture, in situ neural differentiation, and organoid self-organization(regionalization, and cortical organization). Exposure to nicotine induces premature and abnormal neuronal differentiation and migration, and disrupts brain regionalization and cortical development. | Modeling of the neurodevelopmental disorders under environmental exposure; brain disease studies and drug screening. | [89] |
Table 1. Continued.

| Target organ | Cells | Microfluidic platform | Major findings | Application | Ref. |
|--------------|-------|-----------------------|----------------|-------------|------|
| Heart        | Human iPS cell-derived cardiomyocytes | Microfluidic platform integrating two independent yet interpenetrating sensor arrays for field potential and impedance readouts. | Real-time, non-invasive data acquisition of both cardiac electrophysiology and contractility. Treatment with norepinephrine affected cardiac tissue response. | Early assessment of cardiac toxicity in drug discovery. | [95] |
| Lung fibroblasts | Lung fibroblasts | Brain microvessel-on-a-chip platform with an open design. | Miniaturized design for controlled unidirectional media flow at physiological rates, and multiple high resolution imaging. | Modeling of transcytosis across the blood brain barrier of biologics, viruses, or nanoparticles. | [89] |
| Heart        | Human iPS cell-derived cardiomyocytes | Microfluidic platform to culture the human corneal barrier through upper and lower channels separated by a porous membrane. | Increased expression of cytokeratin 19 (CK-19) intermediate filaments in cells, indicating the strengthening of the barrier function. Increased cell body area rather than nuclei. | Ophthalmic drug development and investigation of the blinking shear stress on the ocular surface. | [94] |
| Bone         | HUVECs | Microchannels and access through four parallel channels separated by molded microposts, and integrating fibrin ECM with the synthetic bone mineral Hydroxyapatite. | Hydroxyapatite affected the mechanical properties of the ECM and enhanced sprouting of angiogenesis. | Modeling of the microenvironments for bone vessel sprouting. | [90] |
| Eye          | Primary mouse retina | Microfluidic device composed of PDMS thin-film layer with molded microchannels and access through holes, a tubing support layer, and a media cylinder. Suction channels gently apply negative pressure. | Staining with toluidine blue, transport of locally applied cholera toxin beta, and transient response to lipopolysaccharide. Availability of point access for signaling studies. | Modeling of eye pathophysiological and ophthalmic drug development. | [93] |
| Human corneal epithelial cells (HCE-T) | Human corneal epithelial cells (HCE-T) | Microfluidic platform to culture the human corneal barrier through upper and lower channels separated by a porous membrane. | Increased expression of cytokeratin 19 (CK-19) intermediate filaments in cells, indicating the strengthening of the barrier function. Increased cell body area rather than nuclei. | Ophthalmic drug development and investigation of the blinking shear stress on the ocular surface. | [94] |
| Heart        | Human iPS cell-derived cardiomyocytes | Bioelectronics-integrated microfluidic platform with channels enabling rapid modulation of medium oxygenation. | In hypoxic cells, onset of arrhythmia and narrowing of action potentials. Multiplexing of extra- and intracellular bioelectronics with improved mapping capabilities. | Investigation of the electrophysiological responses to hypoxia, disease modeling, and drug development. | [96] |
| Muscle       | Neonatal rat ventricular myocytes | Heart-on-a-chip microdevice consisting of 3D cardiac microtissues and microelectromechanical system-based microfluidic chips. | Correlation between particle displacement and frequency of external electrical stimulation. Observation of a pharmacological response to isoproterenol, a β-adrenoceptor agonist. Maturation of cardiomyocytes and generation of functional cardiac tissues. Response of the electrical-stimulated cardiac tissues to drug treatment of verapamil and isoprenaline. | Cardiac pathophysiological modeling and pharmacological investigation. | [97] |
| Bone         | Lung fibroblasts | Bone-on-a-chip microfluidic device integrating the bone tissue physiology. Enhanced proliferation, differentiation and ECM production. Enhanced osteogenesis upon chemical and mechanical stimulation through an intermittent flow. Detection of primary cilia on cells. | Modeling of bone tissue physiology and drug screening. | Modeling of bone physiology and high-throughput bone-related drug screening. | [92] |
| Eye          | Human iPS cell-derived cardiomyocytes | Microfluidic platform to culture the human corneal barrier through upper and lower channels separated by a porous membrane. | Increased expression of cytokeratin 19 (CK-19) intermediate filaments in cells, indicating the strengthening of the barrier function. Increased cell body area rather than nuclei. | Ophthalmic drug development and investigation of the blinking shear stress on the ocular surface. | [94] |
damage-free manufacturing have intensified.\[112\] This fact confirms that, more generally, the combination between hydrogel and microfluidics has a high potential,\[121,122\] and attracts increasing interest among scientists who search for more bio-integrable microfluidic systems.\[123–125\]

The following sections will report on how microfluidics can be applied to biofabrication and cell culture, describing recent studies that delineate the potential of such a technique for the generation and maintenance of artificial biological systems. In particular, we will briefly discuss the three main domains where microfluidics has already contributed or can contribute most, being: the biomimicking of the physicochemical conditions of cell environment (replication of the cell microenvironment); the construction of multi-cellular assemblies (biofabrication); and the distribution and control of culture media in cell and tissue culture (perfusion) (Figure 1).

### 3.1. Replication of the Cell Microenvironment

Screening the cell microenvironment provides information on how intrinsic factors of the cell combine with environmental cues to produce diverse cell phenotypes.\[126\] The cell microenvironment is determined by a multitude of factors, including:

| Target organ | Cells | Microfluidic platform | Major findings | Application | Ref. |
|--------------|-------|------------------------|----------------|-------------|------|
| Human bronchial smooth muscle (BSM) cells | Airway musculature-on-a-chip device based on BSM lamellae. | Replication of asthmatic bronchoconstriction and bronchodilation via muscle tissue treatment with a cholinergic agonist and interleukin-13. Pharmacologically induced reversal of the asthmatic phenotype. | Drug discovery via analysis of tissue protein expression, structure, and function. | \[100\] |
| Primary mouse myoblast cells | Microfluidic platform with two main channels that are connected by parallel groove for the compartmentalized culture of motor neurons and muscle cells. | Axonal extensions form functional neuromuscular junctions. Investigation of effects of biochemical cell stimulation. Promotion of axonal growth and innervation upon administration of Ciliary cell line-derived neurotrophic factor (CDNF). | Pathophysiological modeling of the neuromuscular junction, and drug development. | \[101\] |
| Spinal cord explant | Microfluidic device with support pillars to integrate a 3D skeletal muscle construct. | Cell uniaxial alignment, cell differentiation, skeletal muscle tissue formation. Estimation of the passive tension of the engineered muscle tissue bundles suspended between two pillars. Dose-dependent effect of cardiotoxin on the engineered muscle tissue architecture. | Structural and functional muscle tissue modeling, preclinical drug discovery, and development. | \[102\] |

Table 1. Continued.

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**Figure 2.** Cell culture approaches. Static culture of cell monolayers and 3D tissue constructs in plastic dishes (top, left). Microfluidic platforms for cell monolayers and microtissue culture (top, middle). Integrated microfluidic tissue culture (top, right), where scaffolds inserted in the microfluidic platform are crossed by microfluidic channels. Microfluidic flows can support integrated heterocellular cultures while regulating the nutrients’ diffusion and the biomechanical cell stimulation. Microfluidic hydrogels can be generated by photopatterning of hydrogel matrices or removal of sacrificial inks from pre-designed channel networks (bottom, left). Cells can be seeded in the matrix or the microchannel space (bottom, right). \[107,118\]
Cell–cell interactions, soluble factors, mechanical cues, features of the ECM, and various physical conditions (such as temperature, pH, tension of CO₂ and O₂). Variations in these factors can alter the behavior of the cells in relation to processes which are connected to their homeostasis or specialized functional activities. In particular, the cell phenotypes that can be affected are the proliferation, differentiation, damage, repair, apoptosis, migration, adherence, and crosstalk with other cells.[127] Microfluidic chips are an ideal platform to study the cell microenvironment, as they can reproduce complex and dynamic features of the cellular biological milieu. For instance, in the microfluidic devices, it is possible to manipulate the spatial and the spatiotemporal distribution of signaling molecules within both monolayers and 3D tissue architectures.[128,129] This ability has been widely exploited to understand fundamental cellular processes, such as chemotaxis and morphogenesis.[129]

Importantly, microchips can also precisely control the spatiotemporal oxygen distribution in 3D cell cultures,[130–133] becoming essential in investigating the hypoxic microenvironment which is a crucial pathological feature in many diseases, including cancer.[134–138] Through microfluidics, hypoxic tissue models can be produced by recreating physiologically-relevant low and cycling oxygen levels that are not attainable in traditional macroscopic cell culture.[139,140] In a recent study, breast tumor spheroids were monitored over time while being exposed to an anticancer treatment and time-varying oxygen profiles switching between 0% and 10% O₂, providing important insights into cell response to changing oxygenation states of the environment.[140] The O₂ level in the microchips can be controlled with various technologies, being: On-or-off-chip mixers that regulate the O₂ diffusion from liquid or gas; channels with chemical reactions that generate O₂ gradients; electrolytic reactions that locally produce O₂; cell stimulation that increases the O₂ cellular uptake.[135] It is important to notice that microfluidic oxygen control can model not only pathologies, but also differentially oxygenated physiological environments.[139–142] These environments are an optimal scenario for the functional study of cells whose activity is considerably affected by the oxygen availability, such as neurons and muscle cells.[143–150]

Physiologically relevant in vitro models should be built on criteria that meet the mechanobiological needs of the cultured cells. Microfluidics provides scientists with control also over mechanotransduction, the process that links mechanical forces with biological responses. In fact, microchips can adequately replicate the mechanical loading conditions that cells experience in their native environment.[151] The physical cues are typically delivered in the form of shear flow, compression, and stretch or strain.[154] Microfluidic modeling of mechanotransduction has been adopted for the investigation of neural, vascular, bone, cartilage, and mechanically active tissues that comprise of cells equipped with specialized contractile apparatus (namely, smooth, skeletal, cardiac muscle cells, and myofibroblasts).[11] Because of their contractile nature, these cells are inherently subjected to mechanical stimuli of various entities and forms. The response of the cells to such a stimulation drives the tissue maturation and guarantees the achievement of appropriate tissue function. For example, microfluidic systems can replicate sequences of physical stresses that cardiac cells experience during hemodynamic loading and unloading.[153]

These stimuli, especially if sustained, change the gene expression, morphology, and contractile ability of cells, promoting the physiological heart tissue development and cardiac function. Finally, microfluidic technologies can also integrate elements for electrical stimulation of cells,[156,157] that is typically used to study cells with electrically excitable membranes (primarily neurons, cardiomyocytes, and skeletal muscles).[157–160]

Upon selection of suitable natural or synthetic matrices, microfluidics can generate biofidelic cell microenvironments that include systems to apply controlled biophysical stimuli or temporally and spatially-varying delivery of biochemical factors.[129] On the one hand, this approach makes it possible to examine the mechanisms by which specific cell phenotypes are produced while performing experiments in platforms that consider many cues as a whole. On the other hand, microfluidics can also help to search for the appropriate conditions that generate the desired cell phenotypes. As a consequence, this discipline has become essential to develop organ-on-chip models and biofidelic microtissues.[161] In organ-on-chip models, cells are cultured into continuously perfused chambers, and can assemble in multi-cellular configurations that mimic the physiology at a tissue or organ-level.[162] To reach a high level of biomimicry one needs to design and implement cell–cell and cell–ECM interactions in organ/tissue-on-a-chip systems. These models can also replicate tissue-tissue interfaces and enable the investigation of the tissues’ physio-pathological development by means of high-resolution, real-time imaging, and the biochemical and genetic profiling of the component cells.[163–166]

The simulation of the cellular microenvironment emerges as a crucial feature of microfluidic chips.[167] The level of functional replication achieved by these chips can hardly be realized through other bi- or tri-dimensional macroscopic cell culture systems run in traditional polystyrene containers (dishes, well-plates, or flasks).[168] In fact, only microfluidics can master fluid flows in such a way that the microenvironments’ characteristics and the vascular circulation effects can be reproduced. Although the transition to microfluidic systems requires some adaptation of the cell culture protocols,[168,169] microfluidics has notable advantages. It facilitates the simultaneous manipulation and analysis of the cultured cells, and can be applied to the study of single cells, cell populations, and intact tissues. Moreover, microfluidic devices allow for tight geometrical control of the generated tissue constructs. By applying and modulating the biochemical gradients and mechanical stimuli (such as shear stresses and interstitial flows), they closely mimic the physicochemical conditions found in tissues. Thus, it is evident that microfluidic systems allow for exceptional discoveries that strongly deepen our understanding of tissue biology, but this fine environmental conditioning can also be exploited to achieve better tissue maturation and functional constructs in vitro.[170]

3.1.1. Relevance of Biochemical Gradients

Although helpful in identifying and describing biological processes, in vivo models only elicit gross perturbations of the investigated biological signaling patterns with a limited spatiotemporal resolution. In contrast, microfluidic systems provide
the opportunity to dissect complex biological processes and understand the developmental and functional mechanisms of tissue, as they can deliver precisely controlled spatiotemporal concentrations of biochemicals.\cite{173} By controlling the regimen of cell exposure to the key biological regulation factors, microfluidic technologies allow for a focused analysis of the cell response to dynamical but well-defined microenvironments.\cite{172}

Cells can be exposed to stable and highly controlled concentration gradients when microfluidic systems for continuous flow are employed. While in macroscale fluidics inertial forces are predominant, viscous forces dominate microfluidic regimes. Hence, microfluidic systems typically generate a laminar flow, which prevents intermixing. This key feature explains the ability of microfluidics to produce local concentration gradients that do not mix easily. As mixing the fluids could be challenging, microfluidics platforms could be endowed with passive or active mixing systems that include microfluidic network, vortex, acoustic, or magneto-hydrodynamic mixers.\cite{173} The concentration profiles of soluble compounds within microchannels could be accurately defined through these technologies. Moreover, two or more streams of fluids can also be injected into the microchannels with different flow rates.\cite{174} This method, termed hydrodynamic flow focusing, can precisely modulate the biochemical concentration profiles while continuously adapting them over time. Such a technique can serve to expose only specific target cells within an aggregate to certain biochemicals. The strategies to create such continuous gradients include hydrogel or ECM-based networks, “Christmas tree” networks, flow splitters, and T junctions. Principles, methods, and instrumentation of such a microfluidic application are extensively reviewed elsewhere.\cite{175}

The microfluidic spatiotemporal control over the distribution of signaling molecules within the engineered tissues can tremendously enhance the understanding of cellular processes with relevance for tissue development and homeostasis, as well as, paracrine cell communication and functionality of hetero-cellular systems.\cite{25,171,176} Accurate spatiotemporal regulation of cell genetic expression and microenvironment are both critical to morphogenesis.\cite{25} Customized microfluidic cell-culture systems for developmental biology have importantly advanced the understanding of morphogenesis.\cite{177,178} To such a purpose, microfluidic platforms have converged with microengineering systems to improve control over the tissue formation processes. In most approaches of bottom-up tissue engineering, the understanding of mechanisms behind tissue formation and maturation is used to replicate tissue to replicate tissue development in vitro. The crucial phenomena that take place during tissue development, such as tissue patterning, morphogenesis, and morphostasis, are dramatically affected by external cues of chemical or mechanical type.\cite{177,178} For instance, morphogens are signaling entities that trigger specific cellular responses according to dose and duration of the exposure. Morphogens form gradients as they diffuse from their source according to patterns that are defined by their initial concentration, properties of the environment, and kinetics of interaction with the target cells. The production and release of a morphogen can vary over time, its vehiculation can occur through different types of carriers (like vesicles, or lipoproteins), and its binding to cells can be regulated by the level of expression of the specific receptors. Various dose-time thresholds determine the cellular response and create different temporal gene expression profiles which culminate in distinct cell fate.

