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Biofabrication of Living Actuators

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Keywords

bioactuators, skeletal muscle, cardiac muscle, biohybrid, soft robotics, biofabrication

Abstract

The impact of tissue engineering has extended beyond a traditional focus in medicine to the rapidly growing realm of biohybrid robotics. Leveraging living actuators as functional components in machines has been a central focus of this field, generating a range of compelling demonstrations of robots capable of muscle-powered swimming, walking, pumping, gripping, and even computation. In this review, we highlight key advances in fabricating tissue-scale cardiac and skeletal muscle actuators for a range of functional applications. We discuss areas for future growth including scalable manufacturing, integrated feedback control, and predictive modeling and also propose methods for ensuring inclusive and bioethics-focused pedagogy in this emerging discipline. We hope this review motivates the next generation of biomedical engineers to advance rational design and practical use of living machines for applications ranging from telesurgery to manufacturing to on- and off-world exploration.

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1. INTRODUCTION

Actuators convert energy into motion and are central to life. In humans and many other biological creatures, three major tissue types control involuntary and voluntary movement within the body: cardiac, smooth, and skeletal muscle (1–3). Cardiac muscle is present in the heart and is responsible for spontaneous and continuous pumping of blood, enabling systemic oxygenation and nutrient supply. Smooth muscle is present in the walls of many organ systems including blood vessels and the gastrointestinal, urinary, respiratory, and reproductive tracts, aiding in the active directional transport of materials throughout the body. Skeletal muscle, which is distributed throughout the body and constitutes approximately 40% of body weight (4), powers all voluntary movement such as sitting, walking, and chewing. The assembly, maturation, maintenance, and repair of these three types of tissues are critical to maintaining physiological conditions and promoting human health and quality of life (**Figure 1**).

The convergence of engineering and biology has driven the rapid rise of fields such as tissue engineering: the biofabrication of multicellular systems that replicate the morphology and function of native organs (5–8). Applications of mammalian tissue engineering in human health, such as high-throughput disease modeling and regenerative medicine, have been of significant interest across a range of tissue types including muscle (9, 10), skin (11), liver (12), and reproductive tissues (13). While the impact of tissue engineering on medicine continues to grow, there has been an increasing interest in deploying engineered tissues as functional components in human-made machines.

Biological tissues include extremely sophisticated, efficient, and robust examples of the key components desired in most engineered systems: sensors (skin, eyes, ears, noses), processors (central and peripheral nervous systems), and output transducers (muscles, fat). Living creatures leverage a range of specialized cell types to integrate parallel sensory inputs (such as optical, thermal, and mechanical cues in the environment) and generate a coordinated functional output (producing force, secreting chemicals, changing colors) with tightly regulated feedback control (14). Because these biologically derived sensors, processors, and transducers are living, they can

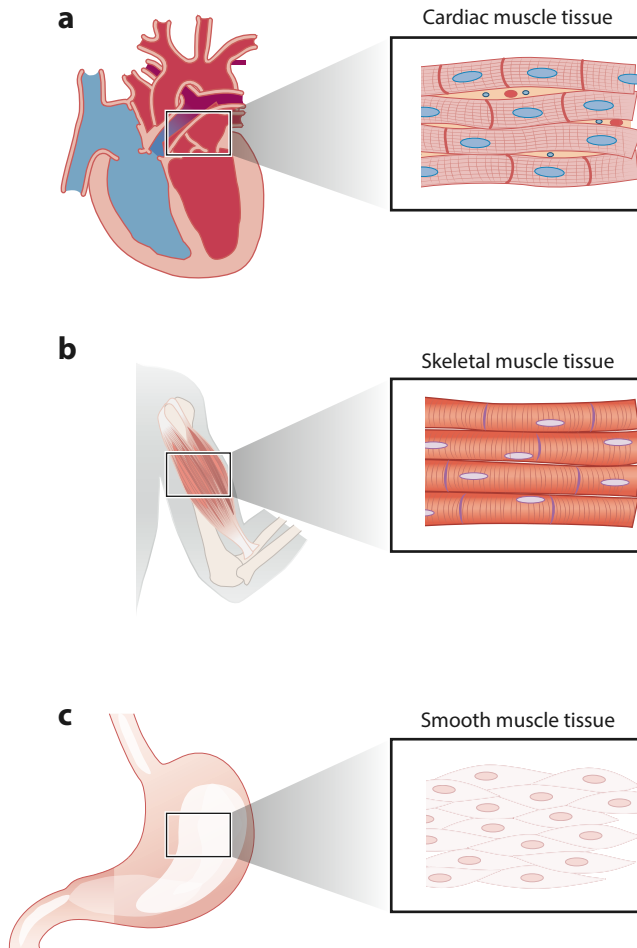


Figure 1

Biological actuators. (a) Cardiac muscle tissue in the heart is responsible for pumping blood throughout the vascular system. (b) Skeletal muscle tissue powers all voluntary movement. (c) Smooth muscle tissue aids active transport of materials throughout the body.

adapt to changing surroundings. The ability to build engineered systems that are adaptive to and resilient in dynamic unpredictable settings would be a paradigm-shifting advance in machine design, motivating the rise of biohybrid robotics (15–19).

This review outlines key advances in integrating biofabricated living actuators with engineered machines by highlighting muscle-powered soft robots that swim, walk, pump, and grip. We discuss active areas of ongoing investigation including integration of upstream sensors and processors, reproducible manufacturing and scale-up, design of robust abiotic/biotic interfaces to enable untethered deployment, and predictive computational modeling. Furthermore, we emphasize the need for multidisciplinary discussions of bioethics, inclusive and immersive pedagogical techniques, and equitable workforce development to ensure the long-term positive societal impact of this emerging field. By sharing both the potential impact and risks of deploying living actuators in real-world settings, we hope to motivate ethical and rational design, biofabrication, and practical application of biohybrid machines (**Figure 2**).

