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Microfabrication and microfluidics for muscle tissue models

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Abstract

The relatively recent development of microfluidic systems with wide-ranging capabilities for generating realistic 2D or 3D systems with single or multiple cell types has given rise to an extensive collection of platform technologies useful in muscle tissue engineering. These new systems are aimed at (i) gaining fundamental understanding of muscle function, (ii) creating functional muscle constructs in vitro, and (iii) applying these constructs to a variety of applications. Use of microfluidics to control the various stimuli that promote differentiation of multipotent cells into cardiac or skeletal muscle is first discussed. Next, systems that incorporate muscle cells to produce either 2D sheets or 3D tissues of contractile muscle are described with an emphasis on the more recent 3D platforms. These systems are useful for fundamental studies of muscle biology and can also be incorporated into drug screening assays. Applications are discussed for muscle actuators in the context of microrobotics and in miniaturized biological pumps. Finally, an important area of recent study involves coculture with cell types that either activate muscle or facilitate its function. Limitations of current designs and the potential for improving functionality for a wider range of applications is also discussed, with a look toward using current understanding and capabilities to design systems of greater realism, complexity and functionality.

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Introduction

Muscle cells are the most abundant cell types in the body, serving numerous critical physiological functions. At the same time, muscle is subject to a variety of incapacitating or fatal disorders such as Duchenne muscular dystrophy or hypertrophic cardiomyopathy, affecting skeletal and cardiac cells, respectively. Although much of the muscle research is based on *in vitro* studies, limitations of traditional culture systems call for more suitable platforms in order to engineer more physiologically relevant models to investigate muscle cell and tissue biology. The field of muscle tissue models and engineering has evolved in parallel across cell types – cardiac or skeletal. While different in many aspects, starting with their morphology (mononucleated cardiac or multinucleated skeletal muscle cells) or their electrical properties (paced or not, with or without electrical coupling), all muscle cell types share some features (such as their ability to generate active contraction or their substrate stiffness-dependent differentiation efficiency) resulting in the parallel and interdependent development of their study platforms. The main challenges consist of identifying and manipulating the cues that guide differentiation towards contractile lineages, understanding how contraction is impaired in muscular disorders and how to rescue it, and engineering *de novo* surrogates for tissue repair or other, non-medical applications.

Microdevices (used here to represent all micro- and nano-fabricated platforms) have been increasingly deployed in the study of muscle and are beginning to displace the existing conventional assay platforms. Our focus will be on microfluidic devices or 3D printed bioreactors, with an emphasis on 3D culture systems, the 2D platforms being covered already (Théry 2010). Made popular by George Whitesides in the late 90's (Xia and Whitesides 1998), soft lithography makes possible the rapid fabrication of PDMS-based biocompatible microdevices for use in bioengineering and biology. Besides their small dimensions reducing the amount of medium and cells per experiment and their optical clarity amenable to live cell imaging, microdevices allow for the controlled formation of 3D tissues and the ability to apply various well-defined cues, be they chemical, mechanical or electrical. Over the past few years, these devices have proven to be extremely valuable tools to investigate cell behavior in the context of chemotaxis (J. Li and Lin 2011), mechanotransduction (Polacheck et al. 2013) or stem cell research (Kshitiz et al. 2011).

In this review, we will first describe the microdevices that have been employed to shape some of the cues necessary to differentiate or enhance the differentiation of pluripotent cells (embryonic, induced pluripotent, mesenchymal, etc.) into muscle cells (skeletal or cardiac) or their progenitors and offer a better control of the microenvironment to help unravel some of the underlying mechanisms of differentiation. We then introduce systems that allow the formation of physiologically relevant 3D muscle tissue constructs and discuss how they can be used to gain insight in fundamental biology or improve drug screening assay capabilities. Muscle cells have also been used to power micro-robots and this will be the focus of the third section. Finally, we will present some of the latest platforms enabling the investigation of the interactions between muscle cells and relevant partners such as neuronal cells and vascular networks.

1. <u>Control of muscle differentiation in microdevices</u>

Differentiation of functional tissues from pluripotent stem cells (PSC) is an attractive strategy to investigate the underlying biology and physiology, generate patient-specific pathological cells or engineer replacement tissues. Muscle tissue is no exception and a variety of pluripotent cell types have been proposed as potential candidates for myogenic differentiation in this context (Fishman et al. 2013), each having some advantages, along with drawbacks. For example, while very well characterized and exhibiting particularly promising results in *in vivo* muscle regeneration assays, satellite cells (SCs) show decreased proliferative capabilities and quickly lose their ability to form myofibers *in vitro*. On the other hand, embryonic stem cells (ESC) and mesenchymal stem cells (MSC, whose myogenicity is still debatable), present great pluripotency but a risk of tumorigenicity. Finally recent breakthroughs in producing induced pluripotent stem cells (iPS) have offered a new pool of candidates, combining qualities such as accessibility, and their autologous and non-immunogenic nature. However iPS cells require genetic manipulation and further analysis to exclude possible tumorigenic effects.

Various types of stimuli including, but not limited to, biochemical (e.g. soluble factors or ECM), mechanical (e.g. cyclic strain) or electrical have been shown to induce differentiation of source cells into myocytes. And while traditional assays have been key to emulating or gaining insight into myogenic differentiation, it is becoming increasingly apparent that these factors need to be cultured in a microenvironment that replicates some aspects of the *in vivo* system (Hazeltine, Selekman, and Palecek 2013). In recent years, microfluidic and microfabrication technologies have provided a powerful tool to address this issue. In this section, we present the different platforms that have been developed to recreate the chemical, mechanical and electrical microenvironment of pluripotent cells in order to better mimic the endogenous condition under which they naturally differentiate into muscle cells or to enhance the induction efficiency.

a. Chemical

Chemical cues can be provided to the cells in the form of soluble factors or as part of components embedded in the extracellular matrix on or in which the cells are cultured. Some of the challenges over the past years have been to find the right factors that would efficiently induce muscle differentiation from PSCs. For instance, to reliably promote cardiac differentiation of hESCs, (Laflamme et al. 2007; Takei et al. 2009) suggested the use of BMP-4 and Activin-4. Other researchers selected a cocktail of BMP-4, bFGF, Activin-A, VGEF and DKK-1 to induce cardiogenesis in mouse and human PSC cells (Kattman et al. 2010; L. Yang et al. 2008). Another example is the use of betaine at a concentration of 10 mM to induce morphological changes and alter the expression profile of the IGF-1 receptor to promote muscle fiber differentiation in C2C12 cells (Senesi et al. 2013). Others (Ryan et al. 2012) differentiated human embryonic stem cells (hESCs) using retinoic acid and found an enhancement of skeletal myogenesis, in particular with expression of myogenic regulatory factors (MRFs) and MyoD after 25 days of treatment. Manipulating the surrounding matrix can also promote myogenic differentiation (Watt and Huck 2013), as suggested in (Hinds et al. 2011) where the composition of the hydrogel (collagen I, fibrin and/or Matrigel) affected the contractility in an engineered muscle tissue. Similarly, a combination of myogenic factors, TGF-β and ascorbic acid embedded within viscoelastic hydrogels promoted myogenic induction of hMSC (Cameron et al. 2014).