Microfluidic systems can replicate the morphogenetic and morphostatic mechanisms, by exposing cells and tissues to precisely controlled spatiotemporal concentrations of morphogens, morphostats, developmental diffusive molecules, and biochemical cues of other types.\cite{177} Through microfluidic gradient generators, it is possible to generate spatiotemporally controlled chemical concentrations over short time periods, controlling cell gene expression and reproducing sequential steps of the tissue formation process.\cite{179–181} Timed hydrodynamic flow focusing can deliver chemical gradients to cells with a one-cell diameter precision over a long time. For instance, spatially restricting the perfusion of the hepatocyte growth factor and transforming growth factor beta 1 for several hours induced local changes in gene expression, epithelial polarity, and morphology in renal cell monolayers.\cite{176} The spatiotemporal specificity of the biochemical environment is also relevant for intercellular signaling. Recently, a reconfigurable microfluidic cell-culture system was developed to facilitate the assembly of 3D tissue constructs via stacking layers containing pre-conditioned microenvironments.\cite{182} Thanks to the high spatial and temporal manoeuvrability in 2D and 3D heterocell culture assays, microfluidics allows scientists to model the sequential paracrine-signaling events such as tumor-cell-mediated macrophage polarization and macrophage-modulated angiogenesis. Pharmacological applications and functional studies of tissue are also critically dependent on spatially and temporally resolved delivery of soluble factors. Controlled biochemical stimulation of neuronal networks was achieved in a micro-chamber with the neuronal cell culture region partially divided in two sub-compartments.\cite{183} Fluidically connecting the two compartments leads to the formation of two parallel neuronal networks with similar electrophysiological activity but functionally independent. In such a microfluidic platform, it was possible to individually stimulate the two networks through the selective delivery of tetrodotoxin, a neurotoxin able to block action potentials. This work highlighted the exceptional potential of microfluidics for functional investigation of neuronal networks. Finally, in 2014 a hydrogel microfluidic chip was proposed for spatiotemporally controlled biomolecule gradients for culture of embryonic stem cells and embryo bodies.\cite{184} This microfluidic system was set up in a 24-well tissue culture plate, and could be used for both adherent (gelatin-based chip) and non-adherent (agarose-based chip) cell culture, demonstrating not only the high versatility of this technology, but also that the efficacy of biochemical control also extends to 3D hydrogel matrices.

In the future, microfluidics could be increasingly applied to improve the formation and manipulation of gradients of multiple bio-active molecules with different functions, with relevance for bottom-up developmental tissue engineering. This might serve to control the architecture of advanced 3D stem cell-based models, regulating cell self-organization, morphogenesis, or stabilization of tissue boundaries and interfaces.\cite{23} Also, microfluidics could be the core technology enabling artificial signaling centers that support morphogenetic gradients to determine local cell fate decisions within engineered tissues.
3.1.2. Relevance of Mechanical Stimulation

Mechanical stimulation can also regulate cell behavior. Biomechanical cues can enhance, nullify, or even reverse the cell response to drugs.[18] While the substrate stiffness, geometric confinement, or topographic cues can act by passively stimulating the cells, there are also active mechanical stimuli that affect tissue microphysiology. Examples of active stimuli are fluid shear stress, interstitial fluid flow, hydrostatic pressure, and connective tissue tensile stretch and compression.

Microfluidic platforms incorporating active biomechanical stimulation can better replicate tissue pathophysiology in vitro. These technologies have found extensive application in musculoskeletal, cardiac, respiratory, intestine, and kidney models. Microfluidic platforms typically provide biomechanical stimuli in the form of fluid shear and interstitial flow. Some systems can also apply mechanical strain to the cells, so as to mimic physiological processes such as breathing, peristalsis, or the blood pumping. Flow and pressure are critical parameters that can determine the nature of the mechanical stimulation and differently affect cellular response. Variations of flow rates are recognized to induce phenotypic modulation of stem cells.[186] For instance, slow flow rates can compromise stem cell proliferation ability and conversely, high flow rates can increase it.[187] Cell differentiation is also strongly conditioned by mechanical stimulation that can be studied within microfluidic devices.[188] More recently, a microfluidic platform was developed, in which three different levels of shear stresses were produced by deflection of PDMS membranes, to induce mechanical forces on stem cells that affected adipogenesis.[189]

Microfluidic devices can also integrate structural systems that dispense mechanical stimulation to engineered tissue. One example is the platforms that support muscle tissue maturation onto supporting posts or through anchorage nodes.[190-198] In these regards, one promising direction in controlling the biomechanical tissue stimulation is represented by magnetic actuation, which guarantees a remote contactless way to interact with the microtechnology that control the tissue development. In a work from Zhao et al., arrays of microtissues were aged and distorted during certain phases of the process (like demolding).[199] Nevertheless, in such a method, large quantities of samples is typically employed because of its operational simplicity. Nevertheless, in such a method, large quantities of samples cannot be generated, and the hydrogel is at risk of being damaged and distorted during certain phases of the process (like demolding).[200-207]

Micro-hydrogels from biopolymers can be produced through various techniques based on molecular association mechanisms or mechanical processes, that include shearing, atomization, extrusion, and membrane emulsification among others.[204] Biomedical micro-hydrogels seeded with cells are conventionally fabricated via relatively direct, rapid, and simple manual procedures.[205,206] One typical example is the micromolding which is typically employed because of its operational simplicity. Nevertheless, in such a method, large quantities of samples cannot be generated, and the hydrogel is at risk of being damaged and distorted during certain phases of the process (like demolding).[200-207]

Microfluidic-based fabrication is an emergent approach in which microfluidics is used to generate micro-hydrogels from different kinds of starting materials and with a precisely controlled morphology.[201,208,209] The fine flow control enables one to master the size and shape of the fabricated structures such as micro-fibers, micro-particles, or building blocks of several other geometries.[201,209] Such a fabrication method is usually implemented through an emulsion-based approach. For instance, many formulations that use microsphere-shaped microgels as building blocks have been prepared from agarose, PEG-based polymers, and alginate.[210-213] Cell-laden microgels obtained in this way serve as fundamental building blocks in the preparation of artificial scaffolds with complex and heterogeneous compositions.[214] Following fabrication, the cell-laden microgels can be assembled to form thick 3D tissue structures.
and this assembly process can be accomplished through microfluidic technologies. For instance, Chung et al. built microfluidic channels containing railed tracks by fabricating grooves on the top surface of the channels.\textsuperscript{[219]} Such a design enabled the researchers to fluidically guide cell-bearing microgels and assemble them inside the microfluidic chambers, generating complex structures composed of more than 50 substructures (each of which was smaller than 50 μm) with zero error. Subsequently, Matsunaga et al. used microfluidic flows to stack cell-laden collagen microspheres into a well-designed tissue on a millimeter scale.\textsuperscript{[216]} These studies demonstrate that microfluidics can control the assembly of small tissues with high precision, highlighting the potential that microfluidics has for future use in the creation of larger actuators composed of multiple biomodules.

According to the material dimensionality, the biostructures formed through microfluidic technologies can be categorized into four main classes.\textsuperscript{[217]} Materials have sizes in every direction at the same order of magnitude are referred to as 0D materials, and they typically present a “particle” appearance.\textsuperscript{[15,217]} For example, micro/nano-particles are 0D materials, as their sizes are all at micro/nano scale, in every direction. Particles can have different designs, such as: spherical, composite, porous, specific-shaped, and core–shell structured designs. 1D materials display a size in one direction that is several orders greater than those in other directions. As such, they present a “fiber” appearance.\textsuperscript{[19,217]} Micro/nano-fibers have one size in a certain direction that can be at millimeter or meter-scale, whereas the sizes in the other directions are confined to the micro/nanoscale. 2D materials are sheet-shaped materials in which the dimensions in two directions dramatically differ from that of the third direction, whereas 3D materials consist of volumetrically constructed forms of materials. Synthesizing particulate biomaterials via microfluidics offers the advantages of reaching highly uniform particle size and controllable morphology. This aspect has rendered microfluidic biofabrication extremely popular in recent years for several biomedical applications, such as drug delivery and spheroid-based cell culture.\textsuperscript{[218]} The two main microfluidic approaches adopted for the production of particulate biomaterials are the droplet-based and the photolithography-combined microfluidics.\textsuperscript{[219,220]} In droplet microfluidics, one liquid is dispersed into another liquid that is immiscible, thus originating independent liquid units within the microfluidic channels.\textsuperscript{[221]} The shapes of the generated particles can be spherical or slight spherical variations, such as cylindrical or hemispherical. However, the shape limitations can be overcome by combining microfluidics and photolithography.\textsuperscript{[222]} In this method, a solution of light-sensitive monomers is injected in the microfluidic channel and then UV-irradiated. A mask reproducing a specific pattern design is present on one side of the chip and causes the partial polymerization of the monomer solution according to the geometry on the covering mask. Although more particle shapes are possible, this approach is only suitable for light-sensitive materials. Microfluidic fibrous materials are predominantly obtained by spinning technology.\textsuperscript{[223]} Fibrous materials can be fabricated with different features and components, and can be used to construct 3D scaffolds with the integrated capability of cell encapsulation. Sheet-shaped biomaterials have also been realized through microfluidic technologies. For example, mosaic hydrogel sheets have been synthesized by means of a digital controlled “textile” technology, in which the flows of a series of highly integrated fluids were orchestrated through particular programs.\textsuperscript{[224]} A variety of cell patterns could be constructed with such a technique, and high-density cell culture was carried out in the unsupported soft material. Finally, within microfluidic platforms, assembled microgel materials with 3D structures and different types of seeded cells can also be fabricated. Multilayered, thick, and branched vascular tissue was created by using a microfluidic device composed of agarose hydrogel.\textsuperscript{[225]} A Ca-alginate layer embedding smooth muscle cells formed from the reaction between the ions diffused from the agarose channel and the introduced alginate-cell solution. Moreover, endothelial cells cultured in this hydrogel layer progressively formed a robust vascular tissue that could also be separated and cultured as independent constructs by removing the agarose. In another work, a membrane-templated microdevice was used for 3D construct self-assembly.\textsuperscript{[226]} The resulting collagen constructs had tunable geometries, encapsulated tissue-specific cells, and generated functional microtissues. Tubular constructs with maximum lengths of 6 mm were also demonstrated.

Despite requiring a careful evaluation of various parameters (such as, the fluid flow rate, the viscosity of the starting materials, and the wettability of the generated substrates),\textsuperscript{[224]} microfluidic manufacturing presents three key features: i) High throughput microtissue production, which is attributed to the continuous generation of micro-sized cell-laden units;\textsuperscript{[221,227,228]} ii) safe and compliant interaction with the materials, which derives from the fluidically-mediated nature of the process;\textsuperscript{[229]} iii) ability to generate complex tissues in combination with 3D bioprinting.\textsuperscript{[230–233]} Microfluidic technologies have been increasingly implemented into 3D bioprinting approaches aiming to fabricate the next generation of tissue constructs with complex and ordered architectures that faithfully mimic the native tissue structures.\textsuperscript{[234]} Extrusion bioprinting is a simple and cost-effective method with rapid processing, but with low printing resolution and speed.\textsuperscript{[235–237]} Combining extrusion bioprinters with microfluidic systems resulted in “microfluidic 3D bioprinting,” a promising tool for the fabrication of heterogeneous biomimetic constructs composed of different biomaterials and types of cells at a high resolution.\textsuperscript{[231,233,238,239]} Microfluidic 3D bioprinting utilizes fluid bioinks that flow through microchannels. This way, the flow, switching, and mixing of the components can be precisely tuned.\textsuperscript{[230,239–242]} Printing occurs in a fluidic environment which guarantees a reduced shear stress because of the shear flow that surrounds the laminar flow core. Furthermore, one can finely control the dimensions, orientation, and morphology of the products\textsuperscript{[243–245]} with a printing resolution that surpasses the current microextrusion resolution (around 50 μm).\textsuperscript{[230,239]} The downsides of microfluidic 3D bioprinting mostly concern the design of the hardware and the choice of bioinks.\textsuperscript{[246,247]} The adopted design and the printed materials have to be carefully selected to confront the reduction of the fabrication precision at the corners of constructs, a technical drawback that derives from the difficulties in maintaining smooth non-fluctuating pressure and flow inside the device. Nevertheless, microfluidic bioprinting has already provided outstanding
results in producing tubular structures and vascularized constructs which may support the survival and integration of tissues following implantation into patients.\textsuperscript{[230,248]} Although this biofabrication approach is currently in its infancy, it is expected to have a major impact on both future organ-on-a-chip models and large size clinical-grade tissue grafts.\textsuperscript{[234,238]}

In summary, microfluidic-based fabrication offers unprecedented opportunities to increase the biomimeticity and complexity of man-made tissues. Interestingly, the following section will show that microfluidics can also be used to engineer systems that can perfuse and maintain viable macroscale tissue.

