

Three-dimensionally printed biological machines powered by skeletal muscle

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Combining biological components, such as cells and tissues, with soft robotics can enable the fabrication of biological machines with the ability to sense, process signals, and produce force. An intuitive demonstration of a biological machine is one that can produce motion in response to controllable external signaling. Whereas cardiac cell-driven biological actuators have been demonstrated, the requirements of these machines to respond to stimuli and exhibit controlled movement merit the use of skeletal muscle, the primary generator of actuation in animals, as a contractile power source. Here, we report the development of 3D printed hydrogel “bio-bots” with an asymmetric physical design and powered by the actuation of an engineered mammalian skeletal muscle strip to result in net locomotion of the bio-bot. Geometric design and material properties of the hydrogel bio-bots were optimized using stereolithographic 3D printing, and the effect of collagen I and fibrin extracellular matrix proteins and insulin-like growth factor 1 on the force production of engineered skeletal muscle was characterized. Electrical stimulation triggered contraction of cells in the muscle strip and net locomotion of the bio-bot with a maximum velocity of $\sim 156 \mu\text{m s}^{-1}$, which is over 1.5 body lengths per min. Modeling and simulation were used to understand both the effect of different design parameters on the bio-bot and the mechanism of motion. This demonstration advances the goal of realizing forward-engineered integrated cellular machines and systems, which can have a myriad array of applications in drug screening, programmable tissue engineering, drug delivery, and biomimetic machine design.

bioactuator | stereolithography

Soft robotic devices composed of adaptable materials permit deformation, locomotion, and control with greater degrees of freedom in a simple, low-power, and cost-effective manner compared with traditional robotics composed of metallic and rigid structures (1–3). Combining biological entities such as cells or tissues with soft materials can yield biological machines with the ability to dynamically sense and adapt to environmental cues and applied stimuli. A biointegrated approach to soft robotics can allow for the realization of biological machines with the ability to interface with the environment and other living systems. An intuitive demonstration of a biological machine is a system that can generate force resulting in net locomotion. To that end, many studies have explored the use of biological components, such as DNA (4), swarms of bacterial cells (5), motile sperm cells (6), and contractile muscular tissues excised from living organisms (7–9) as power sources with the ability to exhibit a dynamic locomotive response across length scales (10–13).

Recent advances in cardiac muscle tissue engineering have yielded dense tissues that form a syncytium, enabling the coordinated propagation of electrical signals and synchronous contraction of engineered muscle (14). This advantageous property has helped to produce machines that include self-assembling microelectromechanical-system-based cantilevers (15), 2D biohybrid “muscular thin films” (16), and “crab-like” robots

(17). These systems were powered by applied electric field stimulation or spontaneous contraction of engineered cardiac muscle, which have also been used as power sources for locomotive machines such as a swimming muscle-elastomer “jelly-fish” (18), a self-propelled swimming robot (19), and a walking millimeter-scale “biological bimorph” cantilever (20, 21), respectively.

Although these soft robots have used cardiac muscle, it should be noted that skeletal muscle is the primary generator of actuation in animals. In vivo, skeletal muscle exhibits organized modular tissue architecture on a range of length scales and supports uniaxial force production. Unlike cardiac tissue, it does not demonstrate significant spontaneous contractility, allowing for more precise control over actuation via external signaling from sources such as electrical stimulation, neural signals, or optogenetics (22). Furthermore, skeletal muscle can interface with multiple other mammalian cell types, such as neurons and endothelial cells, making it an ideal platform for producing locomotion in living cellular systems (11, 12, 23).

In this paper, we present an untethered locomotive biological machine, or “bio-bot,” powered by the contraction of engineered skeletal muscle. The structure of the bio-bot was fabricated from a synthetic hydrogel using stereolithographic 3D printing, which boasts a short fabrication time, potential for scalability, and

Significance

Cell-based soft robotic devices could have a transformative impact on our ability to design machines and systems that can dynamically sense and respond to a range of complex environmental signals. We demonstrate innovative advancements in biomaterials, tissue engineering, and 3D printing, as well as an integration of these technologies, to forward engineer a controllable centimeter-scale biological machine capable of locomotion on a surface in fluid. Due in part to their elastic nature and the living components that can permit a dynamic response to environmental and applied stimuli, these biological machines can have diverse applications and represent a significant advancement toward high-level functional control over soft biorobotic systems.

