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Engineering skeletal muscle – from two to three dimensions

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1. Introduction

Large skeletal muscle defects remain a challenge for patients and surgeons alike. While small injuries resulting in tears and lacerations can be repaired by the body's own regenerative mechanisms, loss of over 20% of muscle will lead to scarring, denervation and loss of function (Turner and Badylak, 2012). This can occur in cases of trauma, tumour, surgery and degenerative disease, which overwhelm the body's repair mechanisms and result in changed anatomy and weakened muscle groups. Loss of limb is another consequence of these disease processes. Restoring mobility and independence is a major reconstructive challenge, for which tissue engineering may offer a solution.

2. A regenerative strategy

Surgeons have a number of techniques at their disposal to address the unique challenges of losing significant muscle bulk, otherwise known as volumetric muscle loss (VML). In principle, the lost muscle is replaced by autologous muscle retrieved either near the site of injury with its own vascular and nerve supply or from a distant site as a free flap. These operations are lengthy and demanding, with variable success and slow recovery. Donor site morbidity and ongoing functional deficits of the flap are inevitable even after successful surgery (Grogan *et al*., 2011; Mertens *et al*., 2014). In the case of amputation, motorized prosthetic limbs promise a return to function, but are dependent on the faithful transfer of nerve signals via muscle to the receiving electrode. Loss of muscle units limits the use of such rehabilitative tools (Kuiken *et al*., 2009; Loeb, 2009).

However, these surgical limitations may soon be overcome by advances in the field of tissue engineering. Promising developments in the engineering of bone, nerve, cartilage, muscle and methods of interfacing these tissues herald a new era of reconstructive surgery, where lost or diseased tissue can be replaced by a biological construct tailored to the individual's anatomy and immune system (Subramanian *et al*., 2009; Amini *et al*., 2012; Hirt *et al*., 2014; O'Connell *et al*., 2016). In the setting of VML, engineered skeletal muscle offers a regenerative solution without associated donor site morbidity. Its potential to correct physical deformity, restore mechanical function, or act as an interface with a robotic device may offer new treatment options for the surgeon in the near future.

3. Anatomy and physiology of skeletal muscle

Skeletal muscle is a richly vascularized, innervated and weight-bearing tissue, which forms 30–40% of human body mass (Janssen *et al*., 2000). It is formed by bundles of parallel fibres that span the entire length of the muscle (Figure 1). Each fibre is a single multinucleated cellular syncytium surrounded by the sarcolemma. Surrounding the sarcolemma is the basal lamina, upon which lie the muscle stem cells. Within the multinucleate muscle fibre are multiple myofibrils that form the cytoskeleton, which are divisible into contractile subunits known as sarcomeres. An overlap of actin and myosin proteins within these sarcomeres enables contraction of the muscle and thus generates movement.

<<Please insert Figure 1 near here>>

Contraction occurs when a nerve signal is transmitted from the motor neuron to the muscle cell membrane at the neuromuscular junction (NMJ). At a NMJ, activation of a motor neuron triggers the release of acetylcholine (ACh) from the presynaptic axon (Figure 2). ACh diffuses across the synaptic cleft to bind to acetylcholine receptors (AChRs) clustered at the motor endplate on the muscle fibre sarcolemma. This triggers an intracellular release of stored calcium, which initiates cross-bridge formation of actin and myosin proteins, shortening the sarcomere and causing a muscle contraction. A single action potential produces a twitch, while multiple action potentials lead to twitch summation and increased muscle tension (Chargé and Rudnicki, 2004). Force generation of skeletal muscle is dependent on recruitment of individual motor units, which consists of a motor neuron and all the muscle fibres that it innervates. The fibres supplied by a motor nerve are spread evenly throughout the muscle bulk, and so its activation will result in an evenly distributed contraction (Figure 1) (Ganong, 2001).

<<Please insert Figure 2 near here>>

In traumatic injury, the muscle anatomy and pattern of innervation is disrupted. The repair process can be divided into three stages: inflammatory, repair and remodelling phase (Turner and Badylak, 2012). Initially, the torn myofibres activate an inflammatory response, recruiting calcium-dependent proteases to disintegrate damaged myofibrils, and then neutrophils and macrophages to digest necrotic material. These cells release cytokines that amplify the inflammatory response and recruit muscle satellite cells to the site of injury. The repair phase is characterized by infiltration of nerves, blood vessels and muscle cells into the wound. The formation of scar tissue by collagen deposition from fibroblasts contracts the gap between remaining muscle fibres and can act as a myofibre conduit. In the remodelling phase there is further maturation of the myofibres and reorganization of the muscle tissue.