3.3. Media Distribution within Tissue

Cells in tissues that exceed a physical size of 200 µm in any dimension cannot rely on simple diffusion mechanisms to obtain oxygen and nutrients or remove waste. Consequently, perfusion and vascularization are imperative for the majority of tissue constructs, and they are considered essential to the successful outcome of tissue engineering.\textsuperscript{[249,250]} In the last decade, methods to implement microcirculation systems have attracted considerable attention because of their potential to improve the culture of 3D tissue mass by increasing its size and architectural complexity.\textsuperscript{[212,251]} For clinical translation, the vascularization of implantable tissue grafts typically occurs via the generation of ‘living vessels.’ This vascularization can be achieved by incorporating vascularizing cells, fragments of blood vessels, or pre-formed vessel-like structures into the tissue. In addition, it is also possible to surgically connect the vascular structure of the implanted constructs with the recipient circulatory system (anastomosis). However, during development and maintenance in vitro, tissues have to be appropriately perfused to constantly guarantee that the cells have been sufficiently exposed to the cell culture medium. Various bioreactor systems for tissue culture under dynamical conditions have been presented.\textsuperscript{[232,252–254]}

In parallel, novel biomaterials and strategies have been developed to structurally control the scaffolds employed as a support for cell growth.\textsuperscript{[205]} In fact, one can direct the internal porosity and interconnectivity of the scaffolds to create matrices that are highly permissive to the fluid flows. In the next subsections, we will briefly discuss how microfluidics has been or could be applied to solve problems connected to tissue perfusion and vascularization.

3.3.1. Tissue Perfusion

An almost exclusive advantage of using microfluidic techniques is the ability to finely perfuse cell culture and tissue models.\textsuperscript{[65]} Thanks to its compartmentalized nature, microfluidics can provide an adequate dynamical flow of media, which is perfused adjacent to or through tissues, guaranteeing a stable supply of nutrients, the removal of waste metabolites, and control of gaseous gradients and oxygenation.\textsuperscript{[65,369]}

Microfluidic perfusion systems have been optimized to achieve low perfusate flow rates, as these allow us to prolong the amount of contact that cells have with reagents in the medium, increase the number of channels, and extend the duration of experiments.\textsuperscript{[61,255,256]} However, a major technical problem is that peristaltic pumps are cumbersome and not suited for high-throughput use, when tens of channels are required.\textsuperscript{[255]} In 2016, Rouunt et al. presented a scalable multichannel microfluidics system that allowed for the assessment of drug effects on tissues.\textsuperscript{[237]} This system was fabricated via 3D-printing, and it employed gas pressure to drive several perfusion experiments in parallel, achieving stable and low flow rates (1–20 µL min\(^{-1}\) per channel) that could be finely controlled (0.2 µL min\(^{-1}\) increments).

Despite the microfluidic system’s high performance in direct perfusion of compact tissue, fluid convection might be still required to efficiently distribute the medium throughout large tissue samples.\textsuperscript{[65,258]} To realize viable thick tissue constructs, the synergy between microfluidics and vasculature engineering has been steadily gaining ground, and the different approaches will be discussed in the next section.

3.3.2. Tissue Vascularization

Microfluidic systems offer the opportunity to engineer cell interactions within limited spaces and with inherent laminar flow. Such technology can spatially control the cells in coculture, the chemical signaling gradients, and the perfusion flow.\textsuperscript{[65]} This ability of microfluidic systems has been extremely advantageous with regard to modelling in vivo vascular micro-environments with fine spatial and temporal resolution.\textsuperscript{[259]} This explains why most research into advancing 3D tissue models has been focused on the vascular domain, both in cancer models and in tissue on its own. Indeed, vasculature has been the most modelled tissue in microfluidic 3D cell culture, followed by brain and liver.\textsuperscript{[65,260]} Since microfluidics is the only platform capable of perfusing microvessels, it can generate important vital flows and related forces. Consequently, several physical and biological studies have been carried out in relation to microcirculatory dynamics, shear responses, and angiogenesis in health and disease.

Engineered tissues should be ideally developed together with integrated vascular networks.\textsuperscript{[261]} Therefore, researchers have employed microfluidic platforms to vascularize tissues.\textsuperscript{[251,262]} The techniques developed so far can be categorized into two main classes; namely, non-living and living vascularization. Non-Living Vascularization: These techniques include prevascularization approaches, which aim to engineer readily available perfusable channels in non-living scaffolds. This prevascularization approach can be achieved by patterning microfluidic materials to create microfluidic networks.\textsuperscript{[208,263–265]}

Engineering micro and meso-vascularization systems within hydrogels relies on three main types of techniques, being: Micromolding, 3D printing, and microfluidic spinning.\textsuperscript{[266]} Various types of hydrogels can be produced and shaped via micromolding, microfluidic spinning, 3D printing, photopatterning, and other methods, to form controllable and immediately perfusable networks.\textsuperscript{[6,208]} These networks can readily distribute solutes by conveying fluid mass along the channels, or by bidirectionally diffusing them between the bulk material and the channels. Non-living prevascularization can be subtractive, additive, or hybrid. In subtractive prevascularization, the
void spaces of the channels arise when physical constitutive components are removed. Typical subtractive methods are based on molding in the presence of needles or dissolvable sacrificial substances. In additive prevascularization, the assembly of the scaffold’s solid phase mostly relies on a progressive construction of the channel network. This process can be achieved, for instance, through a layer-by-layer assembly of micro-hydrogels. In hybrid prevascularization, both an additive material deposition and a subtractive removal of fillers are integrated. For example, living cells can be bio-printed in a 3D space through computer-aided design and a layer-by-layer deposition of cell-laden-matrices. Once these vascular systems have been created, they can be connected to microfluidic platforms, which have great potential in terms of controlling the generated perfusion. In the near future, one can expect that microfluidic vascular networks will enable viable constructs at the mesoscale (that is cm size).

Emerging or hybrid engineering approaches aim to overcome key issues in constructing biomimetic vascularizing systems, including rough geometrical 3D designs and too large vessel sizes. Currently, it is possible to fabricate engineered vascular channels within hydrogels that range in their inner diameters from the microscopic to the millimeter scale. As representative examples, artificial channels of 20 μm mimic the natural capillaries, whereas 10 mm-wide vessels mimic cerebral arteries. Branching networks can be produced within 3D hydrogel volumes. Although much progress has been made, building a complete vascular network, in which both channel sizes and 3D branching architectures are modeled with accuracy on the three axes, remains challenging.

For instance, approaches based on micromolding can shape the channel structures with a high space resolution as they accurately control channel size and planar construction. They are configurable for high throughput production with high manufacturing reproducibility. Channel micromolding in bulk gel by solid objects generated linear channels and crossing networks with channel size > 75 μm. Micromolding by photoresists and sacrificial materials created branched channels with diameters ≥ 20 μm. However, micromolding hardly succeeds in manufacturing complex 3D architectures and large-scale channel networks. In addition, micromolding of new channel designs requires tedious preparation work. Microfluidic spinning can rapidly fabricate hydrogel tubes with tunable sizes in a high throughput manner. Flowing fluids form channels in the diameter size range of 20–600 μm that can be assembled into consecutive hollow tubes for vascular structuring. However, this method is scarcely applicable to the production of branched networks and suffers from a limited choice of suitable materials.

3D printing elicits a higher geometrical freedom in realizing channel configurations, and it is applicable to the creation of large networks. Nevertheless, this approach only allows for the preparation of vessels with relatively large diameters, as the smallest inner diameters of printed vessels in 3D branching architectures fall in the size range of 100 μm. Moreover, the choice of applicable materials is also limited. Channels can be printed directly in the hydrogels via inkjet printing or stereolithography, leading to the formation of zig/zag or branched tubes and networks with vessel diameters ranging from 100 μm to 1 mm. Channels printed with sacrificial materials (such as gelatin, agarose, polyvinyl alcohol, carbohydrate glass, pluronics F-127 ink, etc.) display similar diameter size ranges and have been modeled into simple or 3D branched networks. Printing channels in supporting bath enables the realization of 3D branched networks with considerably smaller vessels. For instance, by using pluronics F-127 as a supporting bath, channels with 18 μm diameters can be printed. Printing by photoablation and photodegradation is based on the localized cleavage of hydrogel polymers induced by light, and can generate branched networks where channels diameter size can be as low as 3 μm.

Importantly, Grigoryan et al. used food dye additives as bio-compatible yet potent photoabsorbers to perform projection stereolithography in photopolymerizable hydrogels. With this technique, the group demonstrated intravascular and multiphase design freedoms in monolithic transparent hydrogels. In particular, they produced entangled vascular architectures from space-filling mathematical topologies, and characterized the oxygenation and flow of human red blood cells. Using this approach, the researchers also investigated fluid mixers, valves, intervascular transport, nutrient delivery, and host engraftment. Lee et al. introduced a method for the 3D-bioprinting of collagen based on the freeform reversible embedding of suspended hydrogels. Collagen gelation was controlled via pH modulation to achieve 20μm filament resolution. This technique allowed the group to create the components of the human heart at various scales, from the capillaries to the full organ, and accurately reproduce patient-specific anatomical features. The collagen provided the mechanical strength necessary to fabricate and perfuse a multiscale vasculature.

**Living Vasculization:** This approach uses biological effectors (i.e., cells) to create the vascular structures. Living vascularization techniques aim to direct cell–cell interactions and mediate vasculogenesis or angiogenesis, the two processes that give rise to vascular networks within the tissue. Vessel-like structures are created by disseminating cells with angiogenic potential throughout the construct. Therefore, it is important to define the biological profile and to understand the behavior of these cells.

Biofabrication involving strategies for living vascularization has dominated the last decade, with important achievements toward the establishment of microcirculatory systems that comprise living cells like the endothelial cells, which are typically used for structural support of channels. In 2012, Miller et al. proposed to vascularize thick and densely populated tissue constructs by means of cylindrical networks that could be lined with endothelial cells and perfused with blood under high-pressure pulsatile flow. To realize such a vascular system, the researchers used rigid 3D filament networks of carbohydrate glass as a cytocompatible sacrificial template. They encapsulated the lattice and the living cells in ECM-based blocks, and then dissolved the lattice in the cell medium. The group demonstrated that the perfused vascular channels not only could form within monolithic ECM, but could also sustain the metabolic function of primary rat hepatocytes seeded within the constructs, preventing necrosis. In 2014, microchannel networks with various architectural features were fabricated within photocrosslinkable hydrogels via a 3D micromolding technique.
that used printed agarose fibers as vascularizing templates.[293] These microchannels were embedded inside various types of synthetic template hydrogels at different concentrations. It was shown that the micro-channeled GelMA displayed improved liquid mass transport, as well as, preserved cellular viability and differentiation ability within the cell-laden tissue constructs. Moreover, the channels were also successfully covered with endothelial cell monolayers, holding promise for future development of living vascularization. Kolesky et al. reported a bio-printing method to produce 3D tissue constructs that included vasculature, multiple types of cells, and ECM.[294] In this study, multi-material bio-printing allowed the group to produce 3D micro-engineered environments with high structural complexity and intricate vascular designs. In particular, to fabricate embedded vasculature, the researchers employed an aqueous fugitive ink composed of Pluronic F127 that can be easily printed and removed under mild conditions. The resulting microchannels were perfused with endothelial cells that could then line the vessels’ lumen.

Microfluidic models have been widely used to investigate the cells that build up the vascular structures in living organisms. The vascularization studies carried out on microfluidic devices culminated in the engineering of networks of perfusable cellular microvessels,[260] which were realized by precisely controlling the vascular microenvironment cues in vitro.[295–297] In certain models, the survival of the surrounding tissue entirely depends on the nutrients supplied by these vessels. In 2016, Sobrino et al. generated vascularized 3D microtumors in an “on-a-chip” platform. The survival of this model relied on the delivery of nutrients through living, perfused microvessels.[298] Such a platform was subsequently optimized to achieve high throughput, driving the flow through the micro-organs via hydrostatic pressure only and not requiring external pumps or valves.[299] In 2014, artery models were assembled from scaffold-free cell sheets and kept in a tubular shape by perfusing them via microfluidic culture.[300] In 2019, Takehara et al. presented a microfluidic vascular-bed device to support the vascular network formation in a skin 3D model.[301] In their device, endothelial cells were co-cultured with fibroblasts within fibrin gels to form endothelial capillaries of heterogeneous sections (5–100 µm in diameter). These biological structures could functionally connect to microfluidic plastic channels and reservoirs with larger (1–10 mm) diameters.

In summary, bio-hybrid robots based on cell and tissue functionality would benefit enormously from a symbiosis with microfluidics. The first results of such a cooperation will be seen in the field of bio-actuation, since it has been the most widely explored field thus far. The following sections will describe how living actuators can be combined with microfluidic devices, and what advantages are gained from this combination.

4. Microfluidics for Bio-Actuators

Tissue engineering primarily seeks to recreate the structures and biological composition of the native tissue in vitro. Nevertheless, the following step, namely to recapitulate the tissue functional characteristics, represents the actual goal to successfully replace natural tissue within physiological systems.[302–304] One vital feature that evolved in a multitude of life forms is the movement. The cells with specialized motile or contractile abilities fulfill the motile functions in unicellular or multicellular organisms, and work as actuators, namely components that allow dynamic systems like the machines to move and interact with the environment. Bio-actuators exploiting contractile or self-propelled cells show promise with regard to mimicking or even surpassing the actuation performance of natural living systems,[305–307] but their development requires intense bioengineering effort to produce highly biomimetic materials that also retain intact functionality and use it in alien conditions. In fact, whereas upon in vivo implantation the functional restoration of biomedical tissue grafts can be supported by the physiology of the recipient,[23,308–310] bio-hybrid actuators have to demonstrate effective integration with non-natural unanimated systems and functionality within artificial environments.