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spatial control. The use of additive manufacturing processes for a myriad of biomedical applications has increased in recent years, owing to the user's ability to rapidly polymerize an assortment of biocompatible materials with controllable geometric and mechanical properties at the micro- and macroscales (24). Skeletal muscle myoblasts were embedded in a natural extracellular matrix (ECM) of collagen I and fibrin matrix proteins, differentiated in the presence of insulin-like growth factor 1 (IGF-1), and self-assembled into a 3D muscle strip capable of contractility and sufficient force generation to power net locomotion of the bio-bot upon electrical signaling (Fig. S1). To our knowledge, this is the first demonstration of an untethered biological machine powered by engineered mammalian skeletal muscle and controlled purely via external signaling, and hence represents an important advance in building biointegrated soft robotic devices for a myriad array of applications in sensing and actuation.

Results and Discussion

Design and Fabrication of 3D Bio-bots. To construct the structure of the bio-bot, we used a modified stereolithography apparatus (SLA), a liquid-based rapid prototyping technology (24), to print a millimeter-scale hydrogel poly(ethylene glycol) diacrylate of M_r 700 g mol^{-1} (PEGDA M_r 700) device composed of two stiff pillars connected by a compliant beam (Fig. 1A and B). We used a linear elastic simulation to determine optimal beam and pillar dimensions that would combine high deflection with a robust mechanical structure (Fig. S2). A liquid suspension of mouse myoblast cell line C2C12 skeletal muscle myoblasts and ECM proteins was added around the pillars of the bio-bot and polymerized via gelation of the matrix proteins (Fig. 1C and D). Embedded cells exerted traction forces on the fibrous proteins via integrin attachments to compact the matrix into a 3D muscle strip over time (Fig. S3D and E and Movie S1), and the capped pillars acted as a physical anchor for the muscle strip. This bio-inspired design mimics the *in vivo* musculoskeletal arrangement in which force transmission occurs from a contracting muscle to bone through a connecting tendon (Fig. S3F).

The ECM contributes to maintaining cellular processes and communication in normal growth and maturation of skeletal myoblasts. Collagen I and fibrin are natural hydrogels that allow for muscle cell proliferation, spreading, and alignment, as well as tissue contraction on a macroscopic scale (25, 26). We tested both matrix proteins for their ability to support the development and organization of embedded myoblasts and provide a compliant system for tissue contraction. The cell-matrix solution consisted of either matrix protein with cell concentrations varying from $1\text{--}10 \times 10^6$ cells ml^{-1} (Fig. S4). We observed that cell traction forces increased with cell concentrations; lower concentrations resulted in less compaction, whereas higher cell concentrations caused muscle strip fracture. We deemed an intermediate concentration of 5×10^6 cells ml^{-1} as optimal.

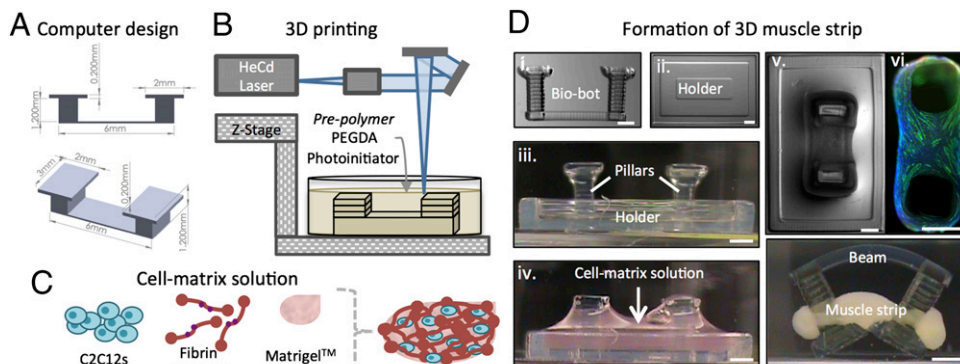
Muscle Strip Differentiation and Robustness. To improve the formation and functional performance of 3D muscle strips, we studied the effect of IGF-1 on myotube formation in 2D and 3D culture. IGFs play a role in skeletal tissue growth in vertebrates by encouraging myoblast proliferation and differentiation (27, 28). Overexpression or exogenous addition of IGF-1 also enhances muscle hypertrophy (29) and decelerates muscle decline in differentiated myofibers (30). In 2D culture, as expected, the fusion of myoblast precursor cells during myogenesis produced elongated, multinucleated myotubes (31). Over 2.5 wk, the average myotube density of myoblast cultures supplemented with 50 ng ml^{-1} IGF-1 significantly increased from $31.8 \pm 8.3\text{--}126.9 \pm 30.3$ myotubes mm^{-2} compared with the control with no added IGF-1 (Fig. S5A and B), and did not change significantly after day 7, when muscle strips were electrically stimulated.