What differs in the case of VML, however, is that the distance bridged by the scar is so great that it forms a barrier that prevents muscle regeneration between the two ends. A relative lack of satellite cell infiltration, perhaps owing to the loss of traditional signalling because of complete destruction of tissue architecture, further contributes to scar formation (Corona *et al*., 2013). The functionless scar tissue inhibits distal innervation leading to muscle atrophy, and impairs contraction of the remaining proximal muscle. The permanent disruption of the highly organized anatomy and contraction physiology lead to grossly changed function, and the muscle never returns to normal performance (Machingal *et al*., 2011; Corona *et al*., 2013).

4. Choosing cells and scaffolds

Efforts to engineer functional skeletal muscle have led to the investigation of a number of stem cells and scaffold materials. In the host environment, cell activity is finely tuned by mechanical, electrical and chemical factors, referred to as the microenvironment or 'stem cell niche' (Wolf *et al*., 2012). The ability to understand

and recapitulate this environment is key to successful tissue engineering, and is largely based on the careful choice of cells and scaffolds.

4.1. Stem cells

Stem cells are defined as cells that can perpetuate through self-renewal and differentiate into mature specialized cells (Reya *et al*., 2001). In the embryo, stem cells are capable of generating all cells that form the three germ layers of the body (Smith, 2001). In the adult, they are responsible for repairing and replenishing adult tissues, and are typically destined to become a particular cell type (Slack, 2008). Satellite cells are skeletal muscle precursors, and are the primary cell responsible in muscle regeneration. They form 2–7% of the nuclei in a particular muscle fibre. In the quiescent state these cells are found between the sarcolemma and basal lamina (Schultz, 1984) but when activated by trauma or micro-injury they divide to become myoblasts (Bischoff, 1986). They migrate to the site of injury and differentiate to form new muscle in a process called myogenesis (Dhawan and Rando, 2005).

The main cell sources for skeletal muscle tissue engineering are satellite cells and myoblasts. Satellite cells have great regenerative capacity *in vivo*, but this quality is challenging to harness *in vitro*. Attempts at *in vitro* expansion have produced cells with decreased proliferative capacity and an increased proportion of differentiated cells (Renault *et al*., 2002; Montarras *et al*., 2005). It is much simpler to use these cells when they have been activated to become myoblasts, which easily proliferate in cell culture and can be maintained and passaged for extended periods (Rossi *et al*., 2011). The future therapeutic model would ideally use myoblasts retrieved from the patient to ensure immunocompatibility, which would then be expanded *in vitro* to tissue engineer the replacement muscle.

Other precursor cells have been investigated for their myogenic potential, including mesoangioblasts (Sampaolesi *et al*., 2006), pericytes (Dellavalle *et al*., 2007), side-population cells (Montanaro *et al*., 2004) and mesenchymal stem cells (Gonçalves *et al*., 2006) to name but a few. Their paracrine effects support local regeneration by regulating the stem cell environment, but it is unknown just how much they contribute to regenerative myogenesis *in vivo*. Other potential sources of stem cells are induced pluripotent stem cells (iPSCs). These are adult somatic cells that have been reprogrammed to a stem-cell like state (Yamanaka, 2009; Inoue *et al*., 2014; Qi *et al*., 2014). Like embryonic stem cells, iPSCs are truly pluripotent and theoretically can differentiate to become any cell type as well as propagate indefinitely. The task of lineage-specific differentiation of iPSCs is currently an area of ongoing research (Dimos *et al*., 2008; Zhang *et al*., 2009; Wu and Hochedlinger, 2011). Their use in tissue engineering has been limited by their potential tumorigenicity (Yamanaka, 2009; Inoue *et al*., 2014). Nonetheless, these cells hold a great deal of potential for the future of regenerative medicine.