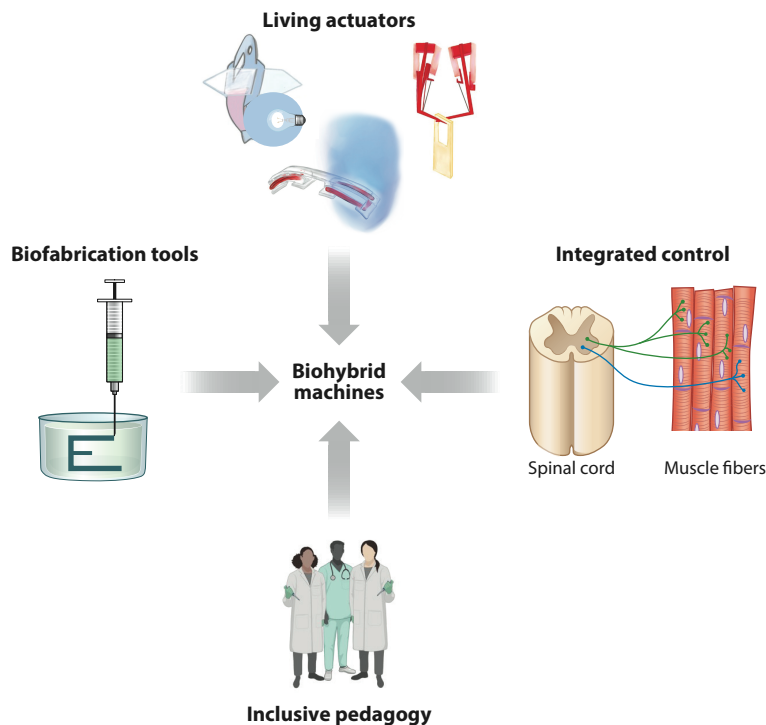


Figure 2

Overview of biohybrid machine design. The field of biohybrid machines has, to date, largely focused on engineering living actuators. Emerging interest in neural control and tools for 3D biofabrication will enable scaling the complexity and size of these systems even further. Pedagogy that cultivates multidisciplinary skill sets and integrates active discussions of ethics will, likewise, ensure a more diverse future workforce. Living actuators illustrations by Radha Raman.

2. ROBOTS POWERED BY LIVING ACTUATORS

A pioneering study by Herr & Dennis (20) in 2004 demonstrated the use of skeletal muscle as a functional actuator in a robot. The centimeter-scale biohybrid machine relied on electrical stimulation of explanted frog muscles, coupled to a compliant skeleton, to drive tadpole-like swimming of the robot across a tank with a top speed of 0.33 body lengths per second. By tuning muscle stimulation parameters, the team was able to regulate swimming speed and direction, enabling top-down control of a biologically powered machine. While the use of biological molecules as nanoscale actuators in machines was, at this time, an emerging area of interest in engineering (21, 22), this study by Herr & Dennis set the stage for deploying tissue-scale biological actuators in robotics. Operating at this macroscale size and force regime expanded potential future applications of biohybrid robots to those traditionally reserved for abiotic machines, ranging from manufacturing to exploration of unknown terrains.

Leveraging explanted muscle as an actuator can advance understanding of biohybrid machine design but is unlikely to be a useful long-term strategy for robotics as it is both unsustainable (i.e., requires sacrificing an animal for each use) and unreliable (i.e., is constrained by natural variability in form and function between different animals). Robust, reproducible, and ethical production of biologically derived actuators must thus leverage advances in 3D biofabrication and tissue engineering to build muscles from scratch. A few significant examples of cardiac-muscle-powered and

skeletal-muscle-powered robots that swim, walk, pump, and grip are described below. Thus far, smooth muscle has not been deployed extensively in biohybrid machines, potentially due to the relatively limited progress in engineering contractile smooth muscle from established cell lines (23).

Importantly, most of the studies highlighted in this review rely on mammalian cell sources, which are constrained by the limitations of our own bodies: they function only at temperatures of 37°C and in environments with precisely regulated humidity, oxygen, pH, and nutrient supply. While these constraints do currently limit the design space and application areas of biohybrid robots, the field's predominant focus on mammalian cells can be attributed to the fact that tissue engineering first evolved with a focus on human health. Many researchers in this field have thus been trained to use mammalian cells, and are in fact motivated to use them, as engineering mammalian muscle could simultaneously advance robotics and fundamental understanding of development, maturation, and remodeling in humans. There are, however, many examples of biohybrid robots powered by nonmammalian cells and tissues, ranging from bacteria to insects to sea slugs, and key examples of those systems are also included in this review. As with any engineered system, the choice of materials will be based on application-specific considerations of performance, longevity, and cost, and it is likely that both mammalian and nonmammalian cell sources will play an important role in this field in the coming years.

2.1. Muscle-Powered Swimmers

Many of the significant advances in biohybrid robotics have been made in the design and fabrication of swimming machines. Cardiac muscle has been a very popular cell source for this application, since many forms of swimming involve continuous rhythmic movement. Cardiac muscle tissue is composed of sheets of electrically coupled cells (**Figure 1a**) and is thus particularly well suited for constant and synchronous contraction that can be paced at different frequencies. Inspiration from the body design of a variety of natural cells and organisms, ranging from jellyfish (24) to stingrays (25) to spermatozoa (26), has generated a variety of biohybrid robots that mimic the form and functional behavior of these naturally existing systems.

In the first generations of biohybrid swimmers, electrical stimulation of engineered 2D sheets of cardiac muscle coupled to flexible elastomers was used as a control mechanism to drive synchronous paced contraction of the actuator and propulsion of the machine. The increasing popularity of genetic engineering tools enabled augmenting the complexity of these control systems by engineering cardiac muscle cells to respond to other stimuli, such as light. Since light offers the advantage of high spatial resolution, this enabled the creation of optogenetic machines where specific regions of engineered muscle could be actuated without activating the whole system, yielding more complex locomotive behaviors. Most recently, a study by Lee et al. (27) created a biohybrid fish, in which each side of the fish's elastomeric body contained a 2D layer of engineered cardiac muscle. The researchers seeded two types of cardiac muscle cells (one responsive to blue light and another responsive to red light) on either side of the fish, generating an agonist–antagonist muscle pair that could be separately controlled by different wavelengths of light (**Figure 3a**). These advances increased the swimming efficiency and speed of centimeter-scale biohybrid robots to ~1 body length per second, matching the performance of natural swimming organisms.

Importantly, cell sourcing of cardiomyocytes for tissue engineering has advanced significantly in recent years. Many early advances in this field relied on harvesting primary cells from neonatal rat hearts, which comes with the disadvantages associated with explanting whole muscles from an animal, as described above. However, recent developments in engineering mature contractile tissues from human induced pluripotent stem cell (iPSC)-derived cardiomyocytes (28, 29) has