Translating these results to 3D, we then studied the effect of IGF-1 addition (beyond that included in the Matrigel basement membrane) on the formation and functionality of fibrin and collagen muscle strips. Differentiated, multinucleated myotubes were distributed throughout muscle strips supplemented with IGF-1 (Fig. 2A and B). Muscle strips cultured without IGF-1 contained populations of both undifferentiated myoblasts as well as multinucleated myotubes at later time points (Fig. 2C and D), signaling that although IGF-1 increased the rate of fusion and maturation, its absence did not hinder muscle strip development. With the addition of 50 ng ml^{-1} IGF-1, the portion of the fibrin-based muscle strip occupied by cells significantly increased (Fig. S5D and E).

Despite cross-linking during polymerization, fibrinogen is extremely susceptible to rapid degradation by cell-secreted proteases such as plasmin (32). *In vivo*, natural inhibitors of plasmin prevent indiscriminate matrix digestion; however, *in vitro*, C2C12s can produce plasmin-activating plasminogen, and a protease inhibitor such as ϵ -aminocaproic acid (ACA) must be added to ensure stability of the system (33). Addition of 1 mg ml^{-1} ACA helped to maintain the structural integrity of the muscle strip, significantly increasing the lifetime of the muscle strip before rupture (Fig. S5C). The bio-bots presented in this study were supplemented with 1 mg ml^{-1} ACA, and 0 (without) or 50 (with) ng ml^{-1} IGF-1.

Hematoxylin and eosin (H&E) staining of histological sections of IGF-1-supplemented fibrin muscle strips revealed an increased peripheral cell density compared with the center (Fig. 2E–H). We located over 75% of cells within $200 \mu\text{m}$ of the edge of the fibrin muscle strip, a distance consistent with the upper limit of diffusion of oxygen within a tissue (34). Finally, we used a colorimetric assay to evaluate cell viability over time. The relative absorbance of muscle strips (normalized to day 0) indicated a $85.76 \pm 10.74\%$ and $79.71 \pm 14.78\%$ viability of cells within the muscle strip after days 6 and 9, respectively (Fig. S5F). Compared with the control muscle strips on day 9, the IGF-1-supplemented muscle strips demonstrated enhanced cell proliferation.

Fig. 1. Fabrication of hydrogel structures and formation of 3D muscle strips. (A) Computer-aided design software was used to design bio-bots with desired dimensions. Measurements are in millimeters. (B) An SLA was used to polymerize hydrogel structures in an additive process. (C) The cell-matrix solution consisted of C2C12 skeletal muscle myoblasts, matrix proteins (fibrin or collagen I), and Matrigel. (D) Fabricated bio-bot (*i*, side view) and holder (*ii*, top view). Cell-matrix solution was pipetted into a polymerized holder containing the bio-bot structure (*iii* and *iv*, side view). Cells and matrix compacted around the pillars to form a solid muscle strip (*v* and *vi*, top view with immunostaining for MF-20, green, and DAPI, blue) and the device was released from the holder (*vii*, side view). All scale bars, 1 mm.



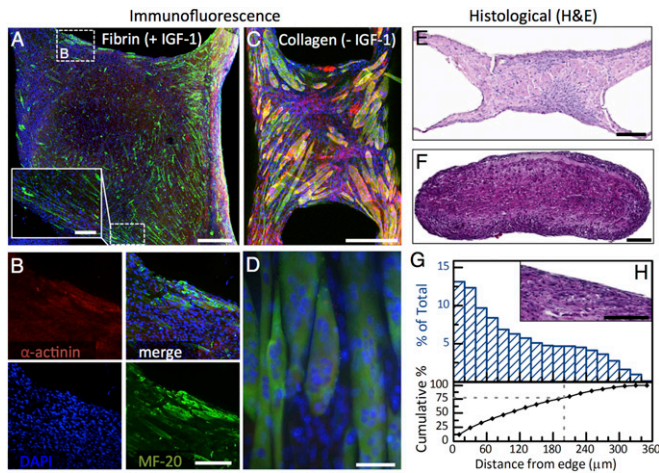


Fig. 2. Biological characterization of muscle strips. (A) Longitudinal fibrin muscle strip slice (day 9). Scale bar, 500 μm . (Inset) Scale bar, 200 μm . (B) Myotubes aligned along the fibrin muscle strip perimeter. Scale bar, 200 μm . (C) Longitudinal collagen muscle strip slice (day 14). Scale bar, 500 μm . (D) Multinucleated myotubes in the collagen muscle strip. Scale bar, 50 μm . (E–H) H&E staining of fibrin muscle strip sections (day 9). All scale bars, 200 μm . (E) Longitudinal and (F) transverse muscle strip sections. (G) Quantitative analysis of transverse sections demonstrated 75% of cells within 200 μm from the edge ($n = 8$). (H) Perimeter of transverse section of the muscle strip.