One of the microtechnologies employed to pattern the spatial distribution of ligands in the ECM is bio-printing. Using an injection based growth factors bio-printer, Ker and co-workers were able to control geometrical and biochemical cues in order to promote musculoskeletal alignment and differentiation of C2C12 cells (Ker et al. 2011). The matrix was printed with factors such as FGF-2, BMP-2 in order to direct cells in muscle or osteoblast lineages in a concentration-dependent fashion. More recently,

Almodóvar and co-workers (Almodóvar et al. 2014) developed engineered biomimetic films that present a gradient of matrix-bound bone morphogenic protein (BMP-2 and BMP-7). The gradients were generated by forming a concentration profile of the ligand in a single microfluidic channel positioned on top of the substrate and by letting BMP bind to the ECM. Upon removal of the device and rinsing of the unbound BMP, C2C12 myoblasts were plated onto the ECM and exhibited differentiation largely toward osteogenic lineage but expression of troponin T was observed with a tuning of BMP concentration, generating a myo-inductive substrate as well.

b. Mechanical

Many tissues are exposed to continuous mechanical stimulation and mimicking the *in vivo* environment has been a major focus in this field. The mechanical forces to which stem cells are subjected are essential to regulate gene expression and cell functionality (Dado et al. 2012). In order to emulate the response of the tissue to mechanical stimuli, as produced by interactions with surrounding cells or matrix, or by muscle contraction, numerous approaches have been introduced. We discuss here two main types of stimulus: passive and active. As passive stimuli, we consider involving mechano-topographical cues and scaffold stiffness. While as active systems, we include stimuli that are dynamic and involve time-dependent stretching or forcing.

i. Passive mechanical stimuli

Cells cultured on ECM substrates are subjected to biomechanical signaling due to the structural dimensions, mechanical proprieties, porosity and topography of the matrix. Material stiffness effects on cell biology have been extensively studied and reported. Early studies (Engler et al. 2006) (Fig. 1a) demonstrated the effects of substrate elastic modulus on hMSCs and identified 10kPa as a stiffness value that promotes myogenic differentiation. Since then, micro- and nanofabrication techniques have been employed to regulate the substrate mechanical proprieties in order to promote and control stem cells fate. An example of these methods is found in the work of Kim et al. (D.-H. Kim et al. 2010), where neonatal rat ventricular myocytes cultured on a PEG nanogrooved substrates were found to exhibit increased cardiac tissue functions, as indicated by electric conduction velocity, expression of Cx43 and tissue geometry (Fig. 1b).

The effects of surface topology have also been studied using methods such as laser cutting (H. Li et al. 2012). MSCs cultured on micropatterned features engraved in a biodegradable polymer substrate (poly(L-lactide-co-e-caprolactone)) resulted in a gene expression profile associated with myogenesis. Alternatively, Kroehne and co-workers (Kroehne et al. 2008) induced collagen orientation in order to enhance muscle cell differentiation (Fig. 1c). Parallel pores (20-50 µm) were created with a unidirectional freezing process and subsequently filled with C2C12 cells, promoting muscle cell orientation and increasing force generation. Various studies have employed hydrogels to create a 3D polymer network mimicking the ECM structure. Recently, viscoelasticity of hydrogels was adopted as a myogenic promoter (Cameron et al. 2014), stressing the time dependent deformation (creep) of polyacrylamide gel (PAM) to induce differentiation of hMSCs toward a smooth muscle lineage.

ii. Active

Active stimuli, mimicking the dynamic environment cells experience *in vivo*, involve the time-dependent loading of cells either plated on top of a 2D substrate or immersed in a 3D hydrogel. Typically strain values for muscle and cardiac differentiation are in the range of 1 to 10 % with a frequency of 0.5 to 2 Hz (Vunjak-Novakovic et al. 2010).These effects have been extensively studied, and recently reviewed

(Ghafar-Zadeh, Waldeisen, and Lee 2011; Hazeltine, Selekman, and Palecek 2013; Simmons, Petzold, and Pruitt 2012).

Generating displacement along one axis is the simplest way to generate uniaxial strain in a deformable membrane on which cells are cultured. One of the most successful microfluidic platforms with uniaxial strain distribution is the "Lung on a Chip" (Huh et al. 2010). This design can be adapted to study stem cell differentiation toward cardiac or muscle tissue. Another approach has been employed, in which the entire PDMS microfluidic chip is subjected to cyclical strain in order to promote the differentiation of murine embryonic stem cells into cardiomyocytes (Wan, Chung, and Kamm 2011) (Fig. 1d). Consistent with the literature on increased cardiomyogenic differentiation by cyclic strain, this platform demonstrated upregulation of α-MHC in the mechanically stimulated embryoid bodies while providing higher levels of control and repeatability of the strain input than more traditional assays.

Biaxial strain offers a more appealing high throughput solution. (Tremblay et al. 2014) developed a microscale biaxial cell stretching device following the *lung on a chip* concept (Fig. 1e), where in a flanked chamber vacuum is applied. (Moraes et al. 2010), instead, developed an array of wells with deformable bottom capable of applying strains in the range of 2 to 15% (Fig. 1f). This platform was able to provide variable strain rates promoting the activation of β-catenin in cardiac progenitor cells. These active mechanical platforms, however, pose challenges for imaging. In some cases imaging is obstructed from the active mechanism or in other cases the vertical membrane displacement creates out of focus images.

Mechanical stimulation is easy to control from an engineering perspective, but biologically the results obtained after stimulation are quite sensitive to the type of stimuli applied. The effects on morphology, proliferation rate and activation can change by applying an uniaxial or equiaxial strain (Watt and Huck 2013), while varying the efficiency of differentiating MSCs into muscle cells . Particular attention must be paid to MSCs that under mechanical strain (10-15% similar to the one for muscle differentiation) may follow the osteogenic differentiation path (Qi et al. 2008).
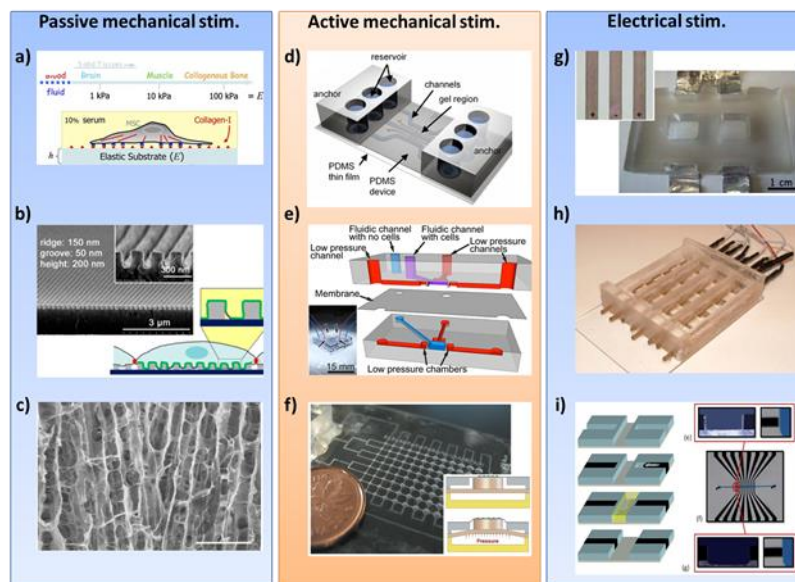