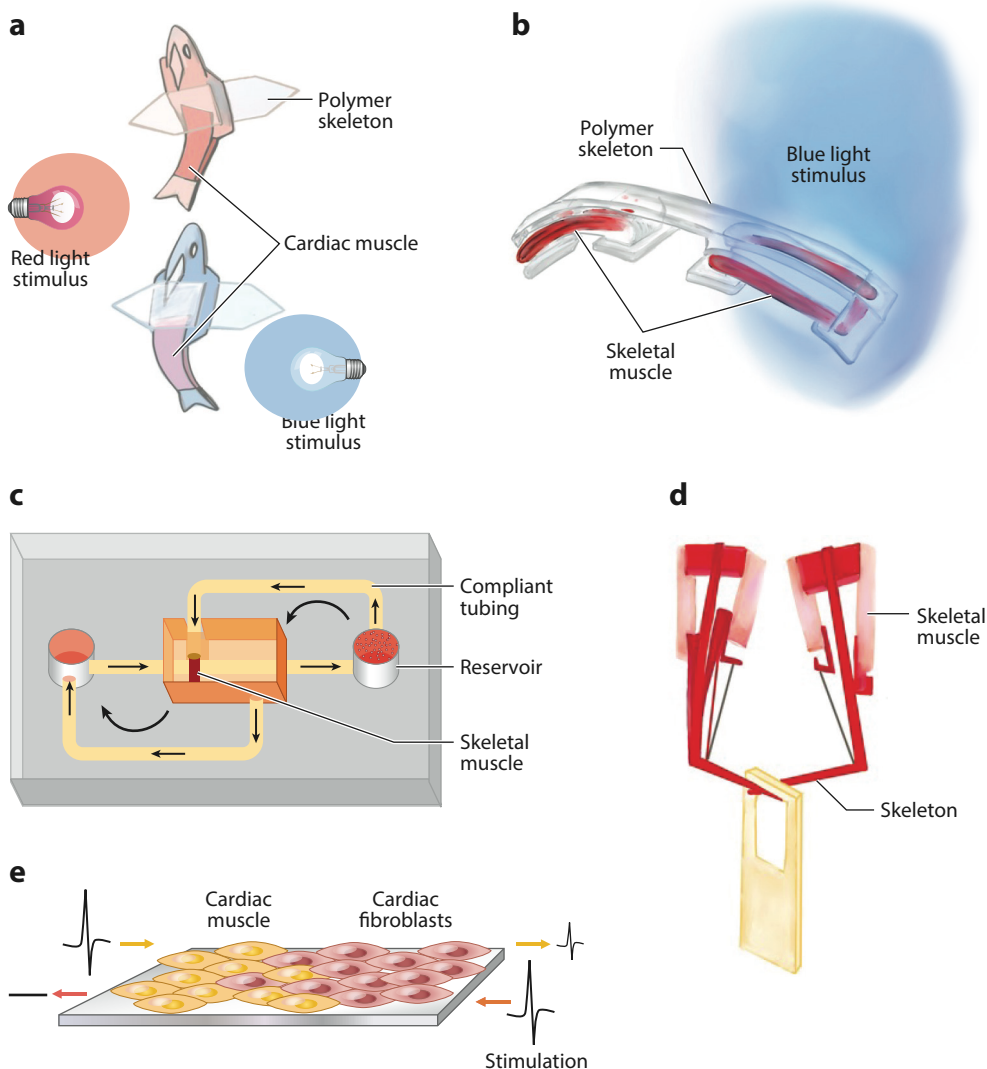


Figure 3

Robots powered by living actuators. (a) Swimming machine powered by agonist and antagonist sheets of cardiac muscle tissue responsive to red and blue light stimulation, respectively. Panel adapted with permission from Reference 23. (b) Walking robot powered by skeletal muscle. Directional locomotion is powered by targeted light stimulation of optogenetic tissue. Panel adapted with permission from Reference 38. (c) Peristaltic pump powered by skeletal muscle. Feedback from the reservoir modulates muscle function. Panel adapted with permission from Reference 47. (d) Gripper robot powered by two agonist–antagonist pairs of skeletal muscle tissues that can be used to pick up and move objects. Panel adapted with permission from Reference 52. (e) Living diode composed of cardiac muscle tissue and cardiac fibroblasts. Panel adapted with permission from Reference 55. Illustrations in panels *a*, *b*, and *d* by Radha Raman.

set the stage for deploying a more sustainable and reliable cell source for cardiac muscle tissue engineering.

While cardiac muscle offers the advantage of synchrony, voluntary movement in mammals is typically controlled by skeletal muscle. In skeletal muscle, individual cells are controlled by an upstream motor neuron. This offers precisely scalable force generation and on/off control within a

hierarchically designed 3D tissue (**Figure 1b**), which are key desirable characteristics in the context of machine design. Aydin et al. (30) led a significant advance in this field by showcasing the first example of a swimming robot powered by skeletal muscle (submillimeter scale) and controlled by upstream motor neurons. Optogenetic control of neurons enabled precise control over swimming trajectory and speed ($\sim 1 \mu\text{m/s}$), again showcasing the value of leveraging genetic engineering tools to enable new functionalities in biohybrid robots. Established sources of mouse skeletal myoblast cell lines enable robust reproducible manufacturing of engineered skeletal muscle, but fabrication of millimeter- to centimeter-scale 3D tissues still requires further advances in manufacturing, vascularization, and innervation, as described in further detail below.

Initial studies in biohybrid swimmers focused on engineering responsive behaviors, such as electrically triggerable and light-triggerable contraction, in the cells themselves. However, concurrent advances in polymer science have enabled creating hydrogels that can be triggered by a range of external stimuli that could be deployed as active elastomeric skeletons for biohybrid machines (6, 31–33). For example, Xu et al. (34) have shown that a cardiac-muscle-powered robot whose skeleton is composed of a light-triggerable hydrogel can undergo a shape transformation that changes the robot from a swimming mode to a cargo-delivery mode. This study sets the stage for engineering biohybrid machines that are capable of more than one output function, and, perhaps more importantly, leverage the state of the art in both tissue engineering and responsive polymer design.

2.2. Muscle-Powered Walkers

Inspired by compelling demonstrations of cardiac muscle as an actuator for swimming robots, several studies have also explored the ability to leverage synchronous cardiomyocyte contraction in the context of walking machines (35, 36). Recently, Kriegman et al. (37) demonstrated design, manufacturing, and functional testing of fully biological submillimeter-scale ball-like walking machines composed of contractile primary cardiomyocytes and epidermal cell progenitors sourced from frogs. Directional locomotion of these robots could be predictably controlled by organizing the relative distribution of contractile and noncontractile cells within the engineered tissue ball. Building on this initial demonstration, Blackiston et al. (38) showed that swarms of such robots, swimming at maximum speeds of $\sim 20 \mu\text{m/s}$, could be deployed to explore new environments. To accomplish this goal, they integrated a fluorescent reporter into their engineered tissues that changed wavelength from green to red when exposed to blue light. Swarms of cardiac-muscle-powered robots were then allowed to randomly explore a petri dish in which a small region was illuminated with blue light. Machines that happened to walk through the blue light-illuminated regime changed color, indicating that biohybrid robots could record and report their experiences as they navigated new surroundings.

Most demonstrations of walking robots have leveraged mammalian skeletal muscle, instead of cardiac muscle, as an actuator, since this architecture better mimics that of most naturally existing legged organisms. We reported the first example of a walking robot powered by 3D engineered skeletal muscle by coupling the muscle to a flexible polymeric skeleton and controlling contraction with an electrical stimulus (39). As described above, the availability of immortalized skeletal myoblast cells, such as the mouse C2C12 line, and established protocols for differentiating contractile muscle fibers from these cell lines (40, 41) were key enabling advances that enabled robust reproducible manufacturing of such machines. We have since shown that these cell lines can be engineered to be optogenetic and that light-triggered contraction of skeletal muscle can be used to control directional locomotion and steering of walking biohybrid robots at linear speeds of ~ 1.5 body lengths per minute and rotational speeds of 2 deg/s (**Figure 3b**) (42, 43). Building on this work, Wang et al. (44) recently showed that multilegged robots powered by several

independent optogenetic muscles demonstrated increased locomotive speed and efficiency, as the actuators could be activated separately or in parallel with localized light stimulation.