4.2 Scaffolds

Scaffolds are designed to mimic the native extracellular matrix (ECM), and must guide cell growth initially through defining geometry, surface properties, porosity and mechanical properties (Bach *et al*., 2004; Dhandayuthapani *et al*., 2011). It is a temporary construct until replaced by native ECM, and so must be biodegradable over a specific time-frame (O'Brien, 2011). Scaffolds can also act as delivery vehicles for growth factors to enhance regeneration, guide nerve growth or induce vascularization (Chan and Leong, 2008; Lovett *et al*., 2009). With these properties in mind, scaffolds for skeletal muscle must facilitate parallel alignment of muscle fibres, enable myogenesis, and promote vascularization and innervation (Razal *et al*., 2009; Quigley *et al*., 2012).

In skeletal muscle tissue engineering, a critical feature of mature muscle is the formation of aligned muscle fibres (Beier *et al*., 2009). A number of techniques have been employed to achieve cell alignment, which can be divided into creating two-dimensional (2D) and three-dimensional (3D) structures (Figure 3). Cell alignment in 2D can be guided by shaping the surface on which cells are cultured. Parallel microgrooves of varying depths and widths have been tested, with grooves ranging from 200 to 600 nm in depth and from 100 to 1200 nm in width inducing myoblast alignment (Clark, 1997; Evans *et al*., 1999; Razal *et al*., 2009). This architecture can be achieved using lithography techniques or, alternatively, through electrospinning nanofibres that are collected in parallel on a drum (Huang *et al*., 2006; Boudriot *et al*., 2006). Another technique for creating cell alignment is by modifying the biomaterial surface through patterning natural or synthetic polymers to guide cell growth.

This can be achieved through soft lithography or printing techniques that essentially deposit ECM proteins in the desired architecture to guide the direction of cell growth (Kane, 1999; Shimizu *et al*., 2010; Cui *et al*., 2013).

<<Please insert Figure 3 near here>>

With 2D techniques, cells are cultured until a confluent sheet of cells and their deposited ECM is formed. In order to transition this technology to create a 3D tissue-like construct, cell sheets can be harvested from the scaffold and then layered (Okano *et al*., 1995; Matsuda *et al*., 2007; Yang *et al*., 2007). While excellent for creating thin tissues, this technique is impractical for skeletal muscle because of the fragility and small size of the sheets. Although valuable in the study of nanoscale contours and its effects on myotube alignment and function, its use for engineering clinically relevant volumes of skeletal muscle seems unlikely.

To achieve cell alignment in a 3D scaffold, the basic shapes that would promote this are meshes or grids of polymers, with built-in in porosity and channels for cell migration and nutrient diffusion. The biomaterials used can be broadly classified into natural or synthetic polymers. Natural 3D scaffold materials such as collagen, fibrin and alginate have excellent biocompatibility, but can be limited by their fragility and potential for immunogenicity (Chan and So, 2005; Lee and Mooney, 2012). Synthetic scaffolds such as poly(lactic-co-glycolic acid) or polycaprolactone have become a major area of research because of the ability to tailor biomaterial characteristics. Although still limited by poorer cell adhesion and proliferation, advances in composite natural and synthetic biomaterials and surface engineering will likely improve biocompatibility (Kurella, 2005; Morra and Cassinelli, 2006). The challenge is to then design a geometry that mimics the natural orientation of skeletal muscle ECM, avoids the pitfalls of a dense matrix that results in a necrotic or acellular core and yet maintains structural properties of a tensile and weight-bearing construct.

One approach is to encapsulate cells within a hydrogel scaffold. Studies using hydrogels mimicking the elasticity of skeletal muscle have demonstrated that there is improved stem cell regeneration and differentiation (Engler *et al*., 2006; Gilbert *et al*., 2010; Salimath and García, 2014). It shows promise as a direct injection delivery of satellite cells into muscle compartments, which would be a useful cell-based treatment for muscular dystrophies (Rossi *et al*., 2011). However, hydrogels do not confer the necessary orientation of cells for myofibre formation when delivered in a matrix form, and can impede efficient cell proliferation and migration because of matrix density (Han *et al*., 2012). Neither do they have suitable mechanical strength for load-bearing (Drury and Mooney, 2003). To address these limitations, Wang *et al*. (2015) created a composite scaffold of a hydrogel shell surrounding a myoblast-laden nanofibre yarn core. This composite fibre successfully aligned myoblasts along the yarn, and could be produced in sheets. However, this approach faces similar limitations to the above-mentioned 2D techniques with regard to size and fragility of layers. Other new techniques have been developed to control the geometry and material characteristics of hydrogels, such as bioprinting.