Figure 1: Microfabrication approaches to control myogenic differentiation: passive, mechanical and electrical stimulation. *Passive mechanical stimulation* (left column): a) role of substrate stiffness in order to induce MSC differentiation (reproduced from (Engler et al. 2006) with permission), b) example of microgrooved substrate to promote cell orientation and differentiation (reproduced from (D.-H. Kim et al. 2010) with permission), c) collagen matrix orientation in order to enhance muscle cell

differentiation (reproduced from (Kroehne et al. 2008) with permission). *Active mechanical stimulation* (middle column): d) stretchable microfluidic chip applying cyclic to 3D-cultured mESC toward cardiac differentiation (reproduced from (Wan, Chung, and Kamm 2011) with permission), e) example of biaxial stimulation performed by applying negative pressure to the culture chamber (reproduced from (Tremblay et al. 2014) with permission), f) microwell array with deformable floor membrane pushed by pillar actuator (reproduced from (Moraes et al. 2010) with permission). *Electrical stimulation* (right column): g) micropatterned electrodes for localized electrical stimulation (reproduced from (Tandon et al. 2010) with permission), h) electrodes embedded in PDMS chamber array (reproduced from (Serena et al. 2009) with permission), i) Injection of electrically conductive material in microfluidic channels for *in situ* electrode fabrication (reproduced from (Andrea Pavesi et al. 2011) with permission).

c. Electrical

Bioelectricity and transmembrane potentials have been known to play an important role in stem cell differentiation and development (Jaffe and Nuccitelli 1977; Robinson 1985; Sundelacruz, Levin, and Kaplan 2009). In cardiac tissue, electric fields on the order of a few mV/mm to several hundred mV/mm were measured during embryonic development. And while the use of electric fields on producing myocyte contraction is ubiquitous, its downstream effect on myogenesis is still poorly understood.

Several protocols were tested on neonatal mouse and rat cardiomyocytes in order to study and promote functional cardiac tissue (Au et al. 2007; Chiu et al. 2011; Tandon et al. 2011). Electrical stimulation not only promotes functionality, but can also induce differentiation of stems cell toward cardiac lineage (Dado et al. 2012; Serena et al. 2009; Zimmermann 2011). Moreover, myogenic potential of muscle precursor cells is improved by electrical excitation (Serena et al. 2008). Distinctive electrical stimulation types or protocols (for instance monophasic versus biphasic stimulation) influence stem cell conditioning and promote muscle differentiation with different efficiencies and specific gene regulations (Pavesi et al. 2014; Pietronave et al. 2013).

Microfabricated platforms such as microelectrode arrays (MEA) allow researchers to apply precise electrical stimulation signals and record electrical activity with cellular resolution. Techniques for metal electrode deposition on glass substrate are widely used to microfabricate electrodes (Tandon et al. 2010). On the other hand, these electrodes have several potential drawbacks. First, in most cases, their opacity compromises imaging. Moreover, they are most commonly planar, and as a consequence a uniform distribution of the electric field across the channel thickness is difficult to achieve. In addition, metal deposited electrodes are generally incompatible with deformable devices or substrates that aim to combine mechanical and electrical stimulation (Lim et al. 2006). An alternative simple methodology to embed electrodes in microfabricated chips is mixing conductive material with PDMS and dedicate channels for the conductive mixture injection (Pavesi et al. 2011). This type of platform can be very useful to understand cellular process driven by electrical stimulation at microscale level

2. Microfabricated devices for physiologically relevant and bio-inspired models

The shortcomings generally encountered in traditional culture assays of muscle tissue encompass the physiological relevance, performance and long term survival. Most commonly plated in culture dishes, muscle cells, regardless of their type, evolve in two-dimensional sheets, on substrates of non-matching mechanical properties, in a randomized configuration far from the well-aligned and organized structures they would assume in their native environment, with the risk to detach from the surface when acquiring contractile capabilities (Cooper et al. 2004). Such geometries also prevent the cells from functioning the way their *in vivo* counterparts would.

To address the performance issue, extensive effort has been devoted to generating substrates to guide the formation of aligned muscle tissue. This was done by fabricating substrates with parallel linear microgrooves(Camelliti et al. 2006; Charest, García, and King 2007; Hosseini et al. 2012; Huang et al. 2006; Hume et al. 2012; Lam, Clem, and Takayama 2008; Lam et al. 2006; Yamamoto et al. 2008; Y. Zhao et al. 2009) or by micropatterning the surface with tracks of ECM molecules(Aubin et al. 2010; Bajaj et al. 2011; Camelliti et al. 2006; Nagamine et al. 2010, 2011; Parker et al. 2008; Salick et al. 2014; Zatti et al. 2012).

An alternative to microgrooves or micropatterning to produce cell alignment is the formation of 3D muscle tissues. These 3D constructs are inherently more physiological because 3D tissues formed between attachment points anchors better than adherent cultures and they spontaneously align along the long axis of the construct, enhancing at the same time their performance. Strategies included providing cells with anchoring points (steel mesh, Velcro or suture anchor)(Dennis and Kosnik 2000; Eschenhagen, Fink, and Watfchow 1997; Hinds et al. 2011; Lam et al. 2009; Rhim et al. 2007; H. H. Vandenburgh, Swasdison, and Patricia Karlisch 1991; H. H. Vandenburgh et al. 1996), to have the cells wrap themselves around fixed posts or mechanotransducer levers (Kofidis et al. 2002; Powell et al. 2002; Swasdison and Mayne 1992; Zimmermann et al. 2006). An alternative to attachment point is to form circular strips around cylindrical posts where alignment occurs circumferentially (Gwyther et al. 2011; Okano et al. 1997; Zimmermann et al. 2002). For a review on existing platforms for cardiac tissue engineering for instance, we recommend the work by Liau et al. 2012 (Liau, Zhang, and Bursac 2012).

To address the problem of throughput in muscle tissue assays, Vandenburgh *et al.* designed a system where small muscular tissues (called "miniature bioartificial muscles" or mBAM) were formed in 96 well plates (Fig 2a), each well containing a muscle construct (H. Vandenburgh et al. 2008). The tissue was formed by seeding primary myoblasts suspended in a collagen-Matrigel mix in wells containing a set of two 4-5 mm long pillars, positioned 4 mm apart. The compliant pillars were made of PDMS. Besides forming a large amount of muscle constructs at once, force measurement could be simply accomplished by monitoring the deflection of the pillars whose known mechanical properties allowed for back calculation of the applied force. This rendered obsolete the need for additional force transducers, while offering a non-invasive way to force measurement. Static tension (also called passive or resting tension) reached approximately 700 μN after 6 days in culture, and electrically-triggered tetanic forces were measured to stabilize around 40 μN after 8 days in culture.

The argument made by the authors is that being able to measure the function of the muscle tissue is as important, if not more so, than monitoring its gene expression in the context of drug screening, since protein synthesis does not necessarily correlates with muscle performance. They term this approach High-content drug screening (HCS), and they illustrate it in a subsequent study where the throughput of the platform described above is put to good use, by testing 31 compounds at 3 to 6 different concentrations on muscle tissues differentiated from *mdx* murine myoblasts, the genetic homolog of Duchenne Muscular Dystrophy (DMD) and by merely measuring the output tetanic force (H. Vandenburgh et al. 2009). While acknowledging that the mBAM do not fully replicate adult *in vivo* tissues, the authors ascertain that it represents a "rapid and cost-effective" intermediate step, bridging the cell-based assays and animal studies.

This platform was later adapted by Hansen et al. to accommodate for the formation of engineered heart tissue (EHT)(Hansen et al. 2010). Neonatal rat heart cells, suspended in a Fibrin-Matrigel hydrogel were casted into rectangular chambers containing pairs of PDMS posts in order to shape muscle constructs they call Fibrin-based mini-EHT (FBME). Once wrapped around the flexible pillars, the 8-mm long tissues were transferred to 24-well plates and the contractile activity of 192 samples was optically

monitored over time, under various chemical treatment condition, including chromanol, quinidine, and erythromycin and doxorubicin.