4.1. Bio-Hybrid Actuators

Biological actuators can be cell- or tissue-based, and can efficiently power micro- and mini-machines. Microsized actuators exploit single or a few living motile cells like bacteria, algae, or sperm.[305,311,312] Actuators exceeding the milli-size can also be built from larger assemblies of contractile cells (i.e., skeletal or cardiac muscle cells).[306] Myogenic progenitor cells of different origins (mammalian and insects, etc.) have been used to construct functional muscle tissues, enabling the motion or locomotion of the resulting bio-hybrid robots. Many muscle tissue-based actuators have been developed according to the bottom-up tissue engineering concept in which cells are assembled with biomaterials to form engineered tissue grafts.[306] The biocompatible polymers that are typically used in skeletal and cardiac muscle tissue engineering are listed in Table 2, which lists general advantages and disadvantages of using materials of natural or synthetic origin.[313–316] Bio-actuators based on muscle tissue have evolved over time through increasingly complex designs to achieve improved performances. In 2016, Morimoto et al. presented 3D cellular constructs of human induced pluripotent stem cell-derived cardiomyocytes for the quantification of the contractile force. Fibers with aligned cardiomyocytes formed and generated contractile force along the fiber direction, which could be accurately measured. In 2018, the same group developed a bio-hybrid robot actuated by an antagonistic pair of skeletal muscle tissues (Figure 3A).[40] In this robot, the balancing tensions of the antagonistic actuators served to prevent the spontaneous tissue shrinkage that typically occur during skeletal muscle tissue development in vitro. Achieving large actuation (≈90° of rotation of a joint) for a long functional lifetime (≈1 week), the robot succeeded in pick-and-place manipulation of objects. Among the most recent examples of bio-actuators, a bio-hybrid pumping machine developed by Li et al. exploited engineered skeletal muscle tissue to generate unidirectional fluid flows through a valveless pumping mechanism.[137] The performance of this bio-actuator was sensibly improved as compared to that of similar systems reported in the past,[138] as it produced flow rates that are at least three orders of magnitude higher. In 2018, a batoid-fish-shaped robot powered by cardiac muscle...
tissue was presented (Figure 3B). The robot was based on a substrate composed of two micropatterned hydrogel layers to provide one mechanically stable structure and one actuation component. The actuation layer consisted of GelMA embedded with carbon nanotubes (CNTs) to culture cardiomyocytes. Electrical stimulation and control of beating cardiomyocytes was provided through gold microelectrodes embedded into the scaffold, leading to self-actuating motions aligned with the direction of the cells’ contractile force.

Muscle tissue-based bio-actuators contain synthetic material that can have a structural or a functional role. For instance, Guix et al. realized a swimming bio-hybrid robot consisting of a ring-shaped construct of skeletal muscle combined with a 3D-printed serpentine spring skeleton (Figure 3C). The skeleton not only provided mechanical integrity to the structure, but also mechanical self-stimulation enhancing the tissue maturation process. By adding specific constituent materials to the bio-actuator composition, it is possible to confer additional functionalities to the bio-actuators, making them more and more intelligent technologies. One example are the microfibers developed by Chen et al., which can transform cell-generated force into macroscopic optical signals. These GelMA microfibers were fabricated via a microfluidic biofabrication technique to contain non-close-packed colloidal arrays for quantitative optical sensing. The contraction of the seeded cardiomyocytes induced structural stretching with synchronous dynamic color variation and wavelength shifts that can serve for dynamic cardiac mechanics sensing. In a previous work, cardiomyocytes were cultured onto synthetic inverse opal hydrogel films. Cell deformations caused cyclic volume and morphology variations in the structure of the substrate film, determining shifting of its photonic band gap and structural colors. Various living materials based on structural color hydrogels have been presented, including 3D dynamic Morpho butterflies. Moreover, by combining structural color hydrogels into microfluidic devices, the group developed a “heart-on-a-chip” platform for microphysiological visualization useful for biological research and drug screening. In recent years, other bio-actuating systems with abilities of cardiomyocyte dynamic displaying have been presented, which included nano-imprinted structural color graphene films with anisotropic microgroove and 2D photonic crystal structures (Figure 3D).

Bio-actuators have also been developed with designs and via technologies that have strong biomedical reverberations, such as tissue regeneration and drug screening. Although 3D bioprinting is still a fairly new technique that has been limited in terms of printable materials and resolution, it is also an emergent manufacturing method with strong biomedical implications. Recently, mini skeletal muscle models were generated via 3D bioprinting of Matrigel and patient-derived skeletal muscle precursor cells (Figure 4). These bioprinted constructs were incorporated between two attachment posts to allow tissue maturation in the presence of a longitudinal tensile force (Figure 4A). Contractile, striated myofibers uniaxially aligned along the longitudinal axis were observed. Upon electrical pulse stimulation the bio-actuators could contract, and were therefore

### Table 2. Biocompatible polymers used in cardiac and skeletal muscle tissue engineering, and their properties.

| Material origin | Advantages | Disadvantages | Polymer examples |
|-----------------|------------|---------------|-----------------|
| Natural         | High biocompatibility; high bioactivity; strong similarity to native ECM; highly efficient in promoting muscle tissue regeneration | Poor mechanical properties (e.g., low viscosity and stiffness); not suitable for generation of load-bearing tissue; limited applicability to 3D bioprinting methods; high biochemical and compositional variability due to the natural origin; difficult degradation control and long-term instability; enhanced risk of immunogenicity. | Collagen, Alginate, Gelatin, Agarose, Hyaluronic acid, Fibrin/fibrinogen, Chitosan, Matrigel |
| Synthetic       | Highly tunable mechanical properties and degradation kinetics. | Poor bioactivity and cell adhesion; limited cell proliferation and differentiation potential; suboptimal tissue formation. | Polyglycolic acid (PGA), Polycaprolactone, Poly-(lactic-co-glycolic acid) (PLGA), N-isopropilacrilammide (NIPAAm)-2-hydroxyethyl methacrylate (HEMA), Polyethylene glycol diacrylate (PEGDA), Polyethylene glycol diacrylate-acrylic acid (PEGDA-AA) |
subjected to actuation experiments aiming to simulate physical exercise and to study the effects of pharmaceutics and other bioactive compounds. First, it was found that repeating high impact stimulation of contractions for 3 h increased interleukin 6 myokine expression and activated the Akt hypertrophy pathway. Second, while the voltage-gated sodium channel blocker tetrodotoxin stopped the electrical pulsed-induced contraction, adding muscle stimulators (such as, caffeine and the drug Tirasemtiv) acutely enhanced the contractile force of the bioactuators (Figure 4B–D). In another study, a different bioprinting technique was applied to create cardiac bio-actuators. Briefly, human cardiomyocytes were printed with collagen through a method in which the polymer gelation was controlled by pH modulation, providing up to 10 μm resolution on printing. Using this method, it was possible to replicate the components of the human heart presenting patient-specific anatomical structures. For instance, cardiac ventricles were printed, which could contract in synchrony, and propagate the action potential directionally. During the peak systole, these hearts deformed resulting in wall thickening up to 14%. Due to their versatility and high level of biomimicry, bioprinted bio-actuators are promising for research and drug testing against muscle wasting diseases.

To control muscle tissue-based bio-actuators, electrical stimulation is typically provided to induce muscle cell contraction. Alternatively, muscle tissue can be generated from genetically modified cells that enable the remote control of bio-actuators via optical stimulation. For instance, skeletal muscle myoblasts expressing a light-activated cation channel, Channelrhodopsin-2, have been used to coordinate a multitude of skeletal myotubes which contracted in response to pulsed blue light in 3D engineered constructs cultured within a high-throughput device. In such a “skeletal muscle on a chip” system, several parameters affecting the maturation, structure, and function of
The tested factors concerned the cell source, matrix composition, microtissue geometry, auxotonic load, growth factors, and exercise.

Raman et al. embedded optogenetic myoblasts within a fibrin hydrogel matrix and injected the cell/gel solution into muscle strip and ring molds. The resulting constructs were used as optically responsive muscle-powered machines (Figure 5A,B). The engineered muscle was actuated through an optical excitation apparatus focusing 470-nm light on the bio-actuator (Figure 5C). Dynamical studies of the two actuator designs were carried out by applying optical and electrical stimulation (Figure 5D–G). The rings were implemented in untethered bio-bots enabling directional locomotion at average speeds of 310 µm s⁻¹ (1.3 body lengths min⁻¹) upon optical stimulation at 2 Hz (Figure 5H).

Due to the intense multi-technological and multi-principle implementation in the tissue maturation devices, muscle tissue engineering has become more and more sophisticated over time. Natural phenomena (like morphogenesis) inspire scientists in developing novel approaches to produce biomimetic and performant muscular replicas in vitro. Interestingly, Mondrinos et al. proposed an approach to model and adapt the mechanical regulation of morphogenesis, in which contractile cells act as sculptors of engineered tissue anisotropy in vitro. Briefly, they patterned the surface anchorage of muscle tissues could be assessed in a combinatorial manner.
cell-laden ECM hydrogels that underwent spontaneous contraction during tissue maturation. The constructs progressively acquired and then maintained anisotropic microarchitecture and specific mechanical properties. This system was then combined with a microphysiological system and a microengineered drug-resistant human lung cancer tissue generating a multior- gan microphysiological system of cancer cachexia. This work clearly demonstrates how the development of bio-actuators is progressing toward physiological complexity, which will impact future biomedical technologies for pharmacological development and pathophysiological research within human in vitro multi-organ systems.

4.2. Microfluidics in Muscle Tissue Engineering

Microfluidics has been consistently applied to the field of skeletal and cardiac muscle engineering. Muscle tissue has unique features, and microfluidic models have enabled us to explore these features by reproducing cell–cell interactions, cell recruitment, or the effect of chemical and biophysical conditioning on the engineered tissue. In fact, several microfluidic platforms have been designed to present specific features promoting a typical reorganization of engineered constructs. Most of these models can be easily adapted to study either skeletal or cardiac muscle by tuning different parameters (such as tissue-specific biochemical gradients, cell source, stimulation modalities and pattern, and force read-out). Importantly, microfluidic platforms can integrate systems for cell electrical and mechanical stimulation that are necessary to recapitulate the natural process of muscle tissue development in vitro, as well as to trigger and control its actuation. In the last decade, several muscle-on-a-chip systems have been developed to model and study the musculature of the heart, skeletal muscles, airways, and others. Some examples are listed in Table 1. These studies demonstrate that microfluidic devices have found extensive application in modeling the musculoskeletal system, but also retain great potential for future advancement.
of the current research efforts focus on improving the muscular disease models that can be used for pathophysiological investigation, drug testing, and bioengineering. However, the different application goals led to the diversification of the approaches to engineer these models. Some microfluidic muscle tissue models only focus on structural replication of native tissues to be involved in cell biology investigations, whereas some other models aim also to reproduce the contractile function of muscles and implement technical paradigms to control and assess the actuation. In the following paragraphs, we trace the lines to categorize the available muscle tissue models according to their envisaged use.

Most of the current microfluidic muscle models can be classified in four different categories. The first category corresponds to basic models, where myoblast cells are let to grow in a microfluidic environment to study their fundamental cellular processes (such as viability or proliferation) also in response to chemical cues (Figure 6A). The second type of microfluidic muscle models is the mechanically robust models for drug screening. As an example, Agrawal et al. engineered skeletal muscle tissues featuring architectural and structural complexities of native muscle within a microfluidic device. They confined a cell-laden gelatin network around two bio-inert hydrogel pillars, thus generating a tissue with bundles suspended between two pillars. During tissue maturation, the engineered tissue bundles were capable of applying passive tension onto the two pillars (Figure 6B). As a proof of concept, this skeletal muscle-on-chip system was perfused with cardiotoxin to assess the toxin effects on the tissue architecture and the passive tension. Cardiotoxin caused muscle tissue disruption and consequent drop of the recorded tensile force.

Microfluidic muscle models of the third type are models designed to respond to external stimulation. Shimizu et al. mixed skeletal muscle cells and a collagen type-I solution. They introduced the solution in a microfluidic channel and let it gelate. After one week of culture, skeletal muscle microtissues formed, which could contract in response to electrical stimulation via rectangular wave electrical pulses (±20 V, 10 ms of pulse width) at 1 and 50 Hz (Figure 6C). The microtissue contraction was estimated via recording the electrical response (Figure 6C). The microtissue contraction was estimated via recording the electric signal transmitted from motor neurons to the innervated muscle fibers from a functional point of view, as this heterocellular communication is crucial for the synaptic function, viability, and maintenance. Duc et al. exploited soft lithography and custom MEAs to engineer a microfluidic model of human neuromuscular junction on a specific pattern of electrodes to stimulate presynaptic axons and record postsynaptic muscle activity (Figure 6F). In this platform the group demonstrated that not only the achievement of a mature and functional neuromuscular modeling via microstructuring of the chip and the cell growth substrates, but also that the electrical activation of motor neurons triggered recordable extracellular muscle action potentials. Such a multi-functional platform is promising for the development of technologies to characterize neural circuits and neuromuscular units with higher sensitivity as compared to the traditional approaches, such as intracellular calcium imaging.

As shown by this collection of studies, microfluidic platforms show promise for muscle disease modeling. While increasing the model complexity and its association with other advanced manufacturing or functional techniques, microfluidics enables a plethora of physiologically relevant tools to mimic the muscle tissue and neuromuscular junctions, and thus study myopathies and neurodegenerative diseases. Considering microfluidics’ contribution to muscle tissue engineering, one can envision that this discipline will strongly sustain the future evolution of bio-actuators. In particular, it might help in four main tasks, being to fabricate small-scale bio-actuators, as well as to generate, vascularize, and control large muscle tissue. The following sections will briefly show examples of studies pursued in these fields.