Optimization of Muscle Force Generation. To optimize force production capabilities of the engineered muscle strip, we studied the effect of varying biological and mechanical environmental cues during muscle differentiation and maturation. By varying the laser energy dose of polymerization of the SLA, we created hydrogel structures with a range of tunable properties and conformations without changing the composition or molecular weight of the material. The elastic modulus of the hydrogel beam (as measured by tensile testing in a hydrated sample chamber) increased logarithmically from 214.6–741.6 kPa with laser energy doses varying from 108.7–512.6 mJ cm^{-2} , due to a higher degree of cross-linking by higher energy doses. With compaction of the muscle strip, traction forces exerted by cells produced an inward force on the pillars, which gave rise to varying degrees of bending in the beam. As expected, the stiffer hydrogel structures offered a greater resistance to bending; thus, beams with higher elastic moduli exhibited a lower deflection in response to passive tension forces exerted by the muscle strips (Fig. 3A and Fig. S6A).

Using Euler–Bernoulli linear bending theory (SI Methods and Fig. S6C), we derived a formula relating the observed hydrogel beam deflection to the muscle-generated passive tension force. An increase in beam stiffness resulted in an increased tension in the muscle strip at rest. For elastic moduli of 214.6, 319.4, 411.2, and 489.3 kPa, the passive tension averaged 860.6 ± 47.2 , 992.7 ± 34.3 , $1,103.6 \pm 45.8$, and $1,146.0 \pm 69.0$ μN , respectively, in fibrin-based muscle strips cultured with IGF-1. We then used finite-element analysis software (ANSYS) to model and simulate global displacement of the beam and pillars in response to an applied force (Fig. 3C and D). The simulated deflection values differed 18–19% from actual measurements (Fig. S6D), validating our methods to extract passive tension and predict muscle force output.

The bio-bot pillars provided uniaxial constraint for cell alignment during compaction, allowing the myotubes to mature in a macroenvironment that mimics the native organization of functional skeletal muscle. The increase in passive tension generated by the muscle strip with increasing hydrogel stiffness indicated that the forces exerted by cells could be modified in relation to mechanical environment of the muscle. Others have validated an increase in force output with dynamic mechanical stimulation (35); here, we also demonstrate that a static mechanical cue imposed

during muscle development contributes to improved functionality, providing further evidence that many types of mechanical stresses are required for muscle development (36). Bio-bot hydrogel structures with a beam stiffness of 319.4 kPa were selected for subsequent experiments, as they combined the advantages of sufficiently high passive tension forces with deformable structures suitable for locomotion.

To determine an optimal matrix system for engineered muscle functionality, we compared the passive tension forces generated by collagen- and fibrin-based cell–matrix systems. In muscle strips containing the same number of cells, we observed a significant increase in passive tension in those containing fibrin (629.3 ± 8.2 μN) compared with collagen (534.2 ± 5.8 μN) (Fig. S6E). Advantageously, fibrin polymerizes relatively quickly compared with other ECM proteins, and it can undergo large deformations without breaking (37, 38). The ability to sustain large strains while maintaining structural integrity during muscle contraction was a necessary characteristic for applications in bioactuation.

Examining the effect of varying other biological environmental cues, we observed that fibrin-based muscle strips supplemented with IGF-1 demonstrated a 70.7% increase in passive tension force, from 581.4 ± 20.6 – 992.7 ± 34.3 μN ($P < 0.001$, $n = 4$), compared with the control without IGF-1. We attributed this significant increase in force production to a greater number of differentiated myotubes in muscle strips supplemented with IGF-1, an observation confirmed by immunostaining and other reported variations of 3D engineered muscle constructs (39). We calculated a normalized stress in the muscle strips with IGF-1 by dividing the passive tension by the cross-sectional area of the tissue, which averaged 1.2 ± 0.04 mm^2 ($n = 9$, Fig. S6F). The passive stress in the muscle strips averaged 0.84 ± 0.03 kPa, values that are comparable to those of similar muscle-based 3D cell–matrix systems with collagen or fibrin (22, 26, 40, 41).

Electrically Paced Actuation of Bio-bots. To externally control muscle contraction and bio-bot locomotion, we used a custom-designed setup (42) to stimulate reproducible contraction of