Chen and coworkers developed a similar PDMS pillar-based microfabricated platform for miniature contractile tissue formation (here named "microfabricated tissue gauges" or μTUG). After characterizing the platform on fibroblasts (Legant et al. 2009), they tested the influence of gel density and pillar stiffness on the ability for cardiomyocyte tissue to generate force, measured by the post deflection (Boudou et al. 2012) (Fig. 2b). The primary cardiomyocytes were suspended in 1 or 2.5 mg/ml collagen gels mixed with 0.5mg/mL fibrinogen, and casted around pillars whose stiffness was modulated by changing the ratio of PDMS to curing agent, rendering them "soft" (k=0.20 μN/μm) or "rigid" (k=0.45 μN/μm). Surprisingly, while both the static and dynamic tension slightly increased in the denser (and stiffer) gel, the engineering stress (force over cross-section area) decreased by more than 30%. This is explained by the fact that stiffer gels pose more resistance to compaction, resulting in thicker tissues than in softer gels with poorer cell alignment. On the other hand, stiffer pillars led to an increase in both tension and stress. Moreover, by integrating carbon electrodes on each side of the array of microwells, the authors were capable of electrically stimulating the μTUGs and reported an increase in the performance of the tissue (gel compaction, cell alignment, force generated). Perhaps as important as the ability to non-invasively monitor the tension was the possibility to assess protein expression by immunostaining, an assay impossible to carry out in larger tissues reported in the literature, which require cumbersome and time-consuming histology sectioning. It also allows comparing in real time levels of tension and calcium signaling via fluo-3 imaging. Recently, this same platform was used in an assay where one of the two micropillars was magnetically actuated via an attached nickel bead, which offer the possibility to mechanically stimulate the muscle tissue without causing its contraction(R. Zhao, Chen, and Reich 2014).

This same μTUG platform served as a substrate for light excitable skeletal muscles in a study by Sakar and coworkers (Sakar et al. 2012). By translating the principle of optogenetics, more commonly used in neurons (Boyden et al. 2005; Yizhar et al. 2011), to C2C12 myoblasts, the authors demonstrated how electrical stimulation could be successfully replaced by light. The advantages of optogenetics, as described in this study, are the ability to precisely control the spatiotemporal stimulation pattern. By reducing the cross section area of the excitation beam of light, individual muscle fibers could be actuated while leaving the others unresponsive, resulting in modes of tissue contraction and deformation unattainable with electrical excitation, thereby paving the way towards light-controlled soft robotics. More importantly, the combination of optogenetics and microfabrication could, in the future, decrease complexity and cost thereby enhancing the throughput of drug discovery platforms.

Most of the existing 3D muscle systems, including those presented above rely on some sort of self-aggregation of myoblasts seeded in a hydrogel, giving rise to a muscle construct whose final shape is reminiscent of the original geometry of the chamber and that of the supporting structures, which lead to some inherent heterogeneity. To circumvent this problem, an interesting method was developed in the Asada lab, resulting in very consistent 3D fascicle-like skeletal muscle strips (Neal et al. 2014). In 5-mm PDMS wells containing a rod of desired dimensions, a sacrificial gelatin gel was cast, which, upon removal of the rod following gelation, retained the cylindrical lumen. C2C12 myoblasts suspended in a liquid fibrin gel were subsequently injected into the lumen, allowed to settle and as the temperature was raised, the gelatin melted, leaving behind a perfectly shaped muscle fascicle (Fig 2c). This method proved to be extremely versatile in controlling the initial diameter of the muscle strip and made it easy to investigate the influence of the muscle size together with the gel composition on its differentiation efficiency.
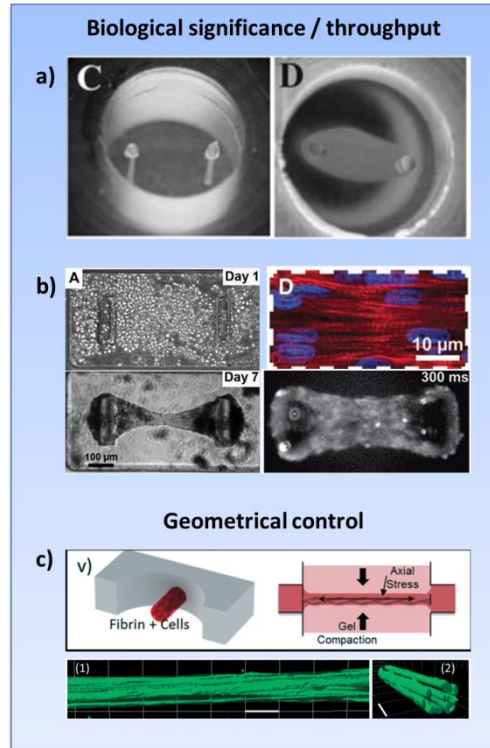
Figure 2: Various designs of microdevices aiming at: (a,b) replicating the physiological morphology of muscle constructs while gaining throughput, (c) enhancing control over the size of the muscle strip, (d) improving the performance of large tissue constructs. a) Rat myoblast-derived muscle tissue formed around PDMS compliant posts (reproduced from (H. Vandenburgh et al. 2008) with permission). b) 3D cardiomyocyte muscle tissue forming around flexible pillars between day 1 (top left panel) and day 7 (bottom left panel), with dimensions small enough to be stained for troponin-T in red and DAPI in blue (top right panel), and displaying calcium activity by calcium indicator fluo-3 imaging (bottom right panel) (adapted from (Boudou et al. 2012)). c): C2C12 muscle bundle formed in a microdevice designed to precisely control the diameter (top panel) exhibiting parallel α-actinin-stained (green) myofibers (bottom panel) (adapted from (Neal et al. 2014)).

Even though the microdevices described above aimed at better recapitulating the physiology of native tissues, the reported levels of engineering stress (= force / cross section area) remain low in comparison to *in vivo* muscle. While using different cell types, the flexible micropillar platforms (Boudou et al. 2012; Sakar et al. 2012; H. Vandenburgh et al. 2008) give rise to tissues capable of generating active stress levels between 0.1-0.6 kPa, with static tension ranging from 0.8 to 3 kPa. The large tissue device(Bian et al. 2012) whose goal was to enhance the performance of the muscular construct reached active stress of 5.8 kPa. These values are however far from the *in vivo* measurements of approximately 250 kPa for skeletal muscles and 50 kPa for myocardium (Claus et al. 2003). Efforts need to be made to match the *in vitro* and *in vivo* properties. One direction would be the ability to decrease the amount of matrix, which tends to increase the cross section area of the tissue while not contributing to force generation. This also applies to undifferentiated cells; treatment with an antimitotic agent such as cytosine arabinoside has been used to reduce the number of proliferating myoblasts and therefore decrease the volume of the inactive cells (Lee and Vandenburgh 2013).