Bioprinting is an emerging tissue engineering technology that holds promise for fabricating skeletal muscle. Customised printers deposit biological ink, composed of stem cells and scaffold polymers delivered in hydrogels, to create a variety of 3D shapes (An *et al*., 2015). The advantage to this technology is the ability to precisely position every element of a construct in a 3D design. Kang *et al*. (2016) demonstrated this concept with a four-material printer that built bone, cartilage and skeletal muscle. Synthetic polymers were printed as a supporting outer scaffold, with rows of cells and temporary sacrificial polymers printed within. In-built microchannels facilitated nutrient diffusion, and the final construct was 1 mm high, 15 mm long and 5 mm wide. This tissue was innervated *in vivo*, and demonstrated positive functional assessment when measuring compound muscle action potentials at 4 weeks. Although limited in size by the lack of vascularization and untested in terms of mechanical strength, these results suggest that bioprinting may be a promising tool for the future of skeletal muscle tissue engineering.

5. Innervation

Of the many challenges of tissue engineering skeletal muscle, a crucial element in creating functional skeletal muscle is innervation. Innervation is essential *in vivo* for long-term survival of muscle and represents the potential for restoring controlled contractile activity. Key to this connection of nerve and muscle is the recreation of the NMJ. Formation of the NMJ is dependent on both muscle and nerve factors that induce AChR clustering and stabilize NMJs. Agrin is a protein synthesized in motor neurons that induces clustering of

AChRs on the muscle cell membrane (Reist *et al*., 1992). It interacts with a number of muscle proteins, in particular Muscle-specific kinase (MuSK) (Glass *et al*., 1996). This kinase is thought to be responsible for the phenomenon of prepatterning, where AChRs cluster in the centre of a muscle fibre even before a nerve is introduced (Lin *et al*., 2001). Once MuSK is activated by agrin, it triggers a series of intracellular events that enhance AChR clustering and stabilize the motor endplate (Wu *et al*., 2010). Other agrin-binding muscle proteins include dystroglycan and laminin, both of which also regulate NMJ formation (Campanelli *et al*., 1994; Denzer *et al*., 1997).

Nerve and muscle co-cultures have been used to study NMJs and to observe the effect of innervation on engineered skeletal muscle. Larkin *et al*. (2005) demonstrated in a monolayer co-culture of myotubes and rat spinal cord explants that the inclusion of a nerve–muscle interface improved contractility, force generation and maturation in the muscle layer. Dharwan *et al.* (2007) implanted myoblasts suspended in a fibrinogen gel around the femoral vessels and nerve in a rat model. After 4 weeks, field stimulation of the neurotized constructs showed a fivefold increase in peak tetanic force when compared with non-neurotized constructs. Similarly, Martin *et al.* (2015) developed a 3D model of NMJ formation but with primary muscle and primary motor neuron cells co-cultured in a fibrin gel. Again, improved myotube morphology was observed in co-cultures, and field stimulation showed a 145% greater twitch force compared to aneural cultures. From these examples, it is clear that introducing a nerve–muscle interface to myoblast cell cultures is a crucial step towards creating functional muscle.

Efforts to enhance NMJ formation and thus myotube maturation have also led to the investigation of biofactors. Zhang *et al*. (2015) investigated the effects of agrin and soluble laminin on NMJ formation in primary mouse myoblasts and PC12 cell co-cultures. While agrin alone enhanced aneural AChR clustering, there was a marked synergistic effect of both agrin and laminin on augmenting AChR clusters. Both the size and number of clusters were significantly increased. It appeared to promote neurite outgrowth and NMJ formation, which correlated with increased contractile activity observed in the muscle monolayer. Such findings are critical for the creation of bioactive scaffolds that could facilitate the delivery of these growth factors to enhance innervation, and the application of these findings in a 3D muscle model would be an exciting step forward.