Since skeletal muscle is designed for adaptive response, we hypothesized that our robots' performance could be improved by increasing the force generation capacity of individual muscle fibers, as well as by increasing the total number of fibers (45, 46). In humans and other animals, this performance improvement is typically accomplished via exercise training. Interestingly, we observed that our living actuators could be exercised by repeated light stimulation and that this significantly improved the force production and locomotive speed of biohybrid robots (42). Pagan-Diaz et al. (47) have since shown that increasing muscle length and width to generate centimeter-scale constructs can also result in improvements in force output of nearly $\sim 6X$ by increasing the total number of actuating fibers.

Unlike cardiac muscle, skeletal muscle is capable of healing after injury by recruiting a quiescent population of muscle stem cells (termed satellite cells) to regenerate muscle fibers that have been damaged. This characteristic enabled us to design the first biohybrid robots that could completely recover from injury through regeneration of new muscle fibers that restored force output to predamage values (48). Interestingly, we observed that healing could be accelerated by targeted light stimulation exercise of the newly regenerating muscle during growth and maturation and have since validated that a targeted exercise strategy can also accelerate healing after traumatic muscle injury in a mouse (49). These studies highlight the potential for cross talk between robotic and medical applications of tissue engineering, as discussed further in a later section.

In the most recent report of walking biohybrid machines, Kim et al. (50) have presented an exciting advance by designing skeletal-muscle-driven robots that integrate onboard electronics. In these machines, a wireless optogenetic device can be activated via a radio-frequency power source, enabling fully remote-controlled on/off behavior, directional locomotion, and tunable speed up to ~ 0.8 mm/s. This study sets the stage for future generations of biohybrid walkers that can be deployed and controlled in an untethered manner for real-world applications.

2.3. Muscle-Powered Pumps

Actuators have functional purposes beyond driving locomotive behaviors such as swimming and walking, motivating exploration of other applications for living actuators such as pumps. In an early demonstration of this capability, Tanaka et al. (51) showed that growing a 2D sheet of cardiac muscle around a hollow elastomeric sphere could generate a heart-like pumping behavior that drove fluid flow through a downstream capillary-like tube at flow rates in the range of 0.01–0.1 $\mu\text{L}/\text{min}$. Pulsatile fluid flow through a hollow flexible tube has also been demonstrated with 3D rings of skeletal muscle stretched around the tube, including examples of muscle explanted from fruit flies as well as muscle engineered from mouse myoblasts (2, 53).

In one of the most recent demonstrations of a muscle-powered pump, Ko et al. (54) leveraged insights from both cardiac and skeletal muscle biology to engineer a hybrid machine that retained desirable performance characteristics of both types of tissue. In cardiac muscle, gap junction proteins (such as connexin 43) enable rapid propagation of action potentials across sheets of cells and coordinate tissue-wide synchronous contraction. By contrast, skeletal muscle fibers are electrically independent from their neighboring fibers unless they are controlled by the same upstream nerve, enabling finely tunable force regulation. Interestingly, skeletal myoblasts present connexin 43 during early differentiation, but this protein is downregulated as myoblasts fuse to form fibers. Ko et al. showed that tethering reduced graphene oxide flakes to myoblasts during early differentiation would sustain expression of connexin 43 in mature muscle tissue, altering gene expression significantly at terminal differentiation. This method improved the force generated by the muscle

in response to electrical and optical stimulation and also increased the resultant fluid flow velocity in the pump by $\sim 2\times$ to a maximum of $\sim 30\ \mu\text{L}/\text{min}$.

While muscle contraction for biohybrid robots has primarily been controlled via upstream electrical and optical stimuli, recent studies of biohybrid pumps have also explored other potential mechanisms to regulate performance. For example, Li et al. (55) recently designed a biohybrid pump with flow loop feedback in which flow generated by muscle contraction drives fluid from a reservoir containing a biochemical stimulant (acetylcholine) toward the muscle, increasing force and frequency of twitch and pumping rate (**Figure 3c**). This study demonstrated a method to regulate the performance of skeletal muscle actuators with biochemical stimuli and, perhaps more importantly, showcased how dynamically living actuators can adapt to changing environments with real-time feedback control.

2.4. Muscle-Powered Grippers

Muscle-powered grippers, though perhaps less popular than their swimming, walking, and pumping counterparts, nevertheless showcase the design flexibility of biohybrid machines. Akiyama et al. (56) made the first steps in this domain by designing a set of microtweezers with hinges actuated by the contraction of explanted muscles from inchworm larvae. More recently, Morimoto et al. (57) engineered skeletal muscle from primary neonatal rat myoblasts, creating an agonist–antagonist muscle pair that could be controlled by electrical stimuli over a rotational range of $\sim 100^\circ$ and be used to pick up, move, and manipulate objects (**Figure 3d**). The authors noted that electrical stimulation, while effective, generated electrolysis in the culture media and reduced the long-term viability and function of the biohybrid machine. These findings align with similar limitations observed in electrically stimulated walkers, swimmers, and pumps and indicate that the next generation of grippers will likely also benefit from the use of noncontact optical stimulation, upstream innervation, or other less-invasive control mechanisms.

In a separate study, Morimoto et al. (58) showed that each arm of the muscle-powered gripper could be encapsulated in an exoskeleton made of collagen. The internal lumen of the exoskeleton could contain culture medium and supply the muscle with key nutrients, but the robot itself could function in air because the muscle was insulated from the ambient environment. While the collagen could not prevent evaporation of water, which caused the muscle to dry out within a few minutes of in-air operation, this study still showcased a proof-of-concept demonstration of untethered function of a biohybrid robot outside a petri dish environment. Building on this demonstration of untethered function in a biohybrid gripper, and extending it to other functions such as walking and pumping, would be a significant step forward for the field.

2.5. Muscle-Powered Computation

While the most obvious application of muscle actuators is in force production, leveraging biological materials in engineered systems enables new use cases for these materials that are not present in natural systems. In a creative example of this line of research, Can et al. (59) constructed living diodes based on a sheet of cardiac muscle cells cultured adjacent to a sheet of cardiac fibroblasts. Since cardiac muscle is electrically excitable and coupled via gap junctions, an electrical stimulus initiated in the cardiac muscle sheet could be amplified and propagated throughout the muscle and pass through to the nonexcitable fibroblast tissue. By contrast, an electrical stimulus initiated in the fibroblast sheet did not initiate or propagate any action potentials. The engineered tissue thus behaved like a diode by transducing electrical signals in a unidirectional manner from muscle to fibroblasts, but not in reverse (**Figure 3e**).

Building on this work, the team has since built a bio-oscillator network composed of nodes of cardiac muscle coupled by bridges of cardiac fibroblasts. Separate nodes of cardiac muscle, which

spontaneously contract, can communicate with each other through fibroblast bridges, which provide pathways for electrical signal propagation. Over time, separate nodes synchronize their beat frequency, and the researchers discuss how such systems can be used to efficiently solve optimization problems (such as the vertex coloring problem) that are typically computationally expensive (60, 61). Extensions of this technology to collective computing could provide new avenues for deploying muscle tissues beyond applications focused on force generation.