3. Microfabrication for self-assembled micro-biobots

So far, we have described microdevices designed to bring the muscle tissues closer to their *in vivo* equivalents. At the other end of the spectrum of muscle tissue engineering, is an active field of research focusing on utilizing contractile cells to emulate the function more than the tissue itself, sometimes at the

expense of physiological relevance. The products of this research are referred to as biological robots or "biobots". When employing muscle cells, those biobots often strive to replicate either locomotive functions such as *in vivo* skeletal muscles or pumping as performed by cardiomyocytes. Examples of such biobots are the fish-like swimmer robot actuated by electrically-stimulated explanted frog skeletal muscles (Herr and Dennis 2004) and the polypod powered by an insect dorsal vessel tissue (Akiyama et al. 2012). Another is the recellularization of decellularized rat heart with cardiomyocytes, recovering pumping abilities (Ott et al. 2008).

a. Locomotion

One example of a muscle-powered walking micro-biobot, was produced by plating dissociated cardiomyocytes on a thin gold 138 μm-long lever, itself mounted on a silicon scaffold (Fig. 3a) (Xi, Schmidt, and Montemagno 2005). The contraction of muscle caused the structure to bend and bring one of the legs of the lever forward while its relaxation caused displacement of the other leg in the same direction, resulting in a net forward displacement of the system at an average speed of 38 μm/s. This fabrication process led to the first demonstration of a self-assembled micro-biobot capable of locomotion. However, with progress in soft lithography and rapid prototyping came various micro-biobots using the same concept of locomotion driven by muscle-powered membrane bending. Using PDMS substrates as the "skeleton", two groups simultaneously developed similar types of microbiobots. One designed a three-legged walker (J. Kim et al. 2007) (Fig. 3b) and the other an ECM-patterned triangular 30-mm-thick membrane that was shown to walk or swim (Feinberg et al. 2007) (Fig. 3c), both powered by cardiomyocytes. The subsequent micro-biobots used the same approach but mimicked different modes of locomotion by adapting the shape of the PDMS substrate, keeping cardiomyocytes as their power source. An eight-lobed thin disk emulated the swimming motion of a jellyfish (Nawroth et al. 2012) (Fig. 3d) while a flagella-like biobot mimicked a sperm cell (Williams et al. 2014) (Fig. 3e).

Among the more advanced fabrication techniques employed recently to engineer the substrate of the biobots, 3D printing by a stereo-lithographic apparatus (SLA) is of particular interest. It allows for more versatility in the shape and material properties than traditionally used PDMS. Such a technique was also employed to pattern complex 3D hydrogel structures, encapsulating various cell types in a way unattainable by other current technologies (Chan et al. 2010). By printing a cantilever mounted on a base structure with the photo-polymerizable hydrogel PEGDA and using it as a support for cardiac cells, a lab at UIUC demonstrated the ability to use this technique to generate forward motion (Chan et al. 2010) (Fig. 3f). Recently, the same group made a significant advance by integrating a self-assembled 3D skeletal muscle strip (as described in the previous section) into a 3D printed hydrogel scaffold (Cvetkovic et al. 2014). C2C12 myoblasts mixed in a fibrin gel wrap around a two-legged cantilever and differentiate into an electrically-excitable muscle bundle (Fig. 3g). Upon contraction, the muscle strip brings the leg together like a limb muscle would cause the flexion of a joint. The asymmetrical geometry of the construct results in a net displacement of the biobot, at a speed of almost 10 mm/min. It should also be noted that, although skeletal muscle cells do not repeatedly contract unless externally stimulated, they have the advantage of having each myofiber electrically decoupled from its neighbors, which, in the future, might provide finer control of the motion than cardiomyocytes, as demonstrated by (Sakar et al. 2012).

While the technological achievements are undeniable, the long term applications remain unclear. While some claim that such biobots are destined to autonomously perform their locomotive tasks (along with some more elaborate ones such as drug delivery or microsurgical procedures) inside the human body, we believe it is more reasonable to think of these platforms as testbeds to a) understand the synergistic effects of the multicellular systems with their substrate and their environment, b) provide

functional readout on the performance of the muscular construct and c) to demonstrate our ability to apply top-down design principles to biological tissues.

b. Pumps

In addition to their use in locomotion, muscle actuators have also been employed to perform pumping. Although extensively reviewed in the past (see, e.g., (Polacheck et al. 2013)), this area of study is worth mentioning in the present context. Two of these systems used a PDMS membrane that was deflected when actuated by cardiomyocytes, either directly plated onto the membrane (Park et al. 2007), or with forces applied via an intermediate push-bar (Tanaka et al. 2006). Combined with a system of nozzle-diffusers or check valves this approach led to a net flow of 0.2 and 2 nL/min, respectively. Another system with a geometry resembling that of a primitive heart consists of a hollow PDMS microsphere with the surface functionalized with cardiomyocytes. Upon contraction, the shell volume shrinks, squeezing fluid out, resulting in an hypothetical flow rate of 47 nL/min assuming ideal valves (Tanaka et al. 2007). Very recently, the Radisic lab designed a microdevice allowing for the study of what they term "biowires", where cardiac cell progenitors were seeded on polytetrafluoroethylene tubing template, resulting in 5-cm long perfusable and contractile muscle cords with diameters ranging from 600 to 800 μm (Nunes et al. 2013; Xiao et al. 2014). While not a pump *per se* since no net flow rate was produced, the potential exists to displace fluid either by the addition of valves or through coordinated peristaltic contraction.

Systems of this type could potentially be integrated into microfluidic platforms such as "organs-on-chips" (Luni, Serena, and Elvassore 2014) to provide constant perfusion of the system in order to supply the tissue with oxygen and nutrients or circulate the secreted factors to distant targets.
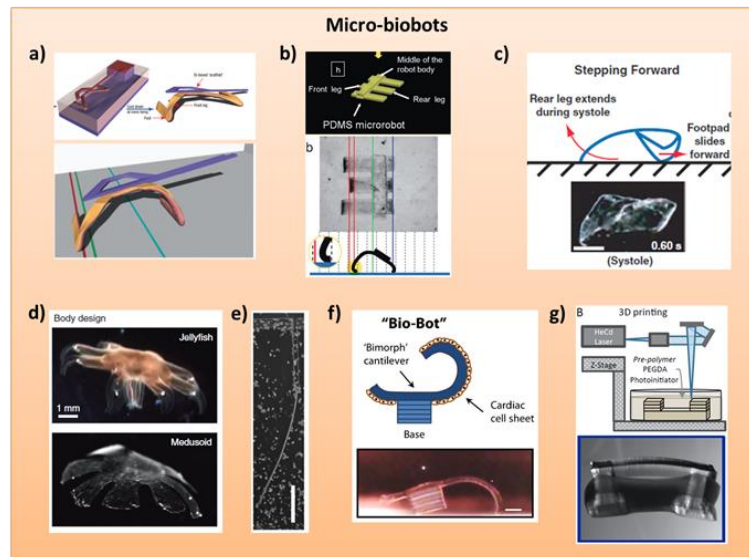

Figure 3: Examples of muscle-powered micro-biobots: a) cardiomyocyte-actuated gold micro-lever mounted on a silicon scaffold (reproduced from (Xi, Schmidt, and Montemagno 2005) with permission). b-c) Walking PDMS-based micro-biobots actuated by cardiac cells, in the form of a tripod (b) (adapted from (J. Kim et al. 2007)) or a thin folded membrane (c) (reproduced from (Feinberg et al. 2007) with permission). d-e) Swimming biobots mimicking the locomotion of a jellyfish (d) (reproduced from (Nawroth et al. 2012) with permission) or a sperm cell (e) (reproduced from (Williams et al. 2014) with permission). f-g) 3D printed micro-biobots actuated by cardiomyocytes (f) (adapted from (Chan et al. 2012) with permission) or C2C12-derived skeletal muscle cells (g) (adapted from (Cvetkovic et al. 2014)).