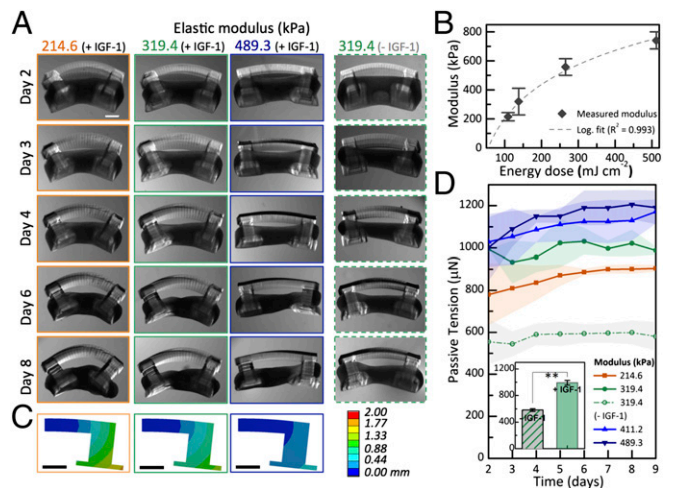


Fig. 3. Analysis of bending and passive tension. (A) Over time, bio-bot beams polymerized with varying energy doses exhibited different bending profiles. Scale bar, 1 mm. (B) Elastic modulus of the beam as a function of energy dose ($R^2 > 0.99$, $n = 5$). Increasing polymerization energy resulted in a higher modulus and less bending. (C) Finite-element analysis simulation demonstrating global deflection of the bio-bot beam and pillars on day 8. (D) Passive tension in the muscle strip as a function of time, modulus, and addition of IGF-1 ($n = 3$, 489.3 kPa; $n = 4$, others). Increasing beam stiffness resulted in a higher muscle strip tension. (Inset) Addition of IGF-1 significantly increased the average tension by 70.7% (for 319.4-kPa stiffness) over time period shown. Statistics represent one-way ANOVA and Tukey’s test, with $** = P < 0.001$. All data are presented as mean \pm SD (shaded region in D).

excitable cells within the muscle strip with a bipolar electrical pulse train (Fig. 4A and Fig. S7). By mimicking signals necessary for the generation of an action potential *in vivo*, electrical pulse stimulation can induce protein expression, contractile ability, cell alignment, and differentiation of skeletal muscle *in vitro* (43–46); here, we harnessed the stimulation to coordinate contraction of multiple myotubes within the muscle strip, which collectively generated sufficient force to visibly deform the hydrogel structure of the bio-bot. Fibrin-based muscle strips supplemented with IGF-1 and stimulated at constant frequencies of 1, 2, or 4 Hz demonstrated a consistent output of 1.01 ± 0.003 , 2.01 ± 0.01 , and 3.95 ± 0.05 contractions s^{-1} , respectively ($n = 10$, Fig. 4B, [Movies S2](#) and [S3](#)), establishing that the bio-bots could be reliably paced with this method. We witnessed twitch contractions below 8–10 Hz and tetanus above this upper frequency limit. In contrast, non-IGF-1-supplemented muscle strips did not respond to stimulation during this time period, a result attributed to fewer myotubes. We predict a response at later time points, as others have shown electrically induced contractility of C2C12s or primary cells in 3D tissue constructs after 14 d (and sometimes up to 30+ d) following formation (26, 47–50). However, IGF-1 treatment presents a simple and physiologically relevant method to enhance differentiation for functional output via electrical stimulation on a shorter time scale (here, as early as day 7). Additionally, we observed that the range of active tension remained above 99.3% of the initial value during 8 min of electrical stimulation at hour 0, and above 98.8% of the initial value at hour 6, revealing a consistent force output from the engineered muscle strips both within a constant stimulation period and at later time points (Fig. S84).

A Kelvin–Voigt viscoelasticity model that correlated the observed cyclic displacement to the contractile force was used to extract the active tension generated by IGF-1-supplemented muscle strips in response to electrical stimulation (Fig. 4C). The range of active tension during contraction decreased with increasing stimulation frequency, from a dynamic fluctuation of 198.68 μN during 1-Hz stimulation to 109.48 μN during 4-Hz stimulation (Fig. 4D). The active tension data followed a positive force–frequency relationship in which the magnitude of

the active tension exerted by the muscle increased, even while the range of pillar motion decreased. Furthermore, as a consequence of muscle relaxation times exceeding the period between electrical pulses at higher frequencies, we observed a temporal summation of force that resulted in a baseline tension increase over time. The muscle strips therefore displayed functional behavior characteristic to physiological skeletal muscle, in which force output increases with frequency before reaching tetanus (11).

Demonstration of Controlled Directional Movement. We aimed to create a biomimetic “crawling” mechanism reminiscent of an inchworm’s movement. Using finite-element simulations, we explored a symmetric and an asymmetric design for the bio-bots (Fig. 5A). For the asymmetric design, we extended the length of one pillar of the bio-bot hydrogel structure, allowing for asymmetric bending of the flexible beam (Fig. S34), hypothesizing that the introduction of deliberate asymmetry within the structure would increase the moment arm between the muscle strip anchor point and the beam, as well as the range of motion between pillars during displacement. It would also change the contact area of the base to the surface for one pillar versus the other. These simulations revealed that as expected, the symmetric structure did not demonstrate significant locomotion. However, an asymmetric structure exhibited nonuniform distribution of stress in the hydrogel structure in response to muscle contraction, corresponding to asymmetric pillar displacements (Fig. 5A and [Movies S4](#) and [S5](#)). Simulations revealed that asymmetric actuation and force generation of the muscle strips by geometric design of the bio-bot would produce greater net displacement over a fixed time and create a more efficient and predictable locomotive mechanism compared with the symmetric design.