4.2.1. Fabrication of Small Bio-Acuators

Biological actuators on a sub-millimeter scale can serve as mini-actuators. To this end, scaffolds for cell culture can be prepared by implementing microfluidic techniques in various ways. One approach to generating small scale bio-actuators
Figure 6. Types of microfluidic muscle tissue models. A) Microbioreactor used for monitoring the proliferation of vascular smooth muscle cells in five identical reactor chambers arranged in parallel. Percentages of surface coverage with cells exposed at variable concentrations of the anti-proliferative chemicals colchicine (left) and curcumin (right). Reproduced with permission.[332] Copyright 2012, Springer Nature. B) Dose-dependent response of engineered muscle strips to cardiotoxin (CTX), demonstrating a drop in the passive tension caused by cytoskeletal disruption. Reproduced with permission.[102] Copyright 2017, Royal Society of Chemistry. C) Bright field photograph of microchannels to culture skeletal muscle microtissues, and histological hematoxylin and eosin staining of cross section of the microtissue on the short and long axis direction. Relative displacement of selected regions of interest in tissues undergoing electrical stimulation at 1 and 50 Hz, as estimated from videos recorded on a microscope. Reproduced with permission.[328] Copyright 2014, The Society for Biotechnology, Japan. Published by Elsevier B.V. D) Photograph and scheme of the biochip for stem cell-bridge muscle model, with microwells aligned multielectrode microarrays. Laser-patterned stem cells (pink) forming a bridge that connected two separated cardiac muscle fibers (green) inside one microwell. Fluorescence imaging of cardiac muscle fibers connected with a stem cell bridge through gap junctions (white arrow), and local conduction velocities of cardiac muscle fibers with different widths, and bridges composed of stem cells, cardiomyocytes, or fibroblasts. Reproduced with permission.[333] Copyright 2012, Royal Society of Chemistry. E) Microchip for the neuromuscular
consists of using droplet-based microfluidic techniques, which have been extensively employed to develop cell-loaded microgels for tissue regeneration purposes.\cite{338} In such an approach, an aqueous solution of polymeric precursors and cells forms droplets to form inside PDMS-based microfluidic chips. The droplets serve as templates to create microgels that can be produced via different polymerization mechanisms. This way, microgels can be prepared in a continuous manner with a precise micro-scaled size, high monodispersity, and control over essential features such as the amount of the encapsulated cells, which can be regulated by determining the initial cell density in the injecting syringes.\cite{119}

Microfluidics has also been used to put embryonic stem cells in cellulose microgels with an alginate shell. Following encapsulation, these cells differentiated into cardiomyocytes.\cite{340} However, when tissue is fabricated via microfluidics, cell survival and activity can be seriously harmed by the extensive purification procedures and the various chemical and physical stresses. These stresses include fluid pressure, shear forces, changes in the pH, contact with surfactants, and hydrocarbon oils. To preserve cell viability and functionality, Choi et al. proposed a one-step microgel production in which a microfluidic device made of a glass capillary was used to generate water-in-oil-in-water double emulsions.\cite{341} The inner aqueous phase of this system consisted of a mixed solution of PEGDA, gelatin methacrylate (GelMA), and a photo-initiator in cell media. The outer phase was represented by a thin oil shell. Photopolymerization occurred under flow off-chip, then microgels were collected in an aqueous solution without surfactants. Finally, the high interfacial tension between the aqueous phase and the oil layer within the solution caused the oil shell to detach from the polymerized core. Cell viability increased by around 60% as compared to a standard two-step production, but a high amount of polymer and a radical photo-initiator were employed. Furthermore, glass capillary devices typically generate only large microgel diameters (around 250 µm), since they are proportional to the diameters of capillaries.

Cell death occurs in microgels with dimensions exceeding 200 µm due to the limited diffusion of oxygen and nutrients; therefore, the one-step microgel production technique could be ameliorated by adopting biocompatible surfactants and oils with more advantageous properties (such as, low solubility in water and gaseous permeability).\cite{342,343} To address these technical issues, Guerzoni et al. encapsulated contractile cells in synthetic PEG microgels that had been fabricated via microfluidics.\cite{344} Their system was based on cardiomyocytes and derived from induced pluripotent stem cells (iPSCs) and primary fibroblasts and acted as mini heart tissues capable of a high beating frequency. The high microtissue functionality was ascribed to the safe microfluidic approach in combination with the formation of an ECM nanofilm (via a layer-by-layer deposition) around the cell membrane prior to encapsulation, which improved cell–cell interactions.

Lin et al. showed another way to employ microfluidics to produce small bio-actuators. They generated bubbles and fabricated them in 3D scaffolds to produce a porous material for cell culture.\cite{345,346} Subsequently, Mei et al. followed the same microfluidic approach to create a porous matrix from type I collagen and gelatin for prolonged cardiomyocyte culture.\cite{347} The main objective in this study was to create a biocompatible material with a high mass transfer efficiency that could preserve the survival and spontaneous contraction characteristics of a co-culture of primary cardiomyocytes and cardiac fibroblasts. They demonstrated that the scaffolds made of pure gelatin had more suitable mechanical properties for myocyte culture and maintained the spontaneous contraction behavior of the cells for a longer time (up to 25 days) than collagen-containing formulations.

In addition to the assembly at the microscale, microfluidics easily allows for the generation and the study of co-culture systems, which represent an important area of recent study in muscle regenerative biology.\cite{348} In fact, it has been demonstrated that culturing myogenic cells with certain other cell types (e.g., fibroblasts and neurons) improves tissue formation, and activates or facilitates muscle function. Therefore, we envision that microfluidics will play a substantial role in designing the next generation of realistic bio-actuators with a high level of cellular complexity and functionality.

### 4.2.2. Generation and Maturation of Muscle Tissue

It has been shown that dynamic culture can not only improve tissue survival and longevity, but also the quality of muscle myofibers in terms of nuclei density, size, and force generation.\cite{349} In the following paragraphs, the contribution of microfluidics to the generation and development of muscle tissues in vitro will be discussed. Some examples will highlight the morphologies that have been most widely explored in microfluidic muscle biofabrication: Microspheres and microfibers (Figure 7).

As is mentioned above, microfluidics can synthesize microscale hydrogels, which can be assembled to make larger tissues composed of several units (Figure 8). Microgels of globular shape (microspheres) based on photocrosslinkable gelatin/GelMA and laden with bone marrow-derived stem cells have been fabricated via microfluidics in oil phase (Figure 8A).\cite{350} In addition to cells, the microspheres also contained growth factors. In such systems, the stem cells were able to differentiate to the osteogenic lineage, also displaying increased mineralization. Furthermore, the cell-laden microspheres were found to promote and accelerate osteogenesis not only in vitro, but also in vivo. In another study, macrophage-laden microgels were prepared by double flow-focusing microfluidics (Figure 8B).\cite{351}
The two flows delineated a core–shell structure in the microspheres during the droplet generation phase. In these microspheres, the macrophages were able to differentiate demonstrating sprouting, and to react to immunological stimulation induced by treatment with lipopolysaccharides. The authors also embedded the microgels into a larger fibroblast tissue construct, providing a proof-of-principle of multiplex tissue fabrication. Microspheroids laden with cells with myogenic potential have been mostly explored for cardiac micro-tissue engineering. Agarwal et al. used a microfluidic flow-focusing device to create core–shell microcapsules for the generation of embryonic spheroids (Figure 8C). They observed a significant increase in the cell pluripotency gene expression as compared to in other 3D culture settings. Moreover, the embryonic cells could be differentiated into beating cardiomyocytes by only adding a small molecule instead of a complex combination of growth factors. Hence, microspheres can be microfluidically fabricated to contain myogenic cells, and, as a subsequent step, they might potentially be assembled into larger structures.

In the muscle, the contractile functional units are represented by the myofibers. In muscle tissue engineering, the scaffolds are characterized by fibrous micro and nanostructuring, as well as, by anisotropic topography. These characterizations are preferred because they guide the alignment of myogenic cells and impart orientational cues for the fiber formation. Simple and chemically homogeneous microfibers can be prepared by conventional methods such as electrospinning wet spinning and melt spinning. Microfluidics-based fabrication also allows for the production of fibers and alginate has been most widely used due to its fast gelation upon ionic crosslinking. Interestingly, alginate is also the most popular choice as a scaffold biomaterial for muscle tissue engineering due to several advantageous properties that include: structural resemblance to the native ECM and excellent biocompatibility; easy tuning of mechanical and gelation properties and biodegradability; non-antigenicity and approval by the US Food and Drug Administration (FDA) for human use. Alginate-based microfibers typically serve as cell-encapsulating building blocks. However, derivatives...
Figure 8. A) Microscope image of a capillary microfluidic device for the production of monodisperse gelatin/GelMA microspheres in the oil phase (top). Under biochemical stimulation, bone marrow-derived stem cells cultured in GelMA microspheres undergo osteogenesis as shown by the increased alkaline phosphatase activity marked by Alizarin red staining after 1, 2, 3, and 4 weeks of differentiation (bottom, scale bar: 100 µm). Reproduced with permission.[350] Copyright 2016, Wiley-VCH. B) Double flow-focusing microfluidic fabrication of macrophage-laden microgels. Core and shell flows are indicated as Aq1 and Aq2 respectively (scale bar: 100 µm). Microscopic images of differentiating macrophages with sprouting formation in microgels treated with lipopolysaccharide (LPS) at day 7 (scale bar: 50 µm). Macrophage-laden microgels were also embedded into a larger fibroblast tissue construct to fabricate multiplex tissue models for immunological studies. Reproduced under the terms and conditions of the CC-BY license.[351] Copyright 2020, The Authors. Published by MDPI. C) Non-planar microfluidic flow-focusing device for one-step generation of core–shell microcapsules...
of alginate (like alginate containing phenolic hydroxyl moieties) can be used to augment cell adhesion on the microfiber surface. Moreover, alginate can combine with cationic polymers (e.g., chitin, chitosan, and poly-l-lysine) to form a polyelectrolyte complex hydrogel useful for the preparation of microfibers. In one crucial study, by using a microfluidic device with double-coaxial laminar flow, Onoe et al. realized core–shell hydrogel microfibers at the meter-scale. Such fibers encapsulated differentiated cells or somatic stem cells, were enriched with ECM proteins, and could be assembled into constructs that replicated the intrinsic morphologies and functions of living tissues, such as, muscle fibers, blood vessels, and nerve networks. In Table 3, some examples of cell-laden microfibers generated by the microfluidic laminar flow method are listed, which include microfibers derived from alginate,[366–375] modified or enriched alginate,[359,376–381] and other biomaterials.[224,281,322,361,382] Some examples of microfluidically generated microfibers are represented in Figure 9 that depicts: Microfibers composed of a single biomaterial whose different diameters affect the alignment of adherent cells (Figure 9A),[159] microfibers whose heterogeneous biomaterial composition increases cell viability (Figure 9B),[363] microfibers with grooved microstructures that facilitate cell alignment (Figure 9C),[378] microfibers encapsulating different cell populations (Figure 9D),[377] microfibers with tailorable internal architectures and hollow cavities, and assemblable into complex 3D geometries (Figure 9E), demonstrating that through multiple-laminar-flow microfluidics, it is possible to prepare multicomponent 3D alginate microfibers in which the morphology of the encapsulated cells can be precisely controlled.[342,379] Zhao et al. fabricated grooved microfibers by using a microfluidic spinning system (Figure 9C).[378] They employed a flow-focusing microchip with multiple parallel channels and introduced different concentrations of sodium alginate into the channels. Upon the ionic crosslinking of the polymers, geometrically defined microfibers formed. These microfibers’ shape and size depended on the viscosity and concentration of the alginate solution, as well as on the flow rates of the polymer and the crosslinker. Consequently, microfibers could be produced in a controllable manner. Furthermore, heterogeneous grooved fibers arose from co-spinning alginate and GelMA, which generated interpenetrating polymer networks. In such an anisotropic scaffold, murine skeletal muscle cells could orient themselves, aligning in an ordered manner and producing fiber-like microstructures. Such fine control over the cells and the biomaterials opens interesting perspectives in tissue engineering for biorobotics. In fact, performant bio-actuators need to have a well-defined morphology with the actuating cells allocated to specific positions to achieve the actuation scheme that is needed to trigger the desired deformations of the 3D constructs.

Microfluidic 3D bioprinting has incredibly contributed to muscle tissue engineering. Constantini et al. have used a microfluidic printing head coupled to a co-axial needle extruder to bioprint hydrogel fibers from photocurable semi-synthetic biopolymer (PEG-Fibrinogen) laden with C2C12 cells (Figure 10). In such constructs, the myoblasts could migrate, fuse, and form long parallel multinucleated myotubes displaying a high degree of alignment along the direction of hydrogel fiber deposition. In fact, after 15 days of culture, long myotubes in the range 300–400 µm were observed by immunofluorescence. Myotubes displayed highly parallel orientation (>90% myotubes within ±10° deviation vs. fiber printing direction) and expressed the myogenic marker MHC, demonstrating the maturation and sarcomerogenesis in the bioprinted microscopic muscles.

In addition to the fabrication approaches, microfluidics has also contributed to muscle development by offering the possibility to generate cultures with different cell types.[384] Co-culture systems have attracted much attention in muscle engineering and regeneration, since the crosstalk among different cell types has proven effective in improving tissue development. For instance, the presence of fibroblasts enhances myogenic cell differentiation and activity, whereas adipocytes can affect the process of energy consumption in myocytes by orchestrating the metabolite release and availability in the microenvironment.[385–388] Other cell types can be co-cultured with muscle cells in microfluidic systems such as endothelial cells and neurons. Endothelial cells are used to generate microvessel structures within the construct volume, which can then be perfused by microfluidic flows.[13,389] Neurons activate and regulate contraction in muscle cells.[390–392] The sections below will discuss microfluidic approaches in the vascularization and functional control of muscle tissue, and the combination of muscle cells with the aforementioned specific cell populations.