4. Heterotypic cocultures in microdevices

While contributing the most to the cellular mass of the body, muscle cells nonetheless never function independently in vivo, and typically interact with fibroblasts, endothelial cells or neurons to name a few. These diverse cell types act synergistically to provide support during development, homeostasis, and repair. They serve to deliver nutrients and remove waste, regulate activity, send input commands or collect data. In this section, we motivate the use of coculture systems integrating neurons or vascular networks to enhance tissue performance and better emulate the function of their *in vivo* counterparts. We then describe the microdevices developed for this field and comment on their competitive advantages.

a. Neuronal coculture

Motor neurons, somatic or autonomic, are natural candidates to coculture with muscle cells. Somatic motor neurons located in the spinal cord innervate skeletal myofibers by forming neuromuscular junctions and trigger contraction upon release of acetylcholine while autonomic motor neurons modulate heart rate via their adrenergic (sympathetic) or cholinergic (parasympathetic) synapses.

Neurons affect muscle development and function in ways beyond simple synapse signal transmission. Secreted factors such as agrin are released as the growth cone contacts the myofiber, leading to the recruitment of AChR that enhances postsynaptic differentiation (Lin et al. 2001). Moreover, studies suggest that levels of contraction of skeletal muscle coculture with neuronal cells can be improved, putatively mediated by neural agrin (Bian and Bursac 2012; Larkin et al. 2006). Conversely, modulation of skeletal muscle activity results in a retrograde signaling cascade causing adaptation of the presynaptic machinery in a pathway yet to be unraveled (Arnold, Christe, and Handschin 2012). The same synergy is true for cardiomyocytes that secrete NGF that enhances synaptic transmission with autonomic neurons (Lockhart, Turrigiano, and Birren 1997; Shcherbakova et al. 2007).

Since the first *de novo* neuromuscular junctions were formed *in vitro* with skeletal or cardiac muscle cells (Chamley, Goller, and Burnstock 1973; Peterson and Crain 1970; Veneroni and Murray 1969) neuron-muscle coculture has consisted of plated muscle explants or progenitor cells in a dish for differentiation before the neurons were seeded on top of or in close proximity to the muscle (Miles et al. 2004; Umbach et al. 2012). Some of the exciting discoveries made with such platforms include the role of terminal Schwann cells on guiding axons during regeneration (Son and Thompson 1995), the influence of neurotrophins on the maturation of NMJ (Wang and Lu 1995), the identification of glia-derived factors enhancing NMJ formation (Feng and Ko 2008; Ullian et al. 2004) or the demonstration of functional NMJ formation with mouse and human ES-derived motor neurons (X.-J. Li et al. 2005; Miles et al. 2004). These platforms, while useful, nonetheless have limitations, starting with the fact that those constructs poorly mimic the physiology of their *in vivo* counterparts (no segregation between cell types, two-dimensional, "infinitely" stiff substrates). Moreover, application of cues, be they chemical or electrical, is often poorly resolved in time or space and rarely cell-specific. These shortcomings naturally call for the use of microdevices, which have the potential to outperform traditional assay platforms in their ability to support, for instance, 3D compartmentalized cell culture.

A plethora of micro devices have been developed to manipulate neurons and axons (see review by (Roy, Kennedy, and Costantino 2013)). Many of these devices are based on the original design by Jeon where two 100-µm high channels separated by micro grooves (3 x 10 µm) through which only axons can pass while blocking the cell bodies (Taylor et al. 2003). This device or variations of it have been used to investigate axon guidance and axonal transport (H. J. Kim et al. 2012; Millet et al. 2010; K. Zhang et al. 2010), nerve injury and regeneration (Hellman et al. 2010; Hosmane et al. 2011; Magdesian et al. 2012; Taylor et al. 2005), generate compartmentalized cocultures (Hosmane et al. 2010; Y. Yang et al. 2009) or

form axonal diodes (Honegger et al. 2013; Peyrin et al. 2011). Other platforms allowed for exposure of 3D culture neurons to precise gradients of chemoattractants and repellents (Kothapalli et al. 2011) or gradients of mechanical stiffness of their collagen substrate (Sundararaghavan et al. 2009). Surprisingly however, there is relatively little literature on the use of microdevices in neuron-muscle interaction. In the following section, we describe the few existing ones.

While not a co-culture system *per se*, this first platform is a good example of how microfluidic devices can be used to investigate the role of neuronal secretion on skeletal muscles by precisely controlling the delivery of chemical cues (Tourovskaia, Figueroa-Masot, and Folch 2005, 2006; Tourovskaia, Li, and Folch 2008) and providing an alternative to existing, more traditional assays (Gautam et al. 1996). In this device, adherent myofibers are formed from C2C12 cells along Matrigel 25µm-wide microtracks regularly spaced by 200 µm (Fig. 4a). After myotube formation, laminar flow streams were applied perpendicularly to the fibers from 4 different inlets, one of which providing a solution of neural agrin. The small dimensions of the device, resulting in laminar flow, allow for localization of the agrin-containing stream to a width of about 100 µm. Besides generating large data sets and consistent conditions across samples, this precise spatiotemporal manipulation of the local chemical environment of the myotubes allowed for a quantitative analysis of the influence of neuron-secreted agrin on the clustering of acetylcholine receptors, the postsynaptic receptors at the neuromuscular junction.

The first microfluidic device allowing for neuromuscular coculture was developed by Takeuchi *et al.* (Takeuchi et al. 2011), adapted from (I. Suzuki, Y. Sugio, Y. Jimbo and K. Yasuda, Lab Chip, 2005, 5, 241– 247.). The device consists of PDMS-based microchambers seeded with 4-8 superior cervical ganglia (SCG) neurons, connected by minute grooves allowing axonal outgrowth and connection with neighboring compartments, flanked by larger chambers containing SCG neurons on one side and ventricular myocytes (VM) on the other (Fig. 4b). The substrate was decorated with a microelectrode array (MEA), used to both locally stimulate neurons and record the activity of the neurons and the muscle cells. Besides recapitulating the known phenomenon that stimulation of sympathetic neurons would cause an increase in the beat rate of cardiomyocytes, the geometrical constraints of the platform result in a very consistent, repeatable and high throughput cell culture system. The compartmentalization also offers the competitive advantage to apply chemical cues locally, therefore stimulating one cell type and leaving the other unperturbed, as illustrated by the local administration of propranolol, a β-adrenergic receptor antagonist blocking the synaptic transmission between the SCG neurons and the cardiomyocytes.

While the previous paper focused on the compartmentalization of neurons and muscle cells, at the expense of a more realistic 3D morphology, Morimoto et al. developed a neuromuscular device in which murine neural stem cell (NSC)-derived motor neurons could innervate a 3D muscle strip differentiated from C2C12 myoblasts (Morimoto et al. 2013). In this device, 7-mm-long strips of C2C12 cells embedded in Matrigel were stamped onto a PDMS substrate and anchored at each end on elevated glass plates, such that upon compaction of the gel, the tissue lifted off the substrate to become a free-standing muscle bundle. Mouse NSC-derived neurospheres were subsequently plated onto the muscle strips and induced into motor neurons. After 14 days in culture, a-bungarotoxin staining revealed the clustering of AChR indicative of the formation of NMJs, confirmed by the contraction of the muscle bundles upon glutamate-induced neuronal excitation (Fig. 4c).
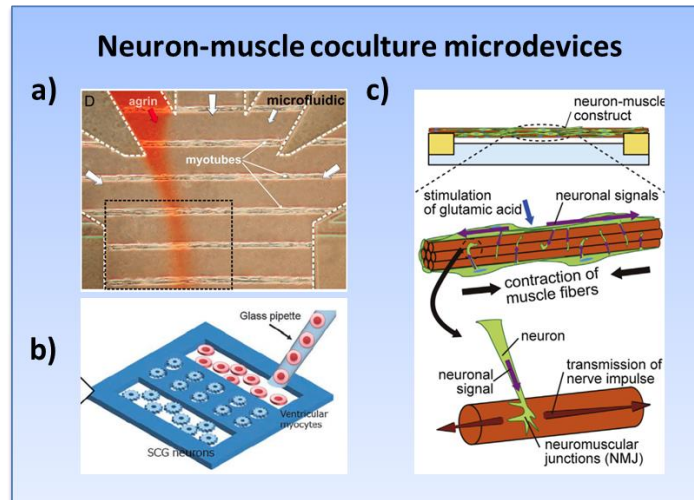
Figure 4: Muscle-neuron interaction microdevices. a) Microfluidic device allowing precise application of neural agrin to arrays of myotubes (reproduced from (Tourovskaia, Li, and Folch 2008) with permission). b) Coculture of spatially segregated sympathetic neurons and cardiomyocytes (reproduced from (Takeuchi et al. 2011) with permission). c) Microfluidic devices designed for 3D muscle strip innervation by motor neurons (reproduced from (Morimoto et al. 2013) with permission).