Consistent with the simulation results, we experimentally observed that for a symmetric hydrogel structure, electrical pacing of skeletal muscle strips attached to bio-bot structures either did not result in net locomotion of the bio-bot across a substrate, or in some cases, resulted in a very small locomotion (Fig. 5B and [Movie S6](#)). Because the hydrogel structure itself was symmetric, we hypothesized that any observed small locomotion of the symmetric structure was attributed to asymmetry in muscle strip formation and force distribution. From a top-view movie, we tracked bio-bot pillar displacements in response to muscle contractions over time and observed that when both pillars displaced equally, the bio-bot did not move. Interestingly, for the minimally locomotive bio-bots, when one pillar displaced more than the other (Fig. 5C), the bio-bot always “crawled” in the direction of the pillar that demonstrated greater displacement in response to muscle contraction. For the case of these symmetric devices, these small velocities were found to be less than $4.34 \mu m s^{-1}$ at electrical stimulation of 1-Hz frequency (Fig. 5D).

We found that muscle strips coupled to the asymmetric compliant hydrogel structure drove inchworm-like crawling locomotion with maximum velocity. Contraction of muscle strips on asymmetric bio-bots resulted in a maximum velocity of $117.8 \mu m s^{-1}$ in response to electrical stimulation of 1-Hz frequency, an increase in velocity of more than 25-fold compared with the symmetric design (Fig. 5E–G, Fig. S8, and [Movie S7](#)). During electrically paced locomotion, the asymmetric bio-bot produced an active tension force of $394.7 \pm 56.6 \mu N$, or 27.9% of the maximum force (Fig. 5G).

Although bio-bot pillar displacement decreased with increasing stimulation frequency, the observed increase in force generation led us to test the effect of stimulation frequency on bio-bot locomotion. The increased number of contractions with increasing stimulation frequency within a given time period resulted in an increased average velocity of the asymmetric bio-bot, which was measured to be 117.8, 132.2, and $156.1 \mu m s^{-1}$ at 1, 2, and 4 Hz, respectively, during the time period shown (Fig. 5H and [Movie S8](#)). At all frequencies, the asymmetric bio-bot moved in the direction of the pillar that demonstrated greater displacement, as predicted (Fig. S8C).

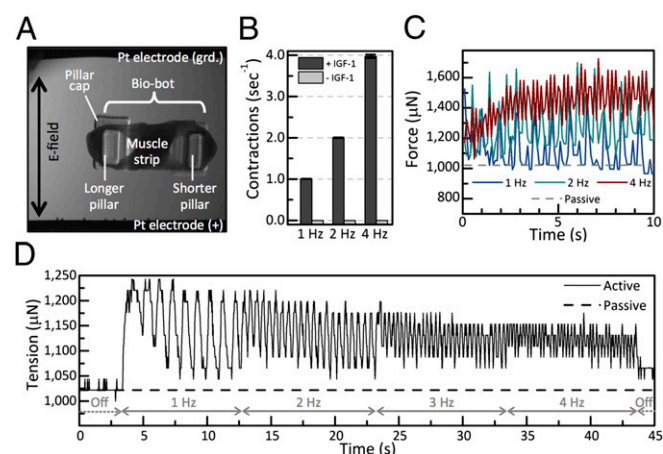


Fig. 4. Electrical control and pacing. (A) During stimulation, bio-bots were placed standing up on the surface of the dish, with the muscle strip’s longitudinal axis parallel to the electrodes and perpendicular to the applied field. (B) IGF-1-supplemented bio-bots were reliably paced at stimulation frequencies of 1, 2, or 4 Hz. Bio-bots not supplemented with IGF-1 did not respond to stimulation at any frequency in the observed time period. (C) A Kelvin–Voigt viscoelasticity model was used to extract active tension generated by the muscle strip in response to electrical stimulation. (D) Muscle strip time-varying active tension was calculated for a bio-bot undergoing varying electrical stimulation (0, 1, 2, 3, 4, 0 Hz) during one experiment, and followed a positive force–frequency relationship.