2.6. Beyond Mammalian Tissue-Based Actuators

Most of the examples outlined above rely on mammalian cells as sources to fabricate living actuators at the tissue scale, rendering them useful tools for advancing fundamental understanding of mammalian biology and for impacting applications in human health. Recognizing the constraints on culture conditions that best preserve the viability and function of mammalian cells, however, many researchers have relied on explanted tissues from nonmammalian creatures, primarily frogs, worms, and insects, to fabricate robots, as outlined in a few of the examples presented above and in other reviews (18). Webster et al. (62) have even extended potential sources of living actuators to less commonly used organisms such as sea slugs (*Aplysia californica*), which typically survive in saltwater environments and can tolerate large temperature changes. As future work extends potential sources for living actuators, and even living sensors and processors, to a broader range of cell sources, more specific taxonomic keys beyond the simple term biohybrid may be required to clarify the exact subset of research being advanced by a particular study (63).

While this review is focused on tissue-scale living actuators, it is important to note that cell-scale actuators from both mammalian and nonmammalian sources are also of significant interest in the field of biohybrid robotics. Mammalian sources of single-cell-based actuators primarily leverage the motion of spermatozoa. A recent study by Rajabasadi et al. (64) coupling spermatozoa to an abiotic drug-loaded hydrogel demonstrated that such single-cell-based biohybrid systems can be used for targeted cargo delivery in applications such as assisted fertilization. Payload delivery has also been a focus in the development of microrobots powered by bacteria. In particular, magnetically steerable bacteria have been of sustained interest in this field, as magnetic control is noncontact, can safely penetrate tissue, and offers high spatiotemporal precision. Akolpoglu et al. (65) have shown that bacterial biohybrid machines composed of *Escherichia coli* loaded with magnetic nanoparticles can navigate through 3D engineered tissues in vitro and accomplish on-demand release of drugs. In an exciting recent advance, Gwisai et al. (66) demonstrated that bacterial biohybrid robots can be safely deployed in vivo and precisely controlled via a directional magnetic field. This study showcased the tissue-penetrating capabilities of such machines in the context of a mouse solid tumor model, demonstrating that bacterial biohybrids accumulate in tumors and could be used, in the future, as a clinically relevant strategy for targeted drug delivery.

In contrast to cell-scale systems, tissue-scale actuators are optimized for generating larger forces, and deformations are, perhaps, better suited to macroscale applications of robotics such as assisted manufacturing, telesurgery, and untethered exploration. However, rendering tissue-scale actuators useful for such real-world practical application requires fundamental advances across multiple domains, as outlined in the next section.

3. ONGOING TECHNICAL CHALLENGES

While the applications of muscle-powered machines highlighted above have developed robust protocols for tissue engineering both cardiac and skeletal muscle, it is important to note that these engineered tissues have yet to completely match the morphology and function of native tissue. For

example, while native skeletal muscle can generate up to 20% contractile strain and ~ 0.5 MPa of stress during twitch, engineered skeletal muscle derived from cell lines (such as the commonly used C2C12 myoblast line) typically falls in the realm of 1–5% strain and ~ 1 kPa of stress (15, 42, 43, 67). Interestingly, some studies have shown that leveraging primary cells to manufacture skeletal muscle actuators can significantly increase the generated force and completely address the strain limitation, matching the performance of native tissue (57). These studies indicate that developing robust reproducible cell lines that more closely mimic the performance of cells matured in the native environment will be the key to driving further improvements in skeletal muscle tissue engineering.

Similar trends are observed in cardiac muscle tissue engineering, where early studies focused on primary cells showcased actuation characteristics that nearly matched those of native tissue (15). While transitioning to a more sustainable and reproducible approach of generating cardiac muscle from iPSCs has met with considerable success (27, 28), these tissues are still significantly less mature than their *in vivo* counterparts, motivating studies that leverage exercise training approaches to promote differentiation and improve performance (68).

While we have focused this discussion on efforts to match the performance of natural muscle, it is also interesting to consider leveraging genetic engineering tools to manufacture actuators that exceed the performance of native muscle. In medical contexts, this type of genetic gain-of-function modification may raise concerns because of the risk of potential off-target effects on other tissues in the body. However, in the context of a robotic system, these concerns are less relevant and could provide a potential avenue for further improvements in actuation parameters. Conducting studies along this trajectory would likely raise potential ethical concerns, as discussed in Section 4.

Of course, practical application of living actuators in biohybrid machines requires more than just controllable force production via muscle tissue engineering, though this was a critical first step in the field. Enabling real-world use of these actuators also requires integrating upstream neural control and processing, reproducible manufacturing and vascularization for scale-up, and methods to enable untethered function in unpredictable environments. Ongoing efforts to address these technical challenges are outlined in detail below.

3.1. Neural Control and Sensory Feedback

Living actuators convert biochemical energy to mechanical output, but pacing muscle and regulating force output require neural control and sensory feedback. This is particularly relevant in the context of skeletal muscle, where on/off control is purely controlled by upstream motor neurons (**Figure 4a**) and critical functional behaviors such as agonist–antagonist pairing and proprioception are regulated by embedded sensory neurons. The challenges associated with innervating engineered skeletal muscle have been extensively outlined in other reviews (69) but are briefly outlined below as they represent a critical technical gap in the field of biohybrid robotics.

Building on established protocols for engineering 3D skeletal muscle from mouse myoblasts, we have fabricated muscle tissues containing an embedded layer of mouse embryonic stem cell–derived motor neurons (70). Encouragingly, we observed that the motor neurons extended neurites toward the muscle to form functional neuromuscular junctions that could be controlled via biochemical stimulation with the neurotransmitter glutamate. However, the nerves appeared to innervate only the regions of muscle that were directly proximal to their soma, leaving most of the muscle fibers in the millimeter-scale tissue uninervated. This and other similar studies have prompted further investigation into mechanisms to enhance nerve growth in engineered multicellular tissues.