b. Vascularization

With diffusion-driven oxygen and nutrient transport spanning distances no greater than about 200 μm, it becomes critical to integrate a vascular network into muscle tissues that would extend beyond those dimensions, regardless of the type of muscle cell or even tissue (Kaully et al. 2009). This is true whether one wants to further increase the physiological relevance of an *in vitro* muscle system for fundamental biology, or build actuators with forces comparable to or even exceeding *in vivo* tissues if that tissue is meant to be implanted into a patient and need to reconnect with the native vasculature. Studies have shown that *in vivo* skeletal muscle regrowth following ischemia was greatly enhanced by the supply of proangiogenic factors or by prevascularizing implantable tissues. The former case is illustrated in studies by the Mooney lab in which those factors were locally released in a well-controlled fashion from engineered polymers (Borselli et al. 2010, 2011). For the latter scenario, Langer and coworkers demonstrated that, when supported by fibroblasts, prevascularized muscle tissues enhanced anastomosis after transplantation, resulting in higher survival of the graft (Levenberg et al. 2005). Similar results were obtained in the domain of cardiac tissue regeneration in cases where engineered scaffolds loaded with proangiogenic factors proved to be better templates for both ES-derived cardiomyocytes or *in vivo* cardiac tissue repair (Madden et al. 2010), where endothelial cell cocultures led to higher survival and better reorganization of cardiomyocytes (Narmoneva et al. 2004), or a combination of both (Dvir et al. 2009).

However, spontaneous revascularization in "adult" or *in vitro* muscle tissue (also true for all tissues), whether occurring before or after implantation, lacks proper organization and structure and therefore has a lower efficiency than normal in vivo vascular networks. This is where microfabrication can bring order to the tissue construct and provide an alternative to enhance tissue function.

The past few years have seen the emergence of microdevices whose goal was to guide the formation of well-controlled microvascular networks (MVN). Those platforms can be divided into two classes. The first class is composed of devices designed to fully guide the formation of highly organized microvessel arrays by prepatterning the substrate that will receive the endothelial cells. This can be achieved by shaping luminal structures, on the device material (Baranski et al. 2013; Liu et al. 2011; Yeon et al. 2012; B. Zhang et al. 2013) or in a hydrogel (Chrobak, Potter, and Tien 2006; Miller et al. 2012; Zheng et al.

2012) (Fig. 5a-b), lumens that are subsequently lined with ECs. The advantage of an extremely well defined geometry is counterbalanced by a vessel formation poorly representative of the natural angiogenesis process and underperforming characteristics. When used to prevascularize implantable patches however (Baranski et al. 2013), those microvessels still outperform randomly grown vasculature in tissue integration.

An alternative to patterning is the spontaneous formation of perfusable vascular networks by angiogenesis or vasculogenesis, wherein cells respectively either sprout from an existing endothelial monolayer or coalesce from a homogeneous single cell suspension. Closer to the *in vivo* formation of vascular network, these methods offer a chance to study the dynamics of vessel formation while generating better performing vasculature. The first noteworthy device to be developed consists of a microfluidic channel, host of a vascularized gel, flanked by medium channels, themselves flanked by fibroblast-containing gel region, there to provide pro-angiogenic factors (S. Kim et al. 2013) (Fig. 5c). In this study, the authors demonstrated the ability to spontaneously form microvessels with characteristics comparable to those *in vivo*, a characteristic not attainable with substrate-assisted vessel formation described above or existing angiogenesis platforms (Song, Bazou, and Munn 2012). Besides showing lower permeability values than in existing platforms, the vascular network responded to flow-induced shear stress in a way *in vivo* constructs would by upregulating NO synthesis and reorganizing their cytoskeleton. When seeded in the surrounding matrix, pericytes were found to elongate at the surface of the endothelium by adhering to the EC-derived collagen IV. In a similar platform, Whisler and coworkers (Whisler, Chen, and Kamm 2013) quantitatively evaluated how the initial cell seeding density, concentration of angiogenic growth factors, the presence of fibroblasts, the composition of the extra cellular matrix and flow-induced shear stress affect the morphology of the vascular network, and provides a tool set to make the most physiologically relevant MVN to be used in a variety of setups, such as in the context of metastasis (Chen et al. 2013).

Contemporarily, a microfluidic device was developed in the George lab, which, while having a different design and requiring interstitial flow for the formation of vascular structures (Fig. 5d), allow for the spontaneous formation of a perfusable network in a 3D hydrogel (Moya et al. 2013). Similarly to the vasculogenesis platforms described above, endothelial cells were suspended in a fibrin gel with the difference that lung fibroblasts were added to the mixture instead of being seeding in segregated chambers.

In the context of muscle vascularization, two strategies have been used so far to generate perfusable muscle tissue. The first one, consisting of forming lumens in the muscle strip before lining it with endothelial cells, was demonstrated in a platform adapted from the design by Vandenburgh (H. Vandenburgh et al. 2008). In this larger version of the original design (Vollert et al. 2014), three opposing pairs of PDMS 14 mm-long posts, positioned 17 mm apart, were decorated with dried alginate wires such that upon addition of calcium following cardiac muscle bundle formation, the alginate would dissolve, leaving behind hollow channels (Fig. 5e). The endothelial cells contained in the original cell mix were found to populate the channels and form a lining resembling a blood vessel. After more than 15 days in culture under constant perfusion, the authors found an increase of cardiomyocytes density inside the tissue, although they were not able to demonstrate an increase of contractile performance.

Another system developed by the Radisic lab used an reversed order, constructing the vasculature before forming the muscle tissue (Chiu et al. 2012). In this work, a PDMS substrate patterned with linear microgrooves and coated with a proangiogenic factor-releasing hydrogel was flanked with artery and vein explants on each side of the platform. The topography of the substrate together with the soluble factor guided anastomosis from one explant to the other, forming a perfusable bridge (Fig. 5f). Subsequently, cardiomyocytes were seeded on top of this vascular bed, and were shown to perform better than the non-

vascularized ones. Moreover, the engineered tissue could be removed from its PDMS substrate, forming a stand-alone perfused cardiac tissue for applications such as transplantation.