4.2.3. Key Considerations for Microfluidic Fabrication of Tissues and Bio-Actuators

When applying microfluidic biofabrication technologies, certain technical aspects have to be considered in relation to the used materials. Furthermore, the parameters adopted to run the fluidics during the biosynthetic processes have to also be considered.[112]

Although cells are the key effectors in tissue formation, they need to be combined with biopolymers which assemble to form biocompatible supporting substrates. The most widely used scaffold materials are polymeric hydrogels which are characterized by a high water content typically ranging between 90% and 99%. Hydrogels feature low Young’s modulus falling in the kPa range, thus explaining their ability to mimic ECM of soft tissues. Biopolymers contribute to the successful tissue fabrication by virtue of their high bio-adherence, biodegradability, as well as their efficient and rapid structural printing into desired patterns via crosslinking. Most biopolymers display high cell-adherence, in particular those deriving from the natural ECM from two aqueous fluids. Zoom-in look of the non-planar design at the flow-focusing junction and gradual coverage of the core fluid by the shell fluid (stained in yellowish). Differential interference contrast imaging of the aggregate morphology of the embryonic spheroids, and immunohistochemical staining of the pluripotency marker Oct-4 (green) with Hoechst nuclear staining (blue); and immunohistochemical staining of cardiac myocyte-specific marker proteins including cTnT (red), α-actinin (green), and connexin 43 (Cx 43, green) within the differentiated microgels. Reproduced with permission.[340] Copyright 2013, Royal Society of Chemistry.
Figure 9. Microfluidic fabrication of microfibers for muscle tissue engineering. A) Generation of microfibers via PLGA spinning with a microfluidic chip. SEM micrograph of fiber surface (scale bar: 100 µm) and their cross section (scale bar: 50 µm). L929 cells (fibroblasts) tend to align well along the longitudinal direction as the fibers become thinner. Reproduced with permission.[359] Copyright 2008, American Chemical Society. B) Scheme of a coaxial
(e.g., collagen, fibrin, etc.) as they retain active sites for cell anchorage. Such polymers typically also support cell proliferation and bioactivity. Polymers with low intrinsic cell-adherence (e.g., PEG) can be chemically modified by introducing motifs for promoting cell attachment. The degradability of polymers is crucial to permit tissue remodeling pursued by the cells themselves, which digest the scaffolding material and replace it with newly secreted matrix over time. Crosslinking can be controlled via various principles, which renders hydrogels extremely versatile and customizable for specific applications. In addition to enzymatic crosslinking reaction, polymer crosslinking can be mediated by chemical and physical methods based on ionic or pH-reactivity and optical or thermal treatment, respectively.\[193-195\] Suitability for fabrication and high compliance to the cell needs are the two desirable features for biofabrication materials, but they can hardly combine in one single material.\[112,196,197\] Hence, to select the appropriate materials for biofabrication, the specific purpose of fabrication has to be clearly defined. This way, the effects of the different properties of the candidate biomaterials on the process can be preliminary predicted and the material suitability well assessed.

Furthermore, the technical setup for microfluidic fabrication has to be defined with consideration of relevant parameters affecting the process of tissue formation. In microfluidics, the fluid behavior is dominated by viscous forces and surface tension, whereas gravitational and inertial forces have a much more limited impact.\[198\] Due to weakening of the inertial effects, laminar flows emerge over chaotic or vortical flows. Microfluidic platforms allow one to exploit the laminar flow regime to precisely establish fluidic patterns. This can be achieved by finely designing the geometry of microfluidic channels, as well as, by adapting the system pressurization, tuning the flow rates, and tweaking the surface tension arising at the interface between different fluids. Since the mixing of molecules under laminar flow regime mostly occurs via diffusive exchange, the development of chemical reactions can be controlled with a high temporal resolution.\[199\] Fast chemical reactions are important for instant to control polymeric crosslinking during cell-laden tissue fabrication, and they enable generation of bioconstructs with specific morphologies and microscale pre-defined features. Moreover, flow rates have been found to differently impact the process of the bioconstruct fabrication. For instance, grooved alginic microfibers were formed within a microfluidic device according to the design of a multifluid laminar flow and variable concentrations of the injected sodium alginate solutions.\[200\] The width and the morphology of the fibers could be controlled by adjusting the flow rates of constituent solutions, namely alginate and CaCl₂.

Biomaterials’ physical properties can affect microfluidic biofabrication as well. In the same work, the effects of the alginate’s viscosity on the fiber fabrication were also investigated, revealing that using solutions with similar viscosities establishes better experimental conditions for fabrication (i.e., stabilization of the laminar flow within the microchannels), yielding to better results in terms of fiber morphology, such as uniform and prominent microgrooved patterns. The accurate choice of biomaterials can lead to produce performant formulations of bioinks. Mixing different biopolymers can help to overcome their individual limitations. As building heterogeneous 3D tissue constructs via microfluidic bioprinting is enhanced by using low-viscosity bioinks, Colosi et al. produced a 3 mm thick synchronously-beating cardiac tissue starting from a printed endothelial cell-laden fibrous construct by using a GelMA bioink that was added with alginate to prevent spontaneous GelMA gelation and maintain a low bioink viscosity.\[201\] Microfluidic bioprinting with capability of high cell viability preservation (80% viable cells) was achieved, enabling the production of the initial fibrous bioconstruct via a coaxial extrusion needle coupled with combining Y-shape microchannel microfluidic device. The endothelialized construct was then seeded with cardiomyocytes to generate the cardiac actuator.

In muscle tissue engineering, one important morphological feature to replicate is the highly ordered matrix architecture that displays the uniaxial aligned cell organization as a crucial functional hallmark. Cell alignment is instrumental to achieve correct myoblast cell fusion to form myotubes which then cluster in an oriented fashion to form myofibers, the functional contractile unit of the tissue. Cell alignment can be induced by applying shear. The possibility to guide cell alignment through shear stresses renders microfluidics a valuable research tool. Moreover, the possibility to generate polymeric cell-laden microfibers makes microfluidic fabrication promising in this field. Coaxial microfluidic extrusion was used to generate muscle precursor cell-laden aligned fibrous constructs that eventually evolved to skeletal muscular tissue composed of myofibers upon in vivo subcutaneous implantation in mice.\[213\] The bioink, composed of alginate and fibrinogen-PEG, degrade over time to be replaced with microfluidic chip for wet spinning of chitosan-alginate hybrid fibers. Optical and SEM imaging of the generated microfibers. Fluorescent imaging of microfibers encapsulating HepG2 cells (live cells in green, dead cells in red; the scale bar: 100 μm), showing that cells in the hybrid fibers were more viable than those in pure alginate fibers, suggesting that cross-linked chitosan provides a better cell environment. Reproduced with permission.\[230\] Copyright 2011, American Institute of Physics. C) The grooved microstructure on the hydrogel fibers provides an anisotropic substrate for murine myoblast cell (C2C12) culture. Grooved fibers with heterogeneous components can be generated via co-spinning GelMA and sodium alginate to form interpenetrating polymer networks. SEM images show the cells on the fibers, and fluorescence images highlight the orientation of F-actin (green) and cell nuclei (blue) within cells on homogeneous and heterogeneous grooved microfibers (scale bar: 100 μm). Reproduced with permission.\[219\] Copyright 2021, Royal Society of Chemistry. D) Microfluidically-derived composite microfibers, containing fluorescent microballoons, are manually assembled into woven structures: The braided strand, helical tube, and knot (scale bars: 200 μm). Microfibers encapsulated MG63 cells (in red) and HUVECs (in green) in the outer and middle layers respectively, as shown by confocal imaging and 3D assembled image by Imaris. (scale bars: 500 μm). Reproduced with permission.\[217\] Copyright 2016, Elsevier. E) Three-compartment alginate microfibers with hollow channels visualized by confocal laser scanning microscopy (scale bars: 200 μm). Four-compartmental core–shell microfibers encapsulating NIH 3T3 cells and HepG2 cells, stained in green and red, respectively. Manually criss-cross weaving and stacking microfibers generates gridding and layer-by-layer scaffold architectures for tissue engineering. Reproduced with permission.\[209\] Copyright 2016, American Chemical Society.
### Table 3. Cell-laden microfibers generated by microfluidic techniques from different biomaterials.

| Material          | Cells                              | Major Findings and Study Significance                                                                 | Ref. |
|-------------------|-----------------------------------|-------------------------------------------------------------------------------------------------------|------|
| Alginate          | Mouse fibroblasts (L929)          | Continuous production of calcium alginate fibers with a microfluidic platform. Fiber size was well regulated by changing flow rates. Cells survived well during the fabrication. | [366]|
|                   | Bovine endothelial cells          | Endothelialized tubes for self-developing capillary-like networks were generated from alginate fibers that were embedded in collagen gels and enzymatically degraded to create channels. Cells attached to channel surfaces and form capillary-like structures. | [367]|
|                   | Endothelial cells (HIVE 78)       | Cell viability was preserved in hollow fiber fabrication. Vascular tissue-mimicking hollow fibers were realized. | [368]|
|                   | Smooth muscle cells (HIVS-125)    | Precise control of inner and outer hollow fiber diameter via coaxial triple cylinder. Two cell populations could be separately located in the hollow core and the gel part, preserving cell viability. | [280]|
|                   | Mouse fibroblasts L929            | Microfluidic system for digital, programmable flow control to fabricate microfibres coded with varying chemical composition and topography (including gas micro-bubbles, nanoporous spindle-knots, and joints) for spatially controlled co-culture of encapsulated cells. | [369]|
|                   | Human hepatocytes (HepG2)         | Fibers containing hepatocytes at the center closely sandwiched by fibroblasts mimicked the hepatic cord structures. High hepatocyte viability and bioactivity (increased albumin secretion and urea synthesis) over 30 and 90 days, respectively. The hepatic constructs were recovered as scaffold-free micro-organoids by enzymatic digestion of the alginate hydrogel. | [370]|
|                   | Rat hepatocytes                   | Continuous fabrication of flat fibers with grooved microstructures whose number and dimensions are controlled by regulation of the slit-shaped channel allowing for mass production of tissue engineering scaffolds. | [360]|
|                   | Glioblastoma multiforme cells     | A 2D fluid-focusing technique with multi-inlets and junctions spatiotemporally controlled the continuous laminar flow. Fibers loaded with magnetic nanoparticles incorporated the drug diclofenac, whose release was controlled by applying a magnetic force. The material demonstrated good cell biocompatibility. | [371]|
|                   | Mouse fibroblasts (3T3)           | Stripe-patterned heterogeneous hydrogel sheets orient cell proliferation, forming arrays of rod-like heterotypic organoids, mimicking in vivo hepatic cord structures. The co-culture increased hepatic functions. | [372]|
|                   | Human hepatocytes (HepG2)         | In situ construction of a 3D fibrous scaffold on a single microfluidic platform. High cell viability and molecule diffusion within the fibrous structure. Limited alginate fiber damage. | [373]|
|                   | Rat cortical cells                | Microfibers with bamboo-like appearance are produced in a one-step strategy combining a droplet microfluidic technique with a wet-spinning process. Microfibers are incorporated with polymer spheres or multicellular spheroids. | [374]|
|                   | Mouse mesenchymal stem cells      | Fibers were produced in a ‘L-shape’ cylindrical-flow channel in the microfluidic platform. Good cell pro- liferation and endothelialization when the matrix was enriched with vascular endothelial and fibroblastic growth factors. Mild host reaction occurred when fibers were implanted in vivo. | [375]|
| Alginate          | Mouse fibroblasts NIH 3T3         | Formation of solid-soft-solid hydrogel fibers, guiding the growth direction of cells inoculated in the soft-core. Linear colonies were formed. Cells aligned along the fiber direction. Neurites elongated generating cellular networks. | [376]|
|                   | Human endocervical cancer (HeLa)  | GelMa increases fibers’ mechanical moduli, enhances stretching performance, and reduces swelling. Robust cell proliferation and gene expression upregulation are observed. | [377]|
|                   | Rat adrenal medulla (PC12)        | Cell viability and ordered alignment, indicating that grooved microfibres have good biocompatibility and orientational function. | [378]|
|                   | Mouse myoblasts (C2C12)           | Tunable fiber morphology and structure via multiple laminar flows. Addition of ECM or GelMa improves cell bioactivity (albumin secretion and urea synthesis) and proliferation. Demonstration of complex architectural cellular constructs. | [379]|
|                   | Mouse fibroblasts (NIH 3T3)       | To increase cell adherence, phenol-substituted amylopectin is used as templates for preparing multicellular spherical tissues (MSTs) and endothelialized curved tubular structures in type I collagen gel. Endothelial cells adhered on the tubular cavity surfaces and formed tubular structures. | [380]|
|                   | Human hepatocytes (HepG2)         | Production of filament-like structures composed of two heterogeneous living cell layers. The Alg-Phe solution was gelable through an enzyme-catalyzed reaction, cross-linking the Phe moieties together. Cells enclosed in the hydrogel tubes were viable and filled the hollow core forming a filament-like structure of HeLa cells covered with a layer of fibroblasts. | [381]|
| Phenolic hydroxyl | Human aortic endothelial cells (HAEc) | The mean orientation of cells and the spatial variation of cell alignment angle directly related to the microfiber diameter. Cell orientation increased as the fiber diameter decreased. | [359]|
| alginate          | Mouse fibroblasts (L929)          |                                                                                                       |      |
Table 3. Continued.

| Material         | Cells                                         | Major Findings and Study Significance                                                                 | Ref.   |
|------------------|-----------------------------------------------|--------------------------------------------------------------------------------------------------------|--------|
| Alginate-Chitosan| Human hepatocytes (HepG2)                     | Chitosan addition in the alginate fibers increases cell viability and adhesion in the chitosan-alginate fibers | [361]  |
| Collagen-Fibrin  | Mouse fibroblasts (3T3)                       | Metre-long core–shell hydrogel microfibres incorporating ECM proteins and various cell types can be fabricated through a double-coaxial laminar flow in a microfluidic device. When transplanted into the subrenal capsular space, fibers encapsulating primary pancreatic islet cells normalized glucose concentration in the blood of diabetic mice. | [281]  |
| Gellan Gum       | Rat cardiomyocytes                            | Heterogeneous structural color microfiber is designed for dynamic cardiac mechanics sensing. Fibers contain non-close-packed colloidal arrays for quantitative optical sensing. Dynamic color variation and wavelength shifts of fibers are induced by cardiomyocytes' beating, which transforms microcosmic cell-generated force into macroscopic optical signals. | [322]  |
| Matrigel Collagen| Rat cardiomyocytes                            | Mosaic hydrogel sheets are continuously produced in a microfluidic device where secondary biopolymers are incorporated within a flowing biopolymer sheet. The sheets have tessellations that can be populated with biomolecules, microparticles and viable primary cells. | [224]  |
| Alginin-peptide  | Rat fibroblasts (NIH 3T3)                     | A tubular microfiber supports in situ synthesis of bacterial cellulose and it is removed after the formation of a cellulose microfiber. Millimeter-scale cellular constructs of various shapes were formed, such as coiled and ball-of-yarn-shaped structures. Cell proliferation increased. | [382]  |

Figure 10. Microfluidics-assisted bioprinting of muscle. A) Schematics of a 3D bioprinter coaxial extruder for muscle fiber printing. B) X-ray microtomographic scan of 3D bioprinted structure composed of unidirectional aligned PEG-Fibrinogen hydrogel fibers. C) Tiled immunofluorescence microphotographs of a large region (300 × 2000 µm) showing multinuclear syncitia formation and MHC expression. D) Myotube orientation is represented in a polar chart where 0° corresponds to the printing direction. Reproduced under the terms and conditions of the CC-BY license[383] Copyright 2017, The Authors. Published by Elsevier.
Figure 11. Vascularization of engineered muscle tissue and inherent microfluidic technologies. A) Skeletal muscle constructs bioprinted at the centimeter scale by creating internal perfusion networks. The bioprinted skeletal muscle was composed of multi-layered myofiber bundles, and it contained perfusable microchannels. Reproduced under the terms and conditions of the CC-BY license. Copyright 2018, The Authors. Published by Springer Nature. B) Innervated 3D bioprinted centimeter-scaled muscle tissue that relies on a perfusion network, fabricated from removal of sacrificial ink. Reproduced under the terms and conditions of the CC-BY license. Copyright 2020, The Authors. Published by Springer Nature. C) An integrated micro-physiological system that replicated perfused cardiac muscle or solid tumor in an optically transparent PDMS chip (top left). Endothelial cells and lung fibroblasts mixed with biocompatible matrices created a robust network of microvessels (displayed in green, top right). Cardiomyocyte spheroids were added (red, bottom left) and by injecting microspheres (red dots and white arrows, bottom right) into the fluidic channels, the efficiency
by newly formed and highly aligned myotubes by 4 weeks post-implantation.