A study by Ko et al. (71) investigated the impact of substrate topography on regulating innervation of skeletal muscle by upstream motor neurons. The researchers first formed highly aligned

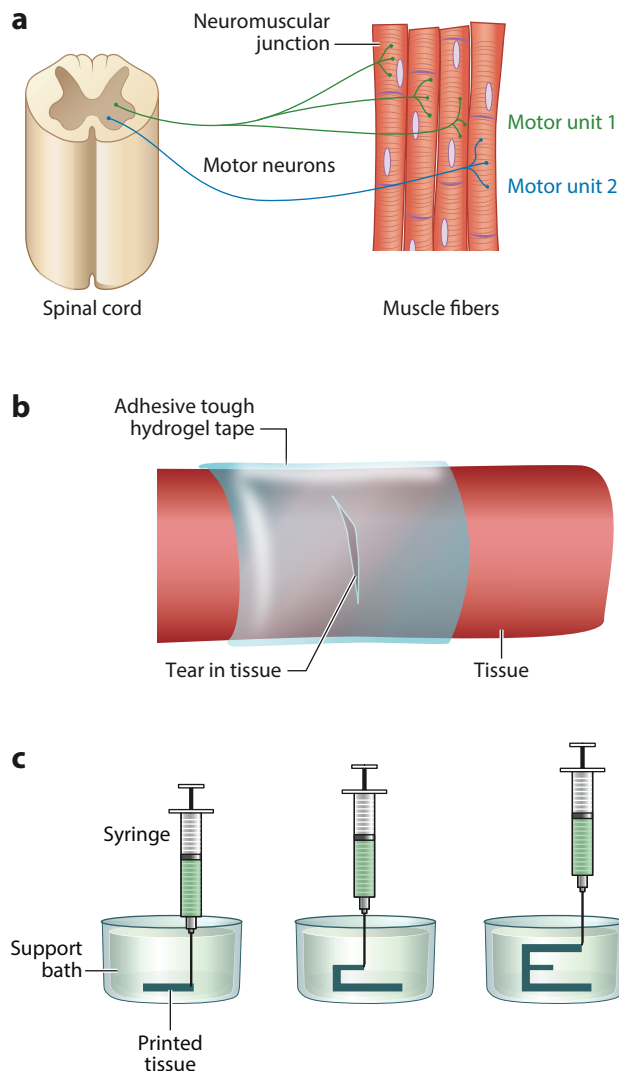


Figure 4

Emerging areas of research in the biofabrication of living actuators. (a) Integrating upstream neural control of muscle can enable precise tuning of force output and complex behaviors such as agonist–antagonist pairing. (b) Bioadhesive interface that enables robust binding to tissues. Panel adapted with permission from Reference 78. (c) Extrusion bioprinting in a support bath enables the printing of soft tissues with high shape fidelity. Panel adapted with permission from Reference 88.

engineered muscle tissue by growing myoblasts on a grooved substrate, demonstrating that this topological cue generated a downstream increase in the expression of acetylcholine receptors on the membrane of mature muscle fibers. Interestingly, they observed improved innervation of aligned muscle fibers by cocultured motor neurons, as compared with randomly aligned fibers grown on an unpatterned substrate. Functional analysis of these cocultures corroborated the formation of more mature neuromuscular junctions, as evidenced by increased contraction of muscle when upstream nerves were stimulated with glutamate. Interestingly, muscle contraction

could be rapidly stopped upon exposure to curare (a neurotoxin) in highly innervated tissues, providing further evidence that the engineered muscle was in fact being controlled by neurons.

While it is important to engineer the interface between motor neurons and skeletal muscle, it is also relevant to think about other elements of neural circuitry, including the elements of the motor cortex that plan and coordinate movement, as well as the interneuron messengers that rapidly relay feedback loops. Recent advances in biology have showcased the ability to generate functional neural circuits between neurons of the central nervous system and peripheral nervous system and skeletal muscle in bottom-up assembly of organoid tissues from stem cells (72). Such organoid-like systems have also lent insight into how neurogenesis and synaptogenesis can be modulated via optogenetic stimulation training, as with muscle (73). However, macroscale engineering of these circuits for centimeter-scale constructs has yet to be demonstrated. Adewole et al. (74) propose a possible strategy for fabricating living electrodes composed of tissue-engineered neural axons encapsulated within a hydrogel cylinder. These constructs can function as electrically conductive biohybrid wires and can be used to relay signals from one end of the wire to the other *in vitro* and *in vivo*. It is possible that this and other hybrid approaches may enable the formation of complex macroscale neural control circuitry for future generations of biohybrid robots.

In engineered systems, control is nearly always accompanied with closed-loop feedback, as this ensures stable and predictable function in dynamic real-world settings. While there have been a few studies showing that intrafusal muscle fibers and sensory neurons can be cocultured with engineered skeletal muscle, robust reproducible cell sources for generating these multicellular tissues and forming functional connections between different cell types remain a challenge (75–77). Guo et al. (78) showcase the future potential and promise of integrating mechanosensation into engineered muscle tissues by coculturing human proprioceptive sensory neurons with intrafusal muscle fibers. Functional synapses between these cell types were observed via immunohistochemical imaging, and electrophysiological analyses revealed that the electrical activity of intrafusal muscle fibers differs significantly from that of their extrafusal (i.e., contractile) counterparts. Future work to advance fundamental biology of intrafusal muscle fibers, upstream sensory nerves, and interneurons will be critical to engineering living actuators that integrate biological control systems and processors.

Operating at the interface of engineering and biology gives researchers additional pathways to attain the goal of closed-loop feedback control of engineered muscle. Rather than relying on biological mechanosensation, Zhao et al. (79) have recently shown that integrating flexible strain sensors with engineered skeletal muscle can reliably record motion and measure contractile force with high accuracy. This approach presents an alternative approach to biohybrid machine design by leveraging advances from both tissue engineering and materials science to advance functional capabilities of living actuators. Further analysis of the longevity and robustness of these abiotic/biotic interfaces will be critical to enable untethered function of biohybrid robots in unpredictable environments, as discussed in the next section.

3.2. Biohybrid Interfaces

Robust longitudinal function of biohybrid devices requires precisely controlling the interfaces between abiotic and biotic materials in these multicomponent systems. In the examples outlined in this review, interfaces between living actuators and flexible skeletons range from biochemical covalent tethers to mechanical friction-based fits. There have been relatively few studies that focus on engineering interfaces in the context of biohybrid robotics, but this topic has been an active area of research for medical devices, and there are likely many translatable insights between fields. For example, Yuk et al. (80) have developed an adhesive hydrogel tape that bonds wet tissues and abiotic devices robustly and can be deployed longitudinally *in vitro* and *in vivo*. By leveraging

covalent cross-linking between amine groups present in the tissue and *N*-hydrosuccinimide ester in the tape, they demonstrate that tissues and devices can be strongly bonded within seconds (**Figure 4b**). Bioadhesives can serve as more than merely passive interfaces between tissues and synthetic materials, as they can integrate conductive polymers and circuitry to incorporate active functionalities such as electrical recording and ultrasound monitoring of tissues (81, 82).

A recent report by Nam et al. (83) showcased how active tissue adhesives can control the performance of a living actuator. In this study, the team designed a mechanically active adhesive gel that contained embedded wires of nitinol, a shape memory alloy that contracts upon electrical stimulation and has an established history of use in medical devices (84). They showed that dynamic mechanical stimulation of disused muscle via actuation of these bioadhesives could prevent atrophy and improve force generation capacity. While the applications of such technologies to regenerative medicine are evident, it is also likely that similar techniques could prove useful for promoting the longevity and robustness of muscle-powered machines. For example, we have recently shown that an actuating gel substrate controlled by external magnetic fields can be used to mechanically stimulate engineered muscle in a noninvasive manner (85). Interestingly, we observed that muscle fibers aligned parallel to the direction of actuation and, as a result, improved synchronicity of contraction. These results serve as a promising indication that such active biohybrid interfaces could be leveraged to program anisotropy of form and function in muscle actuators.