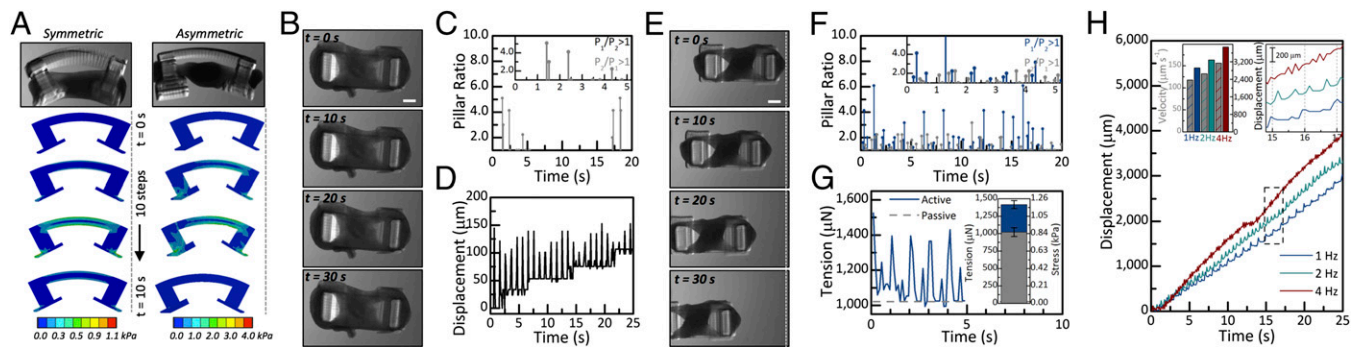


Fig. 5. Simulation and movement profiles of symmetric and asymmetric bio-bots supplemented with IGF-1. (A) von Mises stress of a symmetric and asymmetric bio-bot. The range of one contraction is shown over a period of 10 s (10 “steps” at 1-Hz stimulation). (B and E). Top-view time-lapse images of the symmetric (B) and asymmetric (E) bio-bot’s movement. All scale bars, 1 mm. (C and F) Relative ratios of pillar movement over time during 1-Hz stimulation. The bio-bots moved in the direction of greater pillar movement during contraction. (D) Displacement (μm) of a symmetric bio-bot at 1-Hz stimulation during a 25-s interval when contraction produced maximum displacement along the surface. (G) Active tension force of the asymmetric bio-bot during locomotion. (H) The increased number of contractions with increasing stimulation frequency within a given time period resulted in an increased average velocity of the asymmetric bio-bot. (Inset, Left) Velocity (gray) and total displacement. (Inset, Right) Zoom of boxed region.

Conclusions

Soft robotic devices integrated with biological systems combine the advantages of high-degree-of-freedom compliant response with dynamic sensing and actuation capabilities. We report the design and fabrication of a skeletal muscle powered machine that can be controlled and paced via external signaling, instead of relying on spontaneous muscle contraction. Modeling and simulations were used to study the effect of changing design parameters on functional response, resulting in a more comprehensive picture of the locomotive mechanisms. We improved the force generation capacity and functional performance of this engineered tissue by differentiating muscle strips in an optimized fibrin-based ECM environment supplemented with IGF-1 growth factor. Muscle strips displayed a tunable functional response in relation to the static stress imposed by a 3D printed hydrogel structure, mimicking the *in vivo* mechanical environment of muscle maturation. Finally, electrical stimulation triggered contraction of engineered muscle, driving a biomimetic and controllable directional locomotion.

Although other studies have explored the use of excised and engineered biological components such as cardiac muscle tissues for applications in bioactuation, these studies lack a fabrication methodology that offers flexibility in precisely specifying geometric, material, and biological component design parameters (Fig. S9). Our stereolithographic 3D-printing-based biofabrication system supports the integration of a variety of scaffolding materials and multiple cell types, representing a significant advancement toward engineering biological machines capable of complex and controllable functional behaviors. Furthermore, our biofabrication methodology can readily be modified to demonstrate other mechanisms of locomotion and force output (such as swimming and pumping) while also creating a platform for future studies integrating different biomaterials and cell types. The incorporation of motor neurons resulting in neuromuscular junction formation would lead to more complex mechanisms of functional control of the engineered muscle. Additionally, integration of endothelial cells to engineer an internal vasculature would increase diffusion of nutrients and oxygen to the muscle tissue, thereby increasing efficiency and long-term viability of a living biological machine.

Forward engineering of biological machines can help to advance the understanding of the fundamental scientific and design principles underlying living systems and lead to a quantitative understanding of the way integrated cellular systems sense and respond to environmental signals (1). In the long term, we envision that this system could serve as a basis for target applications such as microscale tissue fabrication for drug screening and tissue- and organ-on-a-chip mimics, dynamic biocompatible microelectronics

and medical implants, and forward-engineered biological machines and systems.