Another important achievement in muscle tissue engineering is fabrication at a high cell density. Cell seeding concentration is of major importance for organogenesis in 3D cell culture models, as it closely packs the cells together, bringing them close to their neighbors and allowing better cell-to-cell communication. Biofabrication at a high cell density becomes even more crucial in muscle tissue generation, as the cell density acts as a key regulator of muscle tissue maturation affecting cell differentiation and alignment. In certain engineered muscle tissue models, the optimal cell seeding densities have been identified, which enable to optimize the dynamical performance of the bio-actuators as measured as peak force generation and myogenic gene expression data. Nevertheless, higher cell concentrations yield to the formation of a more physiologically relevant tissue featuring the phenotypic profile of slow, postural muscle. Novel microfluidic printing devices can regulate the printed cell concentration in real-time. Recently, microfluidic bioprinting at high cell concentrations has been demonstrated by concentrating and dispensing fibroblasts at concentrations up to 10 million cells per milliliter. In this study, microfluidic printing of bladder organoids was also shown, to prove the capability of the technology to handle mechanically sensitive primary cells and further demonstrate the relevance of cell concentration in establishing cell-to-cell interactions.

4.2.4. Strategies toward Muscle Tissue Vascularization

As is the case for other types of tissues, muscle tissue relies on an efficient distribution of oxygen and nutrients for its survival and functionality. Distribution networks can be engineered by sculpting the substrate materials with networks of channels. These fabrication processes can be conducted in a controlled manner upon the conditional removal of sacrificial templates from microfluidic chips. These approaches allow the skeletal muscle constructs to reach a high perfusion rate throughout their whole volume.

Another route to vascularized muscle consists in co-culturing myogenic cells with endothelial cells, which then self-organize into vessel-like structures that can be potentially exploited for artificial fluidic convection. Various approaches can be followed to reconstruct vascularization in microfluidic platforms using endothelial cells. To engineer the vasculature in microfluidic platforms, the vascular cells can be directly patterned on the microfluidic channels by marking a specific area in the device and plating the cells on the marked pattern. Alternatively, one can use temporary molds to secure spaces with structures and create good cell compartmentalization. The molds are then removed so that the vascular cells can be precisely distributed. Another method aims to create patterned microchannels in the device by means of soft lithography. The channels are then filled with hydrogel and cells, which develop the biological vessels. In this approach, the design of the channels determines the geometry (size, orientation, interconnection, etc.) of the newly-formed vasculature. In contrast, the vasculature self-assembly in microchips occurs in the absence of any guidance structure, since, when the cells are seeded into hydrogels, they can autonomously reconstitute 3D vessel networks that display a highly physiological arrangement. Importantly, microfluidic platforms allow us to implement and study the mechanical, chemical or biological factors that trigger the formation of vascular systems from seeded cells. Shear stress and interstitial flows are the main fluid flow forces acting on endothelium on-a-chip models; cells react to mechanical stimulation by drastically adapting their morphology through changing their polarization and alignment. Vasculature is typically conditioned chemically via concentration gradients of angiogenic factors, which play a pivotal role in guiding the direction of angiogenesis or sprouting during vessel formation. The biological factors that affect vessel development can also be assessed through culturing multiple cell types on a single chip, where endothelial cells are flanked by other cell types, such as progenitor, stem or supporting cells. The paracrine signaling that occurs during heterotypic cell interaction enables the establishment of structurally stabilized vessels with highly biomimetic cell niches that include stem cells, mural cells, smooth muscle cells, pericytes, and others.

In the last decade, many relevant studies have been carried out in perfusing and vascularizing engineered muscle tissue (Figure 11). In 2018, skeletal perfusable muscle tissue blocks were bioprinted at the centimeter scale (Figure 11A). Briefly, a cell-laden bioink containing myoblasts, an acellular sacrificing hydrogel, and a supporting polycaprolactone pillar were co-printed in a layer-by-layer fashion. The result was a macroscaled tissue composed of multi-layered myofiber bundles, containing perfusible microchannels. Later on, the same group also innervated this muscle construct while maintaining the perfusion system fabricated upon the removal of a printed sacrificial ink (Figure 11B). Nevertheless, the culture of these muscle constructs was run under static conditions, and no contractile behavior was characterized. These studies report on advanced biofabrication, with important achievements in relation to the engineering of perfusable networks within muscle blocks at a critical size. Nevertheless, although resonant, such bioengineering results could be ameliorated by complementing the generated tissue with a system for dynamical culture.
Microfluidics is essential to create integrated micro-physiological systems for tissue culture. Moya et al. exploited microfluidics to recapitulate the dynamic in vivo features of the microcirculation (Figure 11C). They built a microfluidic platform that replicates perfused cardiac muscle tissues or solid tumors in an optically transparent PDMS chip. In this study, endothelial cells and lung fibroblasts were mixed with biocompatible matrices and microinjected into the central tissue chamber. They were allowed to gel for up to 3 weeks, when a robust network of microvessels developed. To create specific micro-organ systems, additional cells of a different cell type (such as tumor cells or cardiomyocytes) were added to the tissue chamber in the form of spheroids (displayed in red in Figure 11C, bottom left). In particular, cardiomyocytes demonstrated long-lasting viability (around 4 weeks) as sustained by the microvessel network grown in the surrounding tissue. By injecting microspheres (red dots and white arrows in Figure 11C, bottom right) into the fluidic channels, the efficiency of the network's perfusion was verified. Other groups approached the perfusion problem through biomimetic strategies based on purely synthetic manufacturing, without using any vascularizing cells. For example, Figure 11D displays a hierarchical design for microfluidic channels that mimics the natural architecture of vascular systems. This platform was realized by means of a single-mask photolithography process that uses an optical diffruser and produces a backside exposure.

Using microfluidics to distribute gases and nutrients within cell culture has been a subject of interest for many years. Microfluidic strategies to grow and maintain cells first as adherent monolayers and then as 3D assemblies have been investigated for more than one decade. Figure 11E depicts the scheme of a single-mask photolithography process that uses an optical diffruser and produces a backside exposure. For example, Figure 11F displays a microfluidic device to study the mechanobiology of the intimal-medial vascular unit. Here, three parallel microchannels were separated by a thin PDMS membrane with pores to run the co-culture of interacting endothelial cells (green, in live cell staining) and vascular smooth muscle cells (red) under hemodynamic conditions. The muscle cell orientation was analyzed revealing that the aligned growth is promoted under dynamic culture conditions. This collection of studies demonstrates an increasing interest in perfusing and vascularizing tissues by applying micro-controlled fluids. On the one hand, acellular strategies for perfused muscle biofabrication (such as bioprinting) are promising for upscaling tissue constructs, but might become fully effective by synergizing with microfluidic culture. Microchannel architectures that mimic the natural vessel spatial organization or the capillary permeability represent two attempts toward microfluidic vascularization that do not include vascularizing cells, and are applicable to muscle tissue. Other architectural features found in the microvascular networks of natural muscles (such as, bifurcations, tortuosities, and cross-sectional changes) have been replicated on PDMS chips to establish micro-physiological systems. On the other hand, vascular networks generated by cells have also attracted a lot of interest. Endothelial cells and other cell types that support vessel formation and growth have been also extensively studied within microfluidic systems, also in combination with muscle cells and 3D cell configurations. Interestingly, organoids combining endothelial cells and aligned myocardium capable of spontaneous and synchronous contraction were embedded in a specially designed microfluidic perfusion bioreactor to generate an endothelialized myocardium-on-a-chip system. Such a study highlights the promise of culturing functional muscle tissue through microfluidic bioreactors. Altogether, these different approaches set the foundation for future microfluidic endeavors toward muscle perfusion and vascularization.

4.2.5. Functional Control of Muscle Tissue in Microfluidic Devices

In bio-hybrid robotics, the living actuators are mostly controlled by two types of stimuli, through which the contraction and relaxation of muscle cells can be triggered on demand. The first type is electricity, which is provided by placing the electrodes either in the culture medium or in close or direct physical contact with the muscle tissue. The second type of stimulation is light, which can actuate the muscle cells that have been genetically modified to express optically-responsive membrane channels. These optogenetic proteins start membrane depolarization and cell contraction upon illumination at specific wavelengths. Interestingly, microfluidic platforms can be designed to integrate both types of stimulation, as the chips are made of materials that are compatible with both light transmission and electrical convection. Indeed, in addition to its bioreactor-like activity which preserves and grows tissues out of a living physiology, microfluidics can implement biophysical stimulation techniques in microscale models. These biophysical stimulation techniques typically include mechanical, electrical, and fluid flow-induced cues, which can be regulated, combined, and applied simultaneously. Such systems can mechanically stimulate 3D cultures of cardiomyocytes through uniaxial cyclic strain, and they can electrically pace the tissue constructs while also delivering a pharmacological treatment. This way, microfluidic technologies improve tissue development, which enhances muscle performance in terms of functionality and actuation. Importantly, muscle actuators have already been included in synthetic platforms that enable the control of tissue contraction. Such a combination represents a step into the machine bio-integration challenge. Importantly, the translation of microfluidic bio-actuators to robotics might occur also as a natural consequence of their ability to integrate into synthetic structures.

Microfluidic devices and technologies for mechano-electrical stimulation of cells can be efficiently built up in smart, functionally integrated platforms. To control cell bio-actuation, electrical...
stimulation within the microfluidic devices has been investigated. One common technique starts from culturing contractile cells and ECM-like matrices in constructs designed as muscular thin films within a microfluidic channel (Figure 12) [422]. To this purpose, through microcontact printing, the ECM is linearly patterned on a thin PDMS layer. This film lays on a substrate that can orient muscle cells which then form an anisotropic muscle tissue layer. After myocardial tissue was formed, the cell layer was peeled from the surface of the device, eliciting the film deformation. In fact, upon cell contraction, the films bend up, and such a deformation allows one to measure cell contraction forces under flow. One typical application of such functional platforms is drug testing in motor function. For instance, Agarwal et al. presented a high throughput “heart on a chip” model based on such a design, namely thin film cantilevers made of soft elastomer and muscular microtissue [422]. The group could calculate the diastolic and systolic stresses from the deflection of the muscular thin films while working on a reusable and completely autoclavable one channel fluidic microdevice. Their device incorporated a metallic base fitting a heating element for temperature control, embedded electrodes for electrical stimulation and a transparent top surface for recording of the dynamic experiments. Dose-dependent contractility changes in response to the addition of isoproterenol, a non-selective beta-adrenergic agonist of the cardiac system, were demonstrated. In following researches, heart-on-a-chip systems based on actuatable muscular thin films have been realized with patient-derived and genetically engineered pluripotent stem cells to replicate pathophysiology of cardiomyopathies, including those caused by mitochondrial dysfunctions like the Barth Syndrome [423]. These platforms enabled scientists to unravel metabolic, structural, and functional abnormalities associated with the genetic mutations that were relevant for the pathologies.