While most studies in interface design focus on manipulating communication between abiotic and biotic materials, it is also critical to think about interfaces between different types of biological tissues within engineered systems. Sun Han Chang et al. (86) have recently developed a compliant hydrogel that connects two mechanically mismatched tissues (engineered tendon and bone). By forming a continuous bridge between the tissues, which have very different stiffnesses, the hydrogel bridge reduced strain concentrations at the interface between cell types and improved biomechanical performance.

These and other studies indicate that future generations of biohybrid machines must carefully consider not only the biological and nonbiological materials within engineered systems but also the interfaces between them, to enable untethered function of robots that integrate functional biological components.

3.3. Scalable Manufacturing

Tissue engineering has historically relied on traditional techniques of cell culture, such as pipetting, to fabricate multicellular constructs. Advances in a range of tools and techniques for 3D printing with living cells have, however, fueled increasing interest in automated biofabrication technologies that can enhance the scalability and reproducibility of manufacturing engineered tissues (5, 87). While early efforts to 3D print tissues explored a variety of additive manufacturing techniques that proved successful in other application areas, such as stereolithography (88, 89), recent trends have largely focused on extrusion-based bioprinting approaches that enable high-density patterning with cells. In particular, extruding cells into soft gelatinous baths that provide mechanical support during printing has proven to be a robust and adaptable technique for fabricating tissues (90). Lee et al. (91) recently leveraged this approach, termed FRESH (free-form reversible embedding of suspended hydrogels) printing, to demonstrate organ-scale printing of collagen that mimicked a neonatal human heart (**Figure 4c**). Skylar-Scott et al. (92) have also shown that extruding sacrificial materials into a support bath of cells, a variant of FRESH-like printing termed SWIFT (sacrificial writing into functional tissue), can be used to form large tissues with perfusable vasculature. To showcase the promise of these printing approaches for scalable biomanufacturing, the team showed that SWIFT printing could be used to print contractile cardiac muscle that beat synchronously.

Extrusion-based 3D printing has also been used to fabricate engineered skeletal muscle (93). Mestre et al. (94) have shown that C2C12 mouse myoblasts printed in a hydrogel mixture of fibrinogen, gelatin, and hyaluronic acid that mimicked the native extracellular matrix remain highly viable and form aligned mature muscle fibers. Electrical stimulation of printed skeletal muscle produced paced contraction of the fibers within the tissue, indicating that this technique could be used to fabricate functional living actuators. Rising interest in manufacturing large volumes of skeletal muscle for clinical applications has also driven advances in this field, as highlighted in other reviews (see, e.g., 95), including studies showing that 3D printed skeletal muscle can be implanted in rodent models *in vivo* to recover mobility after traumatic injury (96). Printing of living skeletal-muscle-based and cardiac-muscle-based actuators is within reach, and future studies integrating multiple cell types within a single printed construct will enable the design of more complex multiscale hierarchies in the future (97).

Scalable manufacturing must often be coupled with robust strategies for storage and transport to enable practical application across a range of industries. Cryopreservation is the most common technique for long-term storage of cells in a manner that preserves their viability after thawing, motivating the development of novel cryopreservation strategies for storing 3D engineered tissues (98). Interestingly, we have observed that freezing engineered skeletal muscle not only preserves the viability of embedded cells over 2 months but also can improve the alignment of muscle fibers within the tissue, potentially due to changes in pore size within the extracellular matrix (99). Importantly, we observed these improvements in fiber alignment only in tissues that were frozen before differentiation and noted that the microscale hierarchy of tissues frozen after differentiation was disrupted by the printing process. Follow-on studies have since shown that increasing pore size decreases the elastic modulus of the tissue, which in turn upregulates the expression of genes that play key roles in cell migration, extracellular matrix secretion, and cell–matrix adhesion (100). Cryopreservation-induced changes to the physical structure and biochemical composition of the extracellular matrix were associated with improved muscle differentiation. These studies highlight that cryopreservation is not a passive process and that it may have both positive and negative impacts on tissue morphology, maturation, and function. Future studies that leverage cryopreservation not only as a technology enabling long-term storage but also as an active process for tuning tissue performance could add further depth and insight to this emerging field.

Thus, there are many remaining challenges to be addressed to enable reproducible printing, freezing, storage, and revival of living actuators based on skeletal, cardiac, and smooth muscle. With the addition of other, more sensitive cell types (such as motor and sensory neurons), these processes will likely need to be adapted and optimized further to ensure preservation of tissue viability and function. Encouragingly, there have been significant recent advances in long-term organ cryopreservation for transplantation applications (101, 102), and it is likely that these insights can also be translated to applications in preserving and reviving engineered tissues.

While we have focused primarily on technical challenges that limit scalable manufacturing of biological actuators, practical use of these materials in real-world applications also requires considering the cost of making such tissues at scale. Interestingly, recent analyses suggest that the driving cost in biofabrication may not be cells themselves but rather the culture media used to promote their proliferation and maturation. Indeed, culture media accounts for 55–95% of the total cost of manufacturing tissues, largely because of the high cost of the growth factors that are critical to their formulation (103). Emerging advances in methods for cost-effective growth factor production, such as genetic manipulation of yeast to function as protein factories (104), could prove a promising strategy to address this limitation. It is likely that the first real-world use of biological actuators will happen in industries that are more tolerant of high price points, such as telesurgical robots for medicine or locomotive robots for high-risk exploratory missions.

Successful proof-of-concept validation in these beachhead applications will likely drive further advances in cost-effective manufacturing, enabling the deployment of living actuators in lower-margin industries such as consumer goods.

3.4. Computational Modeling

Many of the studies we outlined in prior sections have leveraged computational models of biohybrid robots to predict how generated forces will translate to movement or other functional outputs (27, 42, 47). Modeling the abiotic components of biohybrid machines relies on an established understanding of the mechanical and electrical properties of materials commonly used in engineered systems, such as metals and polymers. By contrast, generating reliable and robust computational tools for predicting the behavior of functional biological materials is a relatively new discipline. Biological materials are difficult to model because they are composed of complex hierarchical structures, have viscoelastic mechanical properties, and are constantly adapting their form and function to changing environments. Encouragingly, there has been increasing interest and focus on developing better models of biological actuators, particularly in the context of robotics.

Zhang et al. (105) have demonstrated that thin elastic deformable cylinders, termed Cosserat rods, can be used to reliably model cardiac and skeletal muscle actuators, enabling the design of new robot skeletons that could improve functional performance metrics, such as walking and swimming speed. In contrast to more traditional models for muscle, such as the Hill model, the Cosserat rod-based approach allows for the separation of rods into active and passive elements, enabling the modeling of more complex phenomena such as partial injury within a tissue. Importantly, the code for this modeling framework is open source and available online (106), and it has been used to design next-generation biological machines, with the accuracy of predicted behaviors confirmed by empirical validation (107). Similar efforts to model other biological materials that could serve as functional components in machines, such as neurons, have also been of increasing interest in the field (108).