To date, however, no microdevice has been designed to take advantage of the spontaneous vessel formation in a muscle-endothelial cell coculture, in a way that has previously been proven successful at a the macroscopic level (Lesman et al. 2010; Levenberg et al. 2005).
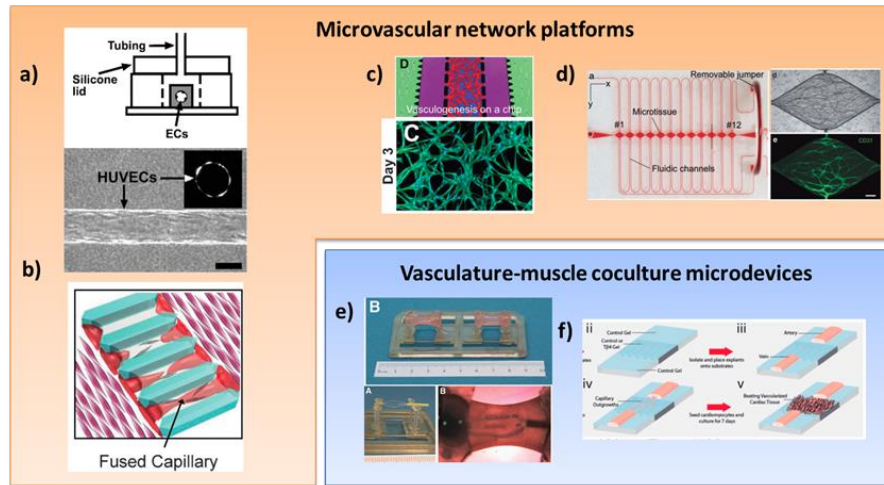

Figure 5: Vasculature microdevices (a,b,c,d) and muscle vascularization platforms (e,f). a) Formation of a endothelialized perfusable lumen in a microdevice (adapted from (Chrobak, Potter, and Tien 2006)). b) microdevice-assisted capillary formation by sprouting angiogenesis (reproduced from (Yeon et al. 2012) with permission). c-d) Examples of microdevice for the formation of perfusable microvascular network by vasculogenesis (adapted from (S. Kim et al. 2013) and (reproduced from (Moya et al. 2013) with permission). e) Precasted lumen endothelialization in a muscle microtissue (adapted from (Vollert et al. 2014)). f) Microdevice promoting the formation of a microvessel bed for the vascularization of muscle tissue (reproduced from (Chiu et al. 2012) with permission).

c. Other potential coculture systems

In the quest to replicate *in vivo* muscle performance and control, further improvements will likely be necessary, and many of these will require the addition of other, non-muscle cell types. Among these, fibroblasts have been shown to enhance myogenesis of C2C12 or cardiomyocytes (Cooper et al. 2004; Radisic et al. 2008) and improve vascularization by secreting factors promoting and stabilizing vasculature (Aubin et al. 2010). Glial cells also have been observed to enhance the clustering of AChR on the postsynaptic side of a NMJ (Cao and Ko 2007; Feng and Ko 2008; Ullian et al. 2004), presumably increasing the activation of the muscle construct. However, no systematic assays have yet been published on using microtechnologies to take full advantage of these observations to enhance neuromuscular tissues or quantitatively and thoroughly investigate the process. Nonetheless, coculturing them with muscle cells in a well-defined and controllable environment as described above with neurons or vascular networks should strengthen the understanding of the associated synergistic mechanisms and at the same time, improve the performance of the engineered tissue.

Conclusion

Muscle tissue engineering has made tremendous progress over the past decade thanks to the ingenuity of many, and the development of new enabling technologies. An ultimate goal of this work is to draw

upon all the features described above in order to produce models that either better replicate human physiology or improve the functionality of *in vitro*, non-physiological systems. In the case of models for physiological tissue, a major aim should be to improve performance of the muscle constructs so that they can better reproduce normal *in vivo* contractile levels of stress. This will likely involve creating systems with natural innervation, vascular supply, and more realistic ECM, both in terms of its elasticity properties and volume fraction within the constructs. All of this should lead to muscle systems that can more realistically mimic *in vivo* function and therefore be more useful as platforms for drug screening, models for muscle regeneration, or studies of basic muscle physiology. In the case of *in vitro*, non-physiological applications, there is much room for improvement, and thus considerable opportunity to expand the realm of muscle-actuated biobots, pumps, or other systems requiring contractile behavior. To enable control of these systems, optogenetic, neural, or other forms of activation will likely be required. Adding sensory neurons that can sample the local environment and direct the motion in response to it through interactions with motor neurons represents a particularly exciting area of research. And as systems of larger scale are needed, vascular networks will become essential in order to meet the high metabolic needs of these tissues. All this appears to be on the not-too-distant horizon, however, so we can expect significant advances in these areas over the next several years.
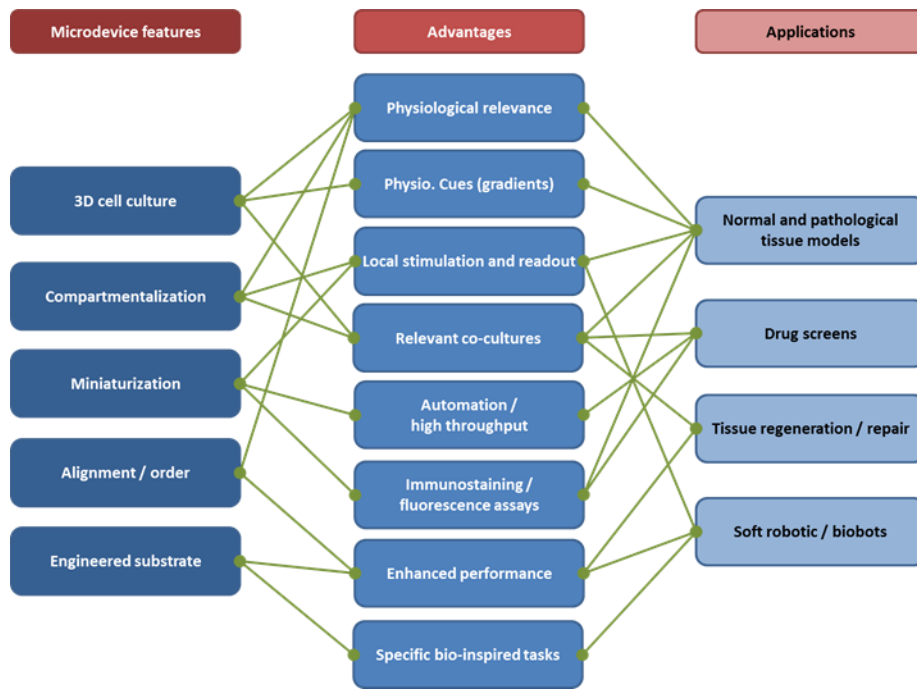

Figure 6: Schematic presenting the specific features of microdevices demonstrated in the literature (first column), resulting in advantages over traditional assays (middle column), that can translate into specific fields of application (right column).

As reviewed here, we can appreciate how microfabrication has allowed the development of microdevices with features absent from traditional assay systems. These features, including compartmentalization of multicellular cultures or alignment and order of the tissue to name a few (Fig. 6, left column), in turn enhance the performance of the tissue culture platform in that, for instance, it better mimics the *in vivo* microenvironment, allow for the application of physiologically relevant cues, or facilitates data acquisition and increases throughput (Fig. 6, middle column).We are now at a turning point where these features should be effectively employed by identifying specific applications that would benefit from the use of microfabricated devices and developing designs accordingly (Fig. 6, right panel).

Also, with increasing ability to precisely form and manipulate tissues, we should not neglect the extraordinary ability for a tissue to self-differentiate / codifferentiate and assemble into complex tissue by exhibiting emergent behaviors as was recently demonstrated for various tissues such as gut or brain (Lancaster et al. 2013). The future of microdevice-aided tissue engineering resides in the ability to harness those properties, often resulting in more *in vivo*-like performance, and combine them with techniques in genetic engineering, imaging and material science.

Acknowledgements

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