Methods

Design and Fabrication of Parts. A commercial SLA (250/50, 3D Systems) was modified for polymerization as previously described (20, 51). Parts generated using computer-aided design software were exported to 3D Lightyear software (v1.4, 3D Systems), which sliced the part into layers. Prepolymer solutions for bio-bots and holders are described in *SI Methods*. For fabrication of bio-bots, an 18×18 -mm-square cover glass was secured to the center of a 35-mm culture dish (both with hydrophilic surfaces) before fabrication. For bio-bot holders, cover glass slides were first treated with 2% (vol/vol) 3-(trimethoxysilyl)propyl methacrylate (Sigma-Aldrich) in 200-proof ethanol (100% EtOH) for 5 min and then washed in 100% EtOH for 3 min, dried, and secured to the center of a 35-mm dish. Following fabrication, each structure was rinsed in PBS, sterilized in 70% EtOH for 1 h, and allowed to reswell in PBS for at least 1 h.

Mechanical Testing. To characterize the mechanical properties of PEGDA M_n 700, dogbone-shaped test specimens fabricated with the SLA were superglued to stainless steel bars, then fixed in custom-fabricated structures in a hydrated sample chamber (*SI Methods*). An ElectroForce BioDynamic test instrument (5100, Bose) with a 1,000-g load cell applied uniaxial tension at each end of the structure at 0.05 mm s^{-1} (Fig. S6B). Control software (Wintest) was used to track load, displacement, and strain ϵ . Elastic modulus E was determined from the linear portion of stress–strain curves.

Cell Culture and Formation of Muscle Strip. C2C12 murine myoblasts were transfected with pAAV-Cag-Chr2-GFP-2A-Puro plasmid to express Chr2 (22). Cells were maintained in growth medium (GM) or differentiation medium (DM) as described in *SI Methods*. During cell seeding, C2C12s suspended in GM were mixed with an ice-cold liquid solution of Matrigel basement membrane matrix and type I collagen or fibrin, as detailed in *SI Methods*, and added to each holder. After 24 h, bio-bots were cultured in DM with antifibrinolytic 6-ACA and human IGF-1 (both Sigma-Aldrich) as noted.

Beam Deflection and Muscle Strip Passive Tension. Side-view images of symmetric bio-bots were taken every 24 h using a stereomicroscope (MZ FL III, Leica Microsystems) with a digital microscope camera (Flex, SPOT Imaging Solutions). To model bio-bot deformation in the passive (bent but unmoving) state, designs were reconstructed using SolidWorks and imported into ANSYS. Appropriate material properties were assigned and a force equal to the calculated passive tension was applied to the base at a distance specified by the measured moment arm. Resultant solutions of total deformation were examined to find maximum beam deflection. Passive tension in the muscle strip was determined using Euler–Bernoulli linear beam theory (*SI Methods*). Stress was calculated by dividing the tension by the muscle strip’s cross-sectional area A , determined from transverse H&E sections.

Electrical Stimulation. A custom-designed electrical setup was built as previously described (42) (Fig. S7). Bio-bots were stimulated with bipolar electrical pulses of 20-V amplitude (21.6-V cm⁻¹ field strength) and 50-ms pulse width. Top-view movies were acquired with a stereomicroscope with a digital microscope camera at 9.2 frames per second (fps). Side-view movies were acquired using a camcorder (Handycam DCR-SR65, Sony) at 30 fps. Electrodes were sterilized in 70% EtOH and rinsed with PBS between experiments.

Characterization of Movement and Force. Active tension exerted by contracting muscle strips upon electrical stimulation was calculated by treating the bio-bot structure as a viscoelastic body governed by a Kelvin–Voigt model (SI Methods). An automated MATLAB script was designed to track the location of a user-specified feature with a normalized 2D cross-correlation and provided X–Y coordinates of a specific point on the bio-bot for each frame. This software tracked the distance between the longer and shorter pillar caps during stimulation from a top-view movie (Fig. S8E). Frequency was measured by manually counting contractions in identical time segments.

Immunofluorescence and Histology. Muscle strips were removed from bio-bot pillars and prepared for immunostaining for myosin heavy chain, sarcomeric

α-actinin, and 4',6-diamidino-2-phenylindole (DAPI), or histological staining with H&E (SI Methods). Muscle strips were imaged with a confocal microscope (LSM 710, Zeiss) or inverted fluorescent microscope (IX81, Olympus). Image stacks were processed using Zen software (2010, Zeiss) and ImageJ.

Statistical Analysis. Results are presented as mean ± SD. All statistical analyses were performed with OriginPro software and represent one-way ANOVA followed by Tukey's Multiple Comparison Test for $P < 0.001$, $P < 0.01$, or $P < 0.05$ as noted.

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