6. Other challenges

As with all types of tissue-engineering, the lack of vascularization limits the size of the tissue. Engineered tissues are often avascular constructs *in vitro* with the goal of vascularization after implantation *in vivo*. This approach requires consideration of the diffusion distance of nutrients, which is approximately 150–200 μm (Griffith *et al*., 2005), and the generation of specific scaffold patterns to accommodate this limitation (Han *et al*., 2012). For this reason, all 3D muscle constructs thus far are less than 1 mm high (Mertens *et al*., 2014). Structural and mechanical cues can be built into the scaffold to guide the growth of vascular stem cells, as well as angiogenic growth factor delivery (Cittadella Vigodarzere and Mantero, 2014). Borselli *et al.* (2010) delivered vascular endothelial growth factor and insulin-like growth factor 1 in an alginate scaffold to mouse muscle damaged by myotoxin and ischaemia. Enhanced muscle regeneration characterized by greater muscle bulk, increased tetanic force and increased capillary density was observed after 6 weeks of implantation. While promising progress is being made, vascularization remains a major challenge to creating volumetric constructs for clinical applications.

Other methods of enhancing myotube maturation are mechanical stress and electrical stimulation. Directed mechanical tension can aid in myoblast alignment and stimulate maturation, augment muscle hypertrophy and increase myoblast proliferation (Vandenburgh and Karlisch, 1989; Kook *et al*., 2008). However, its value in 2D and 3D muscle cultures is unclear and results from stretch-relaxation protocols are varied (Boonen *et al*., 2010). Electrical stimulation is another area of active research, where culturing muscle cells on conductive surfaces influences myoblast proliferation and differentiation (Quigley *et al*., 2012), muscle cell phenotype (Düsterhöft and Pette, 1990), myosin expression (Naumann and Pette, 1994) and contractility (Kaji *et al*., 2010). With the ongoing development of biomaterials, conducting polymers such as polyaniline (Qazi *et al*., 2014), polypyrrole (Gilmore *et al*., 2009) and polythiophene (Breukers *et al*., 2010) may be incorporated into 3D scaffolds for skeletal muscle engineering.

7. Future directions

The tissue engineering of skeletal muscle is still in the earliest stages, but recent developments in scaffold-building technologies and biomaterials have led to significant progress in this exciting emerging field. Advances in 3D bioprinting have opened the doors to custom-made personalized solutions for patients, allowing fabrication of increasingly complex and sophisticated constructs. Furthermore, the promise of establishing NMJs in these constructs has brought engineers closer to creating muscle with controlled contractile activity. This represents a powerful tool in regenerative medicine, with huge scope for applications in biotechnology. For the surgeon, regenerative medicine complements and improves upon treatment options available to patients, and the tissue engineering of skeletal muscle may soon form part of the solution to VML.

Conflict of interest

The authors have declared that there is no conflict of interest

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Figure 1. Schematic diagram of skeletal muscle anatomy, demonstrating the concept of innervation in motor units.

Figure 2. Schematic diagram demonstrating the clustering of acetylcholine receptors (AChR) at the motor endplate of a neuromuscular junction.

Figure 3. Different techniques to achieve myoblast alignment in both two dimensions (2D) and three dimensions (3D).