Despite their popularity in bio-hybrid robotics, electrical and optogenetic control approaches have some disadvantages. For example, if electrical simulation is pursued for a long time, it can trigger muscle fatigue, which exhausts contractile cells and causes them to lose their responsiveness to the stimuli. The optogenetic way requires genetically manipulating cells, and optimal light transmission cannot be guaranteed in every environment (e.g., medium with high opacity). Therefore, the search for alternative control methods has intensified. One emergent approach is to use cellular effectors, such as neurons. These cells can command muscle cell contraction in a more biocompatible and sustainable way, by physiological electrochemical communication. To achieve neurological control of bio-actuators based on skeletal muscles, one has to engineer systems that replicate the neuromuscular junctions [424]. Importantly, the formation, maintenance, and disruption of such biphasic tissue have been modeled in a multitude of microfluidic platforms. Similarly, sympathetic neuron innervation should be studied to understand and control the activity of cardiac muscles. In bio-hybrid robotics, sensory-motor abilities can be achieved by using neural units to command muscle activity. In 2019, Aydin et al. demonstrated neuromuscular actuation in a bio-hybrid swimmer (shown in Figure 13A), paving the way for a new
Figure 13. Neurmuscular actuators and their implementation in microfluidic platforms. A) A scheme and microphotograph of a bio-hybrid swimming robot that implements neuromuscular actuation. Reproduced with permission.[425] Copyright 2019, National Academy of Science. B) A 3D co-culture platform combined with a MEA electrophysiology for the generation of neuromuscular bio-actuators. Reproduced with permission.[426] Copyright 2020, AIP Publishing. C) A 3D functional model of a neuromuscular junction, formed from the self-organization of human muscle progenitors and human iPSCs-derived motor neurons. Reproduced with permission.[427] Copyright 2019, eLife Sciences Publications. D) A 3D bioprint of myogenic precursor cells and neural stem cells to generate innervated muscle tissue with a perfusion channeling network at the centimeter scale. Reproduced with permission.[428] Copyright 2020, Springer Nature. E) An optically excitable 3D motor unit in a microfluidic platform where the tissues were cultured in a compartmentalized setting. Reproduced with permission.[429] Copyright 2016, American Association for the Advancement of Science. F) A microfluidic platform in which an optogenetic motoneuron neurosphere projects neurite extension to the skeletal microtissue. The innervation of the skeletal micro tissues and muscle striation after 10 (left) and 20 days (right) of co-culture are shown by confocal microscopy. Reproduced with permission.[429]
generation of bio-hybrids with multiple embodied functions.\textsuperscript{425} In this robot, a muscle-based bio-actuator was controlled by neural cells capable of stimulating the muscle cyclic contractions. In turn, the actuation drove the deformation of flagellar synthetic appendages, providing thrust for untethered forward locomotion. The same author designed a 3D co-culture platform suitable for the generation of neuromuscular bio-actuators (Figure 13B). The microfluidic tissue chambers are combined with a MEA electrophysiology to study the synergistic interactions in the co-development of muscle tissue and neural networks.\textsuperscript{426} If such neurotized bio-actuators were to be combined with effective designs for microfluidic perfusion, they would be greatly enhanced and brought to larger sizes while maintaining functionality in both neurological and muscular compartments.

Several 3D neuromuscular junction models have been recently reported. Figure 13C presents one system formed by the self-organization of human muscle progenitors and human iPSCs-derived motor neurons,\textsuperscript{427} whereas 3D bioprinting allowed for the generation of centimeter-sized innervated muscle tissues from myogenic precursor cells and neural stem cells (Figure 13D).\textsuperscript{407} In these models, neural and muscle cells were assembled together and kept in the same culture environment. However, compartmentalizing muscle cells and neurons might serve to create cell type-specific microenvironments and more functional tissues. Microfluidic platforms offer the chance to compartmentalize tissues of different types while maintaining a functional connection between them (Figure 13E).\textsuperscript{428} In recent years, neuromuscular junction-on-a-chip systems have emerged with various examples reported, where microfluidic channels serve to connect separate populations of skeletal myoblasts and motor neurons. In 2019, Vila et al. presented a microfluidic platform with distinct chambers for neurons and muscle tissue, in which an optogenetic motoneuron neurosphere projected neurite extensions to a skeletal muscle microtissue (Figure 13F).\textsuperscript{429} The muscle microtissue was innervated after 10 days of co-culture, while the muscle striation appeared after 20 days. Figure 13G shows a micro-physiological 3D model of amyotrophic lateral sclerosis, where a human motor unit was realized with a compartmentalized arrangement of human muscle cells and optogenetic motor neurons derived from iPSCs.\textsuperscript{430} Each unit consisted of a muscle fiber bundle attached to pillar structures and a neural spheroid, located in two different chambers. After the skeletal myoblasts formed the fiber bundle in one compartment, a motoneuron spheroid with collagen gel was injected into another compartment. In this system, the neurite outgrowth occurred after 2 weeks of culture. Motor neurons and human skeletal myoblasts were also compartmentalized in the platform used by Santhanam and colleagues (Figure 13H).\textsuperscript{431} Finally, microdevices with separate chambers for the precise compartmentalization of motoneurons and skeletal muscle tissues, which fit the well size of 24 well plates, have been proposed tools for fundamental research and drug development for neuromuscular disorders (Figure 13I).\textsuperscript{432}

Of note, microfluidic platforms for neuromuscular junction modeling can also include optically excitable cells. Uzel et al. differentiated motor neurons from mouse embryonic stem cells and co-cultured them with myoblast-derived muscle strips within a 3D hydrogel (Figure 14).\textsuperscript{433} The two cell types were physically separated, as the microfluidic device was designed to mimic the spinal cord–limb physical separation (Figure 14A). This configuration not only facilitated the observation of the 3D neurite outgrowth from motor neurons and the subsequent muscle innervation, but also well combined with the use of optically excitable motor neurons derived from photosensitized stem cells. By using this microfluidic platform, the group demonstrated spatio-dynamic and noninvasive control of motor units via optogenetic cell stimulation, showing that force and frequency of muscle-twitching could be modulated by varying the light intensity during irradiation (Figure 14B,C).

This collection of studies demonstrates the current efforts to reconstruct the functional integration of natural motor units in vitro. Microfluidics can meet this challenge by controlling the assembly and spatial organization of different tissue types, and by guiding connective elongated structures (like the axonal projections) through its channels. If transferred and adapted to dynamic systems, microfluidic neuromuscular constructs might represent the enabling technologies of future bio-hybrid robots controlled by neural activity.

5. Conclusions
The growing body of literature at the interface between fluidics and tissue engineering depicts the great potential that microfluidic technologies have when they are applied to bio-hybrid machines, and the next steps are expected in the field of actuation.\textsuperscript{30,97,433–436} In addition to the bio-hybrid intrinsic nature and component materials that are common in soft robotics, microfluidics has the ability to generate microscaled muscles that might be able to work autonomously as micromachines, or grouped and assembled into larger actuators. Moreover, microfluidics can modulate muscle tissue development through various approaches. It can improve cell–cell interaction and the biochemical microenvironment, deliver controlled electro-mechanical stimuli, fabricate fibrous biomaterials as substrates for cell growth, and promote the interaction of multiple cell types.\textsuperscript{137,435,437}

Although there has been recent progress in microfabrication and design, which has rendered microchips extremely performant in several bioapplications, some technical complications...
still persist. One of the major concerns to deal with in the next future is the incompatibility of chip materials with hydrophilic matter. For example, systems made of widely used PDMS pose challenges in relation to their integrability with biomaterials and cells. Importantly, key developments are needed in interfacing the constituent materials with fluids and biological components. This would be a great multidisciplinary advancement, since microfluidics also has bio-integrability issues, along with the engineering of all other bio-hybrid systems, including proper robots.

Impactful innovation is also made in relation to the portability of the microfluidic systems. In fact, scaling down the enabling components of the fluidical systems (like the pumps) would facilitate the use of microfluidic chips for conventional applications. It would also make them similar to bioreactors, which could perhaps then be integrated into robots with an onboard configuration. Such bioreactors with microfluidic abilities could be a formidable tool to create bio-hybrid robots with long functional duration and untethered motion performances. In order to achieve this, alternative methods that can avoid the encumbrance of the fluidic pumps should also be taken into consideration. For instance, thermofluidic pumps, which allow for fluid flow control through thermal gradients and surface tension variations, efficiently performed in microfluidic platforms, and offer a solution toward system miniaturization. Other fluid dynamic principles could also be investigated to achieve sufficient microfluidic portability to complete the translation to robots.

Future microfluidic platforms for skeletal and cardiac muscle tissue engineering will be designed with more portable configurations, rendering them similar to self-sufficient actuators. Muscles integrated with inherent micro-physiological systems and characterized by a highly biofidelic internal organization will become the next generation of actuators. Thus far, microfluidic chips have enabled a detailed characterization of muscle cell behavior, and they can be easily adapted to use with cardiac or skeletal muscle. The technical characteristics of the microfluidic platforms (design, stimulation methods and patterns, force read-out technique, etc.) were originally implemented for highly specific investigations on myoblasts or cardiomyocytes. These adaptations have, over time, enabled the construction of progressively more sophisticated and biomimetic constructs, expanding our understanding of various pathophysiological processes. Muscle tissue engineering platforms combine well not only with the microfluidic technology, but also with bioelectronics, specific microengineering systems, and the culture of other cell types. Their versatility and predisposition to participate in integrated systems renders microfluidic bio-actuators tremendously promising for the development of the next generation of “on-a-chip” devices, like the multi-organs-on-a-chip platforms. The short and mid-term evolution of microfluidic bio-actuators will include adapting to biomedical applications like the in vitro modelling of activatable deformation, agonistic-antagonistic coordination, or mechanobiology during muscle contraction. In addition, microfluidic bio-actuators will become advanced systems for drug testing, bio-hybrid
reconfigurable technologies for medical implantation, or operative modules to enable motion in bio-hybrid robots.

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Conflict of Interest
The authors declare no conflict of interest.

Keywords
bio-actuators, bio-hybrid robots, microfluidics, muscle tissue, tissue engineering

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[1] J. Hwang, Y. Jeong, J. M. Park, K. H. Lee, J. W. Hong, J. Choi, Int. J. Nanomed. 2015, 10, 5701.
[2] A. Y. Chen, C. Zhong, T. K. Lu, ACS Synth. Biol. 2015, 4, 8.
[3] S. Kim, C. Laschi, B. Trimmer, Trends Biotechnol. 2013, 31, 287.
[4] L. Ricotti, B. Trimmer, A. W. Feinberg, R. Raman, K. K. Parker, R. Bashir, M. Sitti, S. Martel, P. Dario, A. Menciassi, Sci. Rob. 2017, 2, https://doi.org/10.1126/scirobots.aao495.
[5] L. Sun, Z. Chen, F. Bian, Y. Zhao, Adv. Funct. Mater. 2020, 30, 1907820.
[6] S. Liu, W. Xu, Front. Sens. 2020, 1, 6.
[7] P. Q. Nguyen, N.-M. D. Courchesne, A. Duraj-Thatte, P. Praveschothunnt, N. S. Joshi, Adv. Mater. 2018, 30, 1704847.
[8] J. D. Sato, M. Kan, Curr. Protoc. Cell Biol. 1998, 00, 1.2.1.
[9] H. Eghbali, M. M. Nava, D. Mohebbi-Kalhori, M. T. Raimondi, Int. J. Artif. Organs 2016, 39, 1.
[10] D. Karami, S. Bahrami, H. Mirshekari, S. M. M. Basri, A. B. Nik, A. A. Aref, M. Akbari, M. R. Hamblin, Lab Chip 2016, 16, 2551.
[11] A. Manigandand, R. P. Amruthavarshini, S. Sethuraman, A. Subramanian, Cells Tissues Organs 2021, 211, 32.
[12] J. Veldhuizen, M. Nikkhah, J. Visualized Exp. 2021, e62539.
[13] W. J. Polacheck, R. Li, S. G. M. Uzel, R. D. Kamm, Lab Chip 2013, 13, 2252.
[14] H. Bo, M. Yoon, M. Gajendiran, K. Kim, Macromol. Biosci. 2020, 20, 1900300.
[15] W. Li, L. Zhang, X. Ge, B. Xu, W. Zhang, Q. Lu, C.-H. Choi, J. Xu, A. Zhang, H. Lee, D. A. Weitz, Chem. Soc. Rev. 2018, 47, 5646.
[16] T. Sun, X. Li, Q. Shi, H. Wang, Q. Huang, T. Fukuda, Gels 2018, 4, 38.
[17] Q. Zhao, H. Cui, Y. Wang, X. Du, Small 2020, 16, 1903798.
[18] L. Wei, W. Li, E. Entcheva, Z. Li, Lab Chip 2020, 20, 4031.
[19] R. G. Dennis, P. E. Kosnik, In Vitro Cell. Dev. Biol. Anim. 2000, 36, 327.
[20] A. W. Feinberg, Annu. Rev. Biomed. Eng. 2015, 17, 243.
[21] A. W. Feinberg, A. Feigel, S. S. Shevokoplyas, S. Sheehy, G. M. Whitesides, K. K. Parker, Science 2007, 317, 1366.
[22] Y. Morimoto, H. Ono, S. Takeuchi, Sci. Rob. 2018, 3, https://doi.org/10.1126/scirobots.aat4440.
[23] Y. Morimoto, H. Ono, S. Takeuchi, APL Bioeng. 2020, 4, 026101.
[24] T. Nomura, M. Takeuchi, E. Kim, Q. Huang, H. Hasegawa, T. Fukuda, Micromachines 2021, 12, 379.
[25] R. Raman, C. Cvetkovic, S. G. M. Uzel, R. J. Platt, P. Sengupta, R. D. Kamm, R. Bashir, Proc. Natl. Acad. Sci. USA 2016, 113, 3497.
[26] R. Cvetkovic, R. Bashir, Nat. Protoc. 2017, 12, 519.
[27] M. S. Sakar, D. Neal, T. Boudou, M. A. Borochin, Y. Li, R. Weiss, R. D. Kamm, C. S. Chen, H. H. Asada, Lab Chip 2012, 12, 4976.
[28] S. R. Shin, C. Shin, A. Memic, S. Shadmehr, M. Miscuglio, H. Y. Jung, S. M. Jung, H. Bae, A. Khademhosseini, X. Tang, M. R. Dokmeci, Adv. Funct. Mater. 2015, 25, 4486.
[29] M. T. Holley, N. Nagarajan, C. Danielson, P. Zorlutuna, K. Park, Lab Chip 2016, 16, 3473.
[30] L. Vanozzi, L. Ricotti, M. Cianchetti, C. Bearzi, C. Gargioli, R. Rizzi, P. Dario, A. Menciassi, Bioinsp. Biomim. 2015, 10, 056001.
[31] V. A. Webster, E. L. Hawley, O. Akkus, H. J. Chiel, R. D. Quinn, Bioinsp. Biomim. 2016, 11, 036012.
[32] J. Yoon, T. W. Eyster, A. C. Misra, J. Lahann, Adv. Mater. 2015, 27, 4509.
[33] S. R. Shin, S. M. Jung, M. Zalabany, K. Kim, P. Zorlutuna, S. bok Kim, M. Nikkhah, M. Khabiri, M. Azize, J. Kong, K. Wen, T. Palacios, M. R. Dokmeci, H. Bae, X. (Shirley) Tang, A. Khademhosseini, ACS Nano 2013, 7, 2369.
[34] G. J. Pagan-Diaz, X. Zhang, L. Grant, Y. Kim, O. Aydin, C. Cvetkovic, E. Ko, E. Solomon, J. Hollis, H. Kong, T. Saif, M. Gazzola, R. Bashir, Adv. Funct. Mater. 2018, 28, 1801145.
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