Computational tools can also be extremely useful for monitoring the behavior of engineered tissues and extracting information about tissue morphology and function in real time. Mohammadzadeh et al. (109) recently published an open-source computational framework for extracting biological information from videos of cardiac muscle tissue. Their software enabled measurement of the functional contraction of sarcomeres in early stages of development and has also enabled tracking of the response of engineered muscle to injury (110). These early studies indicate that both computational measurement and modeling of engineered biohybrid machines promise to improve the reproducibility and predictability of these systems in the coming years.

4. ETHICS, EDUCATION, AND WORKFORCE DEVELOPMENT

As with any new technology, considerations of ethics are of critical importance in weighing the potential positive and negative impacts of an area of research on the global community (111). Living actuators could enable machines that are more efficient, adaptable to changing surroundings, and reliant on sustainably sourced and biodegradable energy sources. It is, however, worthwhile to consider the impact of real-world implementation of materials containing living cells. As engineered multicellular systems become more complex, they may display behaviors that cannot fully be predicted or controlled in untethered settings, indicating a need to develop robust safety policies that integrate input from multidisciplinary stakeholders and the general public (112, 113).

One promising method of integrating safety features with living machines is the ability to precisely control the lifetime of tissues after they have been deployed in a robotic system. Cvetkovic et al. (114) have previously explored some of the factors that may control lifetime in engineered

skeletal-muscle-powered machines. They observed that cells secrete proteolytic enzymes and that three classes of these proteases (plasmin, cathepsin L, and matrix metalloproteinases) degrade the extracellular matrix and reduce mechanical integrity of the tissue. Notably, protease inhibitors added to the culture media could extend tissue lifetime to more than 200 days, a significant improvement from a baseline of only 1 week (99, 114). While these studies have largely focused on extending the lifetime of biological actuators, their findings provide insight into how protease expression can be controlled over time to precisely control tissue survival, with relevance to all forms of engineered tissues including cardiac and skeletal muscle. Lifetime control is, of course, only one of several methods that could be used to modulate performance of untethered biohybrid robots, and the use of genetic engineering tools to control long-term activity and function of engineered tissues will be of increasing relevance to this field in the coming years (14).

Considerations of ethics need to be built into research efforts but must also be integrated with pedagogy focused on training the next generation of biofabrication scientists. Inspired by the established efficacy of project-based approaches in interdisciplinary fields such as biomedical engineering (115), we have demonstrated that lectures coupled with hands-on experiential training in the fundamental lab skills required for biofabrication, followed by an open-ended group research project in tissue engineering, can be an effective method for training undergraduate students for future careers in this emerging discipline (116). We have since extended these efforts to undergraduate students outside of bioengineering programs, graduate students, and other academic community members in the context of classes and workshops at MIT BioMakers campus spaces (<https://biomakers.mit.edu/>). Coupling these efforts with formal evaluation of pedagogical techniques and student outcomes, with a particular emphasis on understanding how such efforts can improve the representation of historically marginalized groups in science (117), will enable crafting and adapting curricula to emerging needs.

For rapidly growing fields such as biofabrication of living actuators, education efforts cannot be constrained to students in the early and traditional stages of the academic pipeline (K–12 education, college, etc.), but must also include learners at all stages of life. While training outside of this pipeline can be harder to establish and grow, it can improve the robustness and equity of the future workforce in the coming years. One promising strategy for workforce development is open-source sharing of biofabrication hardware and software. For example, Sun et al. (118, 119) recently reported modifications for an open-source 3D bioprinter that could improve the mechanical properties of printed hydrogels. Likewise, open-source computational tools that enable precise monitoring of the movement of living actuators and calculation of tissue forces have enabled reproducible and reliable performance tracking across multiple labs (109, 110, 120).

Careful consideration of ethics, education, and workforce development is essential for a holistic view on advancing biofabrication of living actuators to real-world implementation and positive impact. A significant advantage of emerging topics is the ability to learn from the growth and maturation of other fields. In particular, we hope that all researchers focused on living actuators take this opportunity to include historically marginalized groups in their pedagogical, outreach, and workforce development efforts. By ensuring that diversity, equity, and inclusion are integral parts of this emerging discipline, we will set the stage for building a dynamic, productive, and growing community over the coming years.

5. CROSS TALK BETWEEN ROBOTICS AND MEDICINE

While this review has primarily focused on applications of mammalian tissue engineering in robotics, it is important to highlight that fundamental advances in manufacturing multicellular systems benefit all other real-world applications of this field. For example, innervation and vascularization of muscle actuators, while important for improving control and scalability of

robots, could simultaneously help generate more representative models of the *in vivo* environment for applications in high-throughput drug testing. Likewise, robust scalable processes for manufacturing macroscale bioactuators are equally critical in regenerative medicine, which focuses on fabricating patient-specific implantable constructs to restore diseased or injured native tissues. Of course, communication between robotics and medicine must be bidirectional. Researchers in biohybrid robotics have an established history of learning from medically oriented tissue engineers, and new learnings about multilineage differentiation of stem cells and gene editing can, in particular, help enable new functionalities in next-generation living robots. Our hope is that biohybrid robotics will integrate inputs from both roboticists and biologists, enabling the training of a new generation of biomedical engineers that is ideally poised to advance fundamental understanding and practical applications of biological actuators.

6. CONCLUSIONS AND FUTURE DIRECTIONS

Nature has developed a range of efficient, adaptable, and robust actuators across length scales that generate movement and are central to the emergence, maintenance, and long-term survival of all living systems. In this review, we outline significant recent efforts to biofabricate tissue-scale living actuators, with a focus on cardiac and skeletal muscle. While most efforts to deploy these engineered tissues have leveraged muscle actuation to generate force and produce motion, including applications in swimming, walking, pumping, and gripping, emerging studies that leverage such materials for processing and computation promise an even wider range of impact in the coming decades.

We have outlined key technical challenges that must be addressed to enable practical implementation of living actuators in untethered robots, including integration of upstream neural control and sensing, interfaces with abiotic materials and devices, scalable reproducible manufacturing and storage, and predictive computational modeling. We hope that these technical advances will be coupled with dynamically adaptive and inclusive pedagogical and workforce development practices and emphasize the need for careful consideration of bioethics at every stage of education, research, and practical implementation. We further anticipate that, as the field broadens and develops more standardized cell sources and protocols for differentiation, we will be able to make more quantitative comparisons of actuator performance and efficiency across tissue types and application areas.

Over the past decade, biofabrication of living actuators has emerged as an exciting and rapidly expanding area of research that extends the impact of tissue engineering into the realm of robotics. Future work aimed at developing and implementing biological sensors, processors, and actuators as functional components in living machines could address challenges across diverse application areas from manufacturing to telesurgery to on- and off-world exploration. By sharing the potential positive impacts of, and the risk mitigation strategies for, deploying living actuators in real-world settings, we hope to motivate rational design and practical use of next-generation biohybrid machines.

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