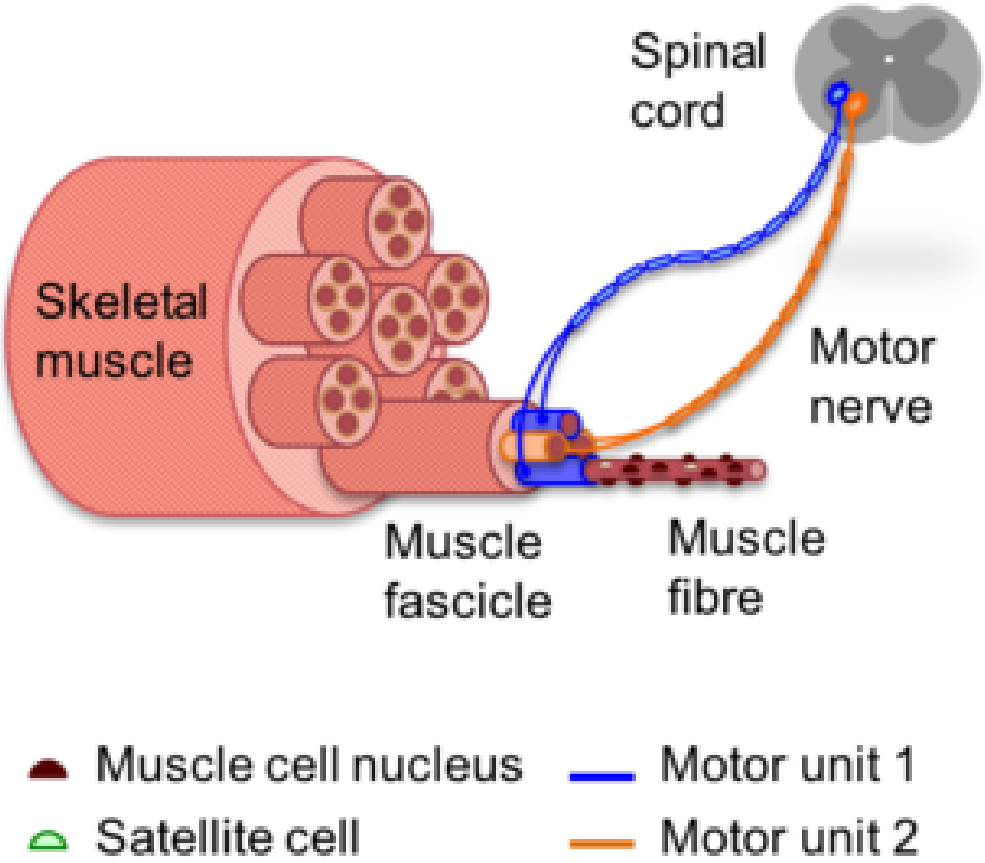

Figure 1. Schematic diagram of skeletal muscle anatomy, demonstrating the concept of innervation in motor units.

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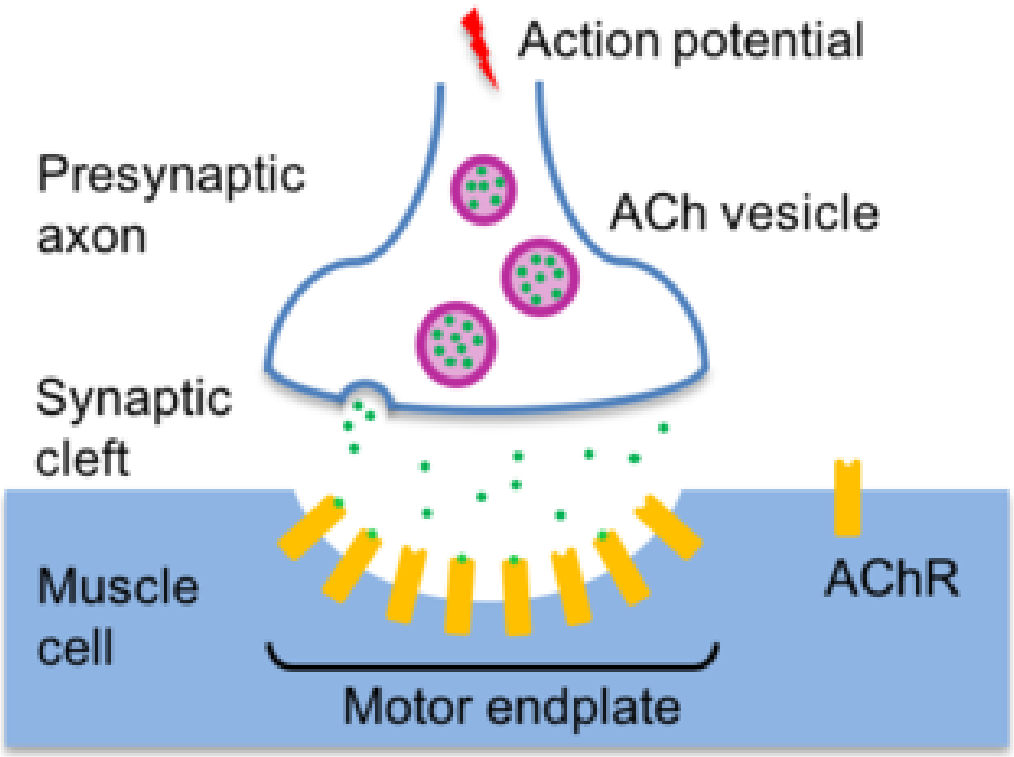

Figure 2. Schematic diagram demonstrating the clustering of AChRs at the motor endplate of a neuromuscular junction.

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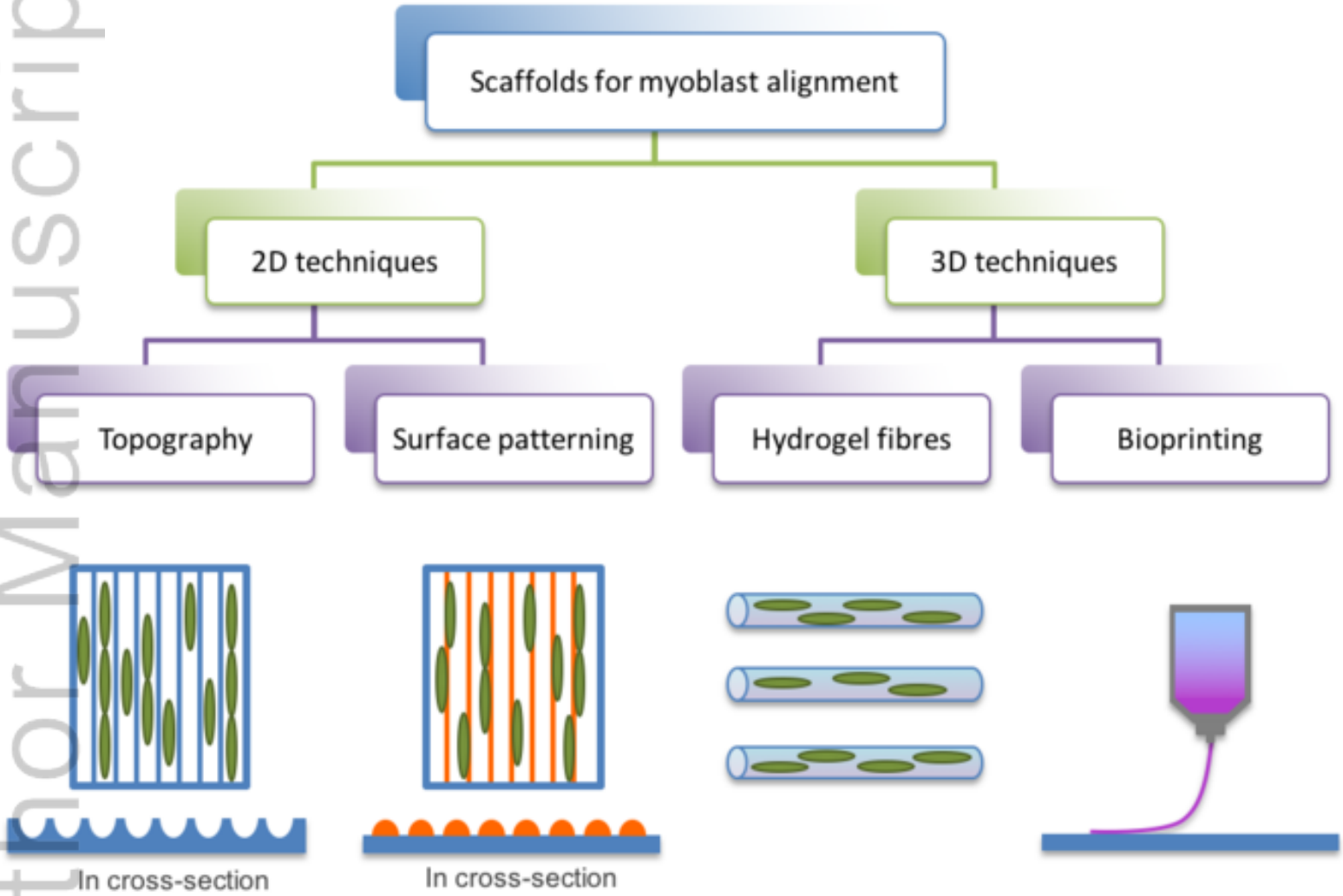

Figure 3. Different techniques to achieve myoblast alignment in both 2D and 3D

TERM_2265_F3